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Influence of dietary microalgal concentrates on growth, survival and health status of *Penaeus vannamei*

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Abstract

The present study evaluated the nutritional value and antimicrobial activities of *Thalassiosira weissflogii* and *Tetraselmis* sp. concentrates. The study also ascertained the effect of microalgae supplemented diets on the growth and survival of *Penaeus vannamei* post larvae (PL 18, mean weight: 19.714 ± 1.62 mg). Microalgae concentrates were prepared by flocculation, and the proximate composition showed no significant difference in the crude protein content between these species (*T. wesissflogii*: 43.07 ± 1.78%; *Tetraselmis* sp. 42.11 ± 2.55%; $p > 0.05$). However, the crude lipid content of *T. weissflogii* was significantly ($p < 0.05$) higher (20.11 ± 1.02%) than that of *Tetraselmis* sp. (10.56 ± 0.27%). Significantly higher ($p < 0.05$) polyunsaturated fatty acid was found in *Tetraselmis* sp. compared to *T. weissflogii*. Significantly higher inhibition against *Vibrio parahaemolyticus* was shown by the *Tetraselmis* extract compared to that of *T. weissflogii*. Further, a 42-day feeding trial was conducted with three different inclusions of *T. weissflogii* (THA) and *Tetraselmis* sp. (TET) concentrates in *P. vannamei* nursery diet (0, 0.5, 1, and 1.5 g kg^{-1} of diet). Significantly higher ($p < 0.05$) average body weight (ABW) was observed in $TET_{0.5}$, $TET_{1.0}$, $TET_{1.5}$, $THA_{1.0}$ and $THA_{1.5}$ compared to the control. The highest ABW was recorded in $TET_{1.0}$ (0.96 ± 0.02 g), which was significantly higher than all other treatments. Significantly higher ($p < 0.05$) weight gain was observed in $TET_{1.0}$ (0.94 ± 0.02 g) compared to the rest of the diets. Significantly higher ($p < 0.05$) average daily gain was observed in $TET_{1.0}$ (22.48 ± 0.55 mg day^{-1}). Haematological parameters of *P. vannamei* fed with microalgae concentrates were higher than those of the control group. The gut microbial analysis showed a significant reduction in total *Vibrio* count in the animals fed with 1% and 1.5% *Tetraselmis* or *Thalassiosira* compared to other treatments. These results indicated the beneficial effect of growth and better antimicrobial property to withstand against the common pathogenic microbe and thus indicating the beneficial effect in the early life stages of shrimp.

Keywords Microalgae concentrate · Nursery feed · Feed additives · Nutraceuticals

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Introduction

Shrimp is a highly traded seafood commodity farmed commercially in at least sixty countries. However, shrimp aquaculture has often been hampered due to mass mortality caused by infectious bacterial and viral diseases, which cause heavy economic loss to the farmers (Thornber et al. 2020; Patil et al. 2021). Most of the problems are encountered during the initial period of first 30 days of stocking the seed. With an effective nursery cycle, overall productivity can be significantly improved by increasing survival and growth rates during the early stages of life (Wasielesky et al. 2013). The nursery-rearing system has become a crucial phase in sustainable shrimp production in many shrimp-farming countries since it enhances productivity and profitability (Crab et al. 2012; Anand et al. 2021). Further, the short duration of the final grow-out phase allows the execution of more rearing cycles each year, which results in more significant economic gain for the farmer (Anand et al. 2021).

Although it is possible to manage higher stocking densities and increase the number of crops per year in shrimp farms through nursery cycles, the health of the shrimps in the nursery system is crucial. The diseases were treated with antibiotics, and the incidence of antibiotic residues coupled with multiple drug-resistant bacteria has become an emerging problem in shrimp aquaculture (Thornber et al. 2020) which is one of the reasons for many shrimp export rejections from India (Geetha et al. 2020). Hence, ensuring the health of the shrimp in the nursery phase is one of the major researchable areas getting attention among the aquaculture researchers.

Microalgae metabolites, including antioxidants, anticoagulants, anti-inflammatory agents, antimicrobials, and anticancer agents, have demonstrated strong biological activity (Kiran and Venkata Mohan 2021; Sandeep et al. 2022). Thus, it is an excellent ingredient for fish and shrimp feed due to their favourable biochemical composition, shape, size, palatability, digestibility, and cell wall composition (Radhakrishnan et al. 2016). Microalgae are the natural food for early life stages of shrimp and fish and hence inclusions of these algae enhance the nutritional value and palatability of the feed while also helping in the economic and sustainable front. Providing proteins with a balanced ratio of the essential amino acids, omega-3 fatty acids, and pigments is an additional significant advantage of using microalgae as fish feed. Several studies have shown that adding microalgae to fish and shrimp diets improves the growth and nutritional qualities (Ju et al. 2012; Chen et al. 2016; Ansari et al. 2021; Sandeep et al. 2022). Some microalgae are rich in long-chain polyunsaturated fatty acids, mainly eicosapentaenoic (EPA), docosahexaenoic, and arachidonic acid, which improve and stabilise nutrition, promote growth and appetites, and are considered essential for high survival rate and normal development of larval and juvenile fish, crustacean, and mollusc (Shan and Lin 2014; Sandeep et al. 2019).

Microalgae have developed several defensive and adaptive strategies to survive in a highly competitive environment, with widely fluctuating chemical and physical parameters, synthesising a tremendous diversity of compounds (Ghasemi et al. 2004). Microalgae pigments possess antimicrobial activity (Hajimahmoodi et al. 2010; Bhattacharjya et al. 2020; Sandeep et al. 2022), and extracts of green algae, diatoms, and dinoflagellates produce a variety active antibacterial activity in vitro (Katircioglu et al. 2006). The antimicrobial properties against pathogenic microbes help to control diseases in the culture systems when the particular microalgae are incorporated into shrimp diet. There were few studies regarding the partial replacement of fish meal and fish oil by microalgae biomass in the diet of shrimps (Ju et al. 2012; Allen et al. 2019). Adding microalgae biomass to the diet will enhance shrimp's growth and overall health (Basri et al. 2015). Similarly, the inclusion

of microalgae concentrates as a dietary source revealed a positive effect on the eastern oyster, *Crassostrea virginica* (Rikard and Walton 2012); winged pearl oyster, *Pteria penguin* (Wassnig and Southgate 2016); and giant clam, *Tridacna noae* (Southgate et al. 2017).

However, conclusive studies on the effect of dietary microalgae concentrate in *P. vannamei* post larvae are not there. The present study evaluates the nutritional composition, antioxidant properties, and anti-*Vibrio* properties of microalgae concentrates on understanding the underlying mechanisms of the effect of microalgae on the shrimp diet. Moreover, the present study holistic approach was used to substantiate the beneficial effect through the haematological parameters and gut microbiology of *P. vannamei* fed with different levels of different microalgae concentrations.

Materials and methods

Ethical statement

The experiments in the present study were carried out according to the guidelines of the Committee for Control and Supervision of Experiments on Animals, a statutory committee of the Department of Animal Husbandry and Dairying, Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India. Further, the approval was obtained from the Institutional Animal Ethics Committee of the ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India.

Microalgae cultures

The microalgae used in the present study, *Thalassiosira weissflogii* and *Tetraselmis* sp., were isolated from the Muttukadu estuarine ecosystem, Tamil Nadu, India, and were maintained in the microalgae repository of ICAR-Central Institute of Brackishwater Aquaculture, Chennai (Sandeep et al. 2019; 2021). They are axenic cultures maintained under laboratory conditions. The pure isolates were grown in Conway (Walne 1966) medium in the indoor microalgae laboratory at 24 ± 1 °C in a light intensity of 4000–5000 lx. Photoperiod was fixed at 14:8-h light and dark periods.

Bacterial cultures

The three pathogenic bacteria used in the present study were collected from culture repository of AAHED (Aquatic animal health and environment division), ICAR-CIBA (Central Institute of Brackishwater Aquaculture), Chennai, India. *Vibrio campbelli* LB1 (Kumar et al. 2021), *V. harveyi* SB1, and *V. parahaemolyticus* AMR2021S14 were used in the study. The bacterial strains were isolates collected from shrimps during disease outbreaks in shrimp hatcheries and farm.

Flocculation of microalgae to make concentrate

A preliminary dose assessment trial was conducted to evaluate the flocculation efficiencies of different alkalis (NaOH, KOH, and NH_4OH) at different concentrations (0.1, 0.5, 1, 10, and 20 mM). Based on the study, the best alkali with the optimum dose was used to flocculate

Thalassiosira weissflogii and *Tetraselmis* sp. cultures. The flocculation efficiency was calculated at 30 min for 3 h after the chemical treatment. The cultures were subjected to intense aeration immediately after adding alkalis for 2 min to allow the pH to stabilise. The flocculation efficiency was calculated by applying the formula of Gerde et al. (2014).

$$\text{Flocculation Efficiency}(\%) = \left(1 - \frac{A}{B}\right) \times 100$$

where A is the OD at 750 nm of the sample, and B is the OD at 750 nm of reference.

Microalgae concentrate obtained after flocculation was deflocculated with 0.1 N HCl by reducing the elevated pH. The number of cells in the culture and flocculated concentrates was estimated using a haemocytometer. The concentrates were kept at 4 °C until use.

Nutrient profiling

The microalgal biomass was harvested in the log phase of the growth and freeze-dried. The proximate composition (g/100 g) of experimental diets and microalgae was analysed according to the standard procedures of AOAC (2005). For moisture analysis, (the initial microalgal sample, final shrimp samples, and diets) samples were oven dried at 105 °C until a constant weight was achieved. The micro-Kjeldahl method (FOSS, Kjeltec™ 8400 analyser, USA) was followed to estimate the crude protein (CP), and lipid was estimated by solvent extraction using Soxhlet's apparatus (SOCS PLUS Six Place Fully Automatic Solvent Extraction System, Pelican, India). Samples were incinerated at 550 °C for 5 h in a muffle furnace to determine the total ash (TA) content. The crude fibre (CF) content of fat-free samples was performed through acid digestion followed by alkaline digestion (Fibre Cap™ 2021, FOSS, USA). Then the digested sample was incinerated in a muffle furnace at 550 °C for 5 h. The subtraction method was employed to calculate nitrogen-free extract (NFE) content of the experimental diets. The proximate composition was calculated based on the following formulae:

$$\text{Moisture}(\%) = \frac{\text{The initial weight of the sample} - \text{The dried weight of the sample}}{\text{The initial weight of the sample}} \times 100$$

$$\text{Crude Protein } (\%) = \frac{\{(\text{Sample titre} - \text{Blank titre}) \times 0.1 \times 0.014 \times 6.25\}}{\text{Weight of the sample (g)}} \times 100$$

where 0.1 is the normality of acid, 0.014 is the molecular mass of nitrogen, and 6.25 is the constant relationship between N and animal protein of sample.

$$\text{EE } (\%) = \frac{\text{The initial weight of the sample} - \text{Final weight of the sample}}{\text{The initial weight of the sample}} \times 100$$

where EE is the ether extract.

$$\text{Ash } (\%) = \left(\frac{\text{Weight of the ash}}{\text{Weight of the sample}}\right) \times 100$$

$$\text{NFE } \left(\frac{g}{100} g\right) = \{100 - (CP + CL + CF + TA)\}$$

where NFE is the nitrogen-free extract, CP is the crude protein (g/100g), CL is the crude lipid (g/100g), CF is the crude fibre (g/100g), and TA is the total ash (g/100g).

The total protein of microalgae biomass was determined following Lowry's method (Lowry et al. 1951) and lipids by Bligh and Dyer method (Bligh and Dyer 1959), and the respective fatty acid methyl esters were prepared and extracted into petroleum ether. Analysis of methyl esters was performed by a gas chromatograph (GC2014 Shimadzu, Japan) on an RTX wax capillary column (100 m length × 0.25 mm I.D × 0.2 µm film thickness). The quantity of fatty acids (mg kg^{-1}) was calculated according to Aziz et al. (2012).

Antioxidant properties

Total phenolic content

Estimation of the total phenolic content (TPC) of the sample was done by Folin–Ciocalteau (FC) assay described by Singleton and Rossi (1985) with minor modifications as per Sivaramakrishnan et al. (2017). Briefly, 200 µl of the extracted sample was mixed with 200 µl FC reagent (0.5 N) followed by 1.6 ml 7.5% Na_2CO_3 and incubated for 2 h in the dark at room temperature. The absorbance was measured at 765 nm using a UV–visible spectrophotometer. Gallic acid was standard, and the phenolic contents were expressed as gallic acid equivalents (mgGAE g^{-1}) of macroalgae extract.

Total flavonoid content

The flavonoid content of the microalgae extract was determined by the spectrophotometric method of Zishen et al. (1999) with slight modification as per Sivaramakrishnan et al. (2017). Briefly, 0.5 ml of sample was mixed with 0.3 ml 15% sodium nitrite, 0.6 ml 10% ammonium chloride hexahydrate, and 3 ml of 1N sodium hydroxide after 5 min. Absorbance was immediately measured at 510 nm. Flavonoid content was expressed as Rutin equivalents of extract (mg RE g^{-1}).

Total antioxidant activity

The total antioxidant activity of methanolic extracts was quantified by Prieto et al. (1999) method with slight modification as per Sivaramakrishnan et al. (2017). Briefly, 0.2 mg/ml of samples in different aliquots was mixed with 1 ml of each of the three reagent solutions (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The samples were incubated at 95 °C for 90 min in a water bath. Absorbance was measured at 695 nm. Total antioxidant activity was expressed as milligrammes of ascorbic acid per gramme of extract.

Antibacterial assay

The microalgal biomass was harvested in the log phase of the growth and freeze-dried. About 25 g freeze-dried microalgal powder was percolated in 60% alcohol (100 ml) and ground with a mortar and pestle. The extraction was repeated until the solvent became colourless. Subsequently, the extract was dried under vacuum at 45 °C to remove the solvent. The dried extract was collected and stored at − 20 °C until used. Bacterial

strains *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* were obtained from the culture collection of Aquatic Animal Health Division, ICAR-CIBA, Chennai, India. The bacterial strains were cultured in nutrient broth (Hi Media, India) for 12 h. Turbidity was adjusted to 0.5 McFarland Standards (10^7 CFU/ml) and confirmed by spectrophotometric reading at 600 nm. McFarland Standards were used to standardise numbers of bacteria when required by a procedure or for the susceptibility test. The antibacterial activity of the methanolic extracts of microalgae was tested against selected bacteria by agar well diffusion method (Holder and Boyce 1994) using nutrient agar media (Hi Media, India). Both methanol and DMSO-dissolved microalgae extracts (50 µl each) were loaded separately into the wells in duplicate. Likewise, negative control (solvent alone) and positive control (streptomycin) were also prepared in duplicate. The plates with bacterial inoculums and extracts were incubated for 24 h at 37 °C. Zones of inhibition formed after treatment with extracts were measured using a Hi Antibiotic Zone Scale (HiMedia Laboratories).

Preparation of formulated feed

Seven iso-nitrogenous and iso-energetic experimental diets were prepared (Table 1) with different levels of microalgae concentrates as additives at three different inclusion levels

Table 1 Ingredient composition (g kg^{-1}) and proximate composition of the experimental diet with graded levels of microalgae concentrates

	Control	$TET_{0.5}$	$TET_{1.0}$	$TET_{1.5}$	$THA_{0.5}$	$THA_{1.0}$	$THA_{1.5}$
Fish meal	170	170	170	170	170	170	170
Dried acetes	80	80	80	80	80	80	80
Dry fish	50	50	50	50	50	50	50
GNOC	40	40	40	40	40	40	40
Wheat	298	293	288	283	293	288	283
Soya	300	300	300	300	300	300	300
Fish oil	15	15	15	15	15	15	15
Soy lecithin	15	15	15	15	15	15	15
Vitamin mix*	10	10	10	10	10	10	10
Mineral mix**	10	10	10	10	10	10	10
Binder	10	10	10	10	10	10	10
Choline chloride	1	1	1	1	1	1	1
Vitamin C	1	1	1	1	1	1	1
Microaglae	0	5	10	15	5	10	15
Proximate composition (g kg^{-1})							
Moisture	91.7 ± 0.6	90.3 ± 1.1	88.9 ± 2.1	89.4 ± 3.4	90.7 ± 0.4	92.1 ± 1.1	90.9 ± 0.11
Crude protein	370.4 ± 4.5	364.2 ± 2.5	373.6 ± 2.6	369.1 ± 2.2	369.2 ± 3.3	365.1 ± 4.9	362.7 ± 3.7
Ether extract	54.6 ± 0.8	53.8 ± 0.6	53.7 ± 0.4	53.1 ± 0.04	53.7 ± 0.8	54.2 ± 0.5	54.0 ± 0.2
Crude fibre	22.3 ± 0.5	21.7 ± 0.4	26.8 ± 0.9	25.0 ± 0.04	26.8 ± 0.09	21.9 ± 0.3	21.8 ± 0.4
Total ash	85.7 ± 3.2	76.9 ± 5.9	83.9 ± 3.9	87.6 ± 4.4	76.9 ± 3.2	90.0 ± 8.5	81.8 ± 2.8
NFE	375.2 ± 4.3	393.3 ± 6.1	373.1 ± 6.6	375.8 ± 5.5	382.7 ± 7.3	376.7 ± 6.9	388.7 ± 0.9

* and ** compositions are as per Ambasankar et al. (2022)

of *Tetraselmis* (0.5%, 1%, and 1.5%) and *T. weissflogii* (0.5%, 1%, and 1.5%). The pH of the microalgae concentrates was brought to the neutral pH with 0.1N HCl before including in the feed. All the dry solid ingredients were ground separately into a fine powder, passed through a 100-µm sieve, and further homogenised. The dough was steam-cooked and cooled. Then vitamin and mineral mixtures were added to this dough and were homogenised again for uniformity.

Experimental animals

Post larvae (PL 18) of *P. vannamei* were stocked at 100 PL/100 L tank (mean weight: 19.72 ± 1.62 mg). The *Penaeus vannamei* post-larvae were procured from a private hatchery (Approved hatchery by Coastal Aquaculture Authority, Chennai, India) from Tamil Nadu, India. The SPF *P. vannamei* post-larvae (PL 18) were screened again for all OIE-listed pathogens in Aquatic Animal Health Laboratory, ICAR-CIBA, Chennai, and healthy PLs were used for the experiment. The animals were kept in a flow through system during the entire period of the experiment. The water temperature during the experiment was 28.55 ± 1.97 °C, which was measured at 11:00 h on every alternate day. The feeding trial was conducted for 42 days. Animals were fed with seven iso-nitrogenous and iso-energetic experimental diets prepared with different levels of microalgae concentrates as additives: *Tetraselmis* (0.5%, 1%, and 1.5%) and *T. weissflogii* (0.5%, 1%, and 1.5%).

Growth performances of *P. vannamei* post larvae

All the animals were individually weighed at the beginning and the end of the experiment. Average body weight (ABW), total biomass, percentage weight gain (WG %), specific growth rate (SGR), average daily gain (ADG), and survival were estimated using the formulae mentioned by Steffens (1989).

$$\text{Percentage of weight gain (\%)} = \{(\text{Mean final weight} - \text{Mean initial weight})/\ \text{Mean initial weight}\} \times 100$$

$$\text{Average daily gain (mg day} - 1) = (\text{Mean final weight} - \text{Mean initial weight})/\text{days}$$

$$\text{Specific growth rate (\%day} - 1) = ([\ln \text{final weight} - \ln \text{initial weight}]/\text{days}) \times 100$$

$$\text{Survival rate (\%)} = (\text{Final number of shrimp}/\text{Initial number of shrimp}) \times 100$$

Haematology

In order to estimate the total haemoglobin count (THC), granular haemoglobin (GH), and nongranular haemoglobin (NGH) counts, haemolymph was obtained from the animals at the end of a 42-day feeding trial. This was performed through the ventral sinus of the first abdominal segment. The proportions of GH, which had both large granular haemocytes (LGHs) and small granular haemocytes (SGHs), and NGH, which had both large

nongranular haemocytes (LNGHs) and small nongranular haemocytes (SNGHs), were estimated in accordance with prior studies (Ananda Raja et al. 2017).

Gut microbiology

According to the process prescribed by Kumar et al. (2017), gut samples were collected and used for microbial investigation. Six animals from each treatment (chosen at random, two from each replication) were taken at the end of the feeding trial, anaesthetized with clove oil (50 L L^{-1}), and then transferred to ice. The aseptically removed gut was weighed and homogenised in a normal saline solution (NSS; 0.89%). Then samples were serially diluted (tenfold) in NSS, and 100 µl of appropriate dilutions was plated on nutrient agar having 1.5% w/v NaCl and 2% agar concentration for total plate count and thiosulfate citrate bile salt sucrose agar for total *Vibrio* count. The gut samples were also processed on starch agar (De et al. 2015) and peptone gelatin agar (Bairagi et al. 2002) for amylolytic and proteolytic bacterial counts, respectively. These culture plates were incubated at 30 °C for 48 h. Colonies in the range of 30 to 300 counted and expressed as colony forming unit (CFU/g for gut).

Statistical analysis

Statistical analyses were performed using R statistical software (version 4.0.4). Data were expressed as mean ± SD. The independent sample *t* test was performed to check the significance ($p < 0.05$) of difference in mean growth characteristics, haematological parameters, and gut microbial counts using R 'stats' package. One-way analysis of variance, followed by Tukey's HSD post hoc tests, was performed to test the significance ($p < 0.05$) of the differences in mean of various parameters separately. All the plots were created using 'ggplot2' package (Wickham 2016) of R statistical software (ver. 4.0.5).

Results

Flocculation of microalgae

Flocculation trials on *T. weissflogii* and *Tetraselmis* sp. revealed that the highest flocculation efficiency (89% and 82%, respectively) was shown by 10 mM and 20 mM NaOH after 30 min (Figs. 1 and 2). Further, no significant difference was found in the flocculation efficiency between 10 and 20 mM NaOH from 30 to 180 min. However, the flocculation efficiency was increased in both concentrations along with time. The results revealed that 10 mM NaOH could help in quick flocculation of *T. weissflogii* and *Tetraselmis* sp. Moreover, the flocculation efficiency of NaOH is significantly higher than that of KOH at 10 mM and 20 mM at different time intervals. The results also showed that NH_4OH did not induce flocculation in all the tested concentrations in both the microalgae.

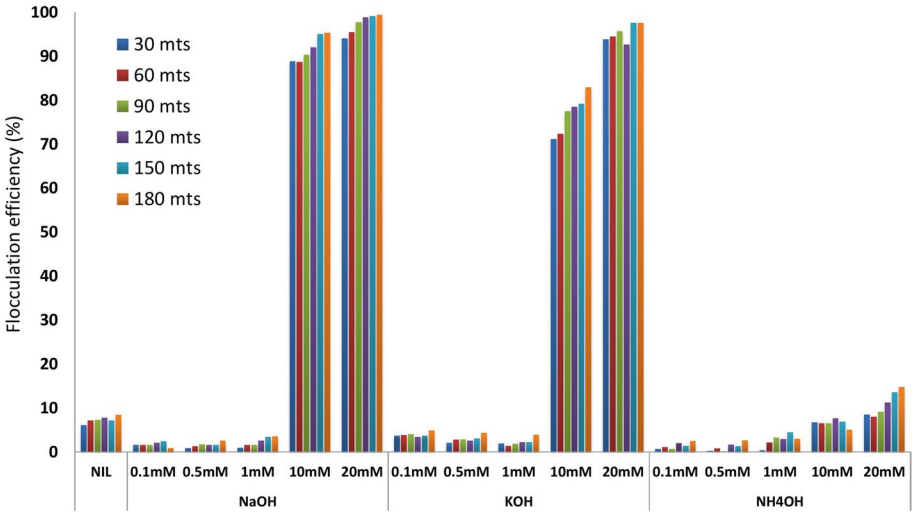

Fig. 1 Flocculation efficiencies of different alkalis on *Thalassiosira weissflogii*

Nutritional composition of microalgae

The proximate composition of *T. weissflogii* and *Tetraselmis* sp. concentrates showed that the microalgae biomass is rich in protein (43.07% and 42.11%, respectively) and the values were not significant between them (Fig. 3). However, *T. weisfloii* has significantly higher lipid content than *Tetraselmis* sp. (20.11% vs 10.56%; $p < 0.05$). No significant difference was observed for other proximate parameters between the two microalgae concentrates. Fatty acid profile analysis indicates that *Tetraselmis* sp. had more than

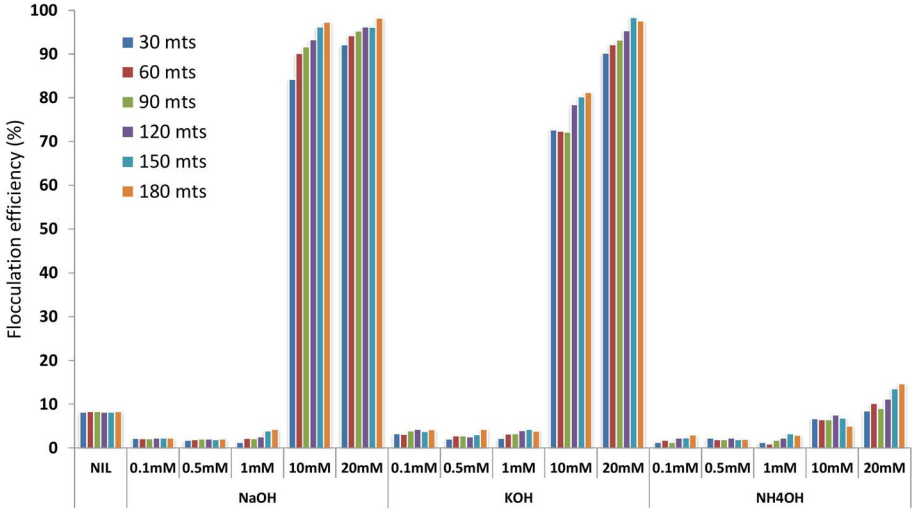

Fig. 2 Flocculation efficiencies of different alkalis on *Tetraselmis* sp

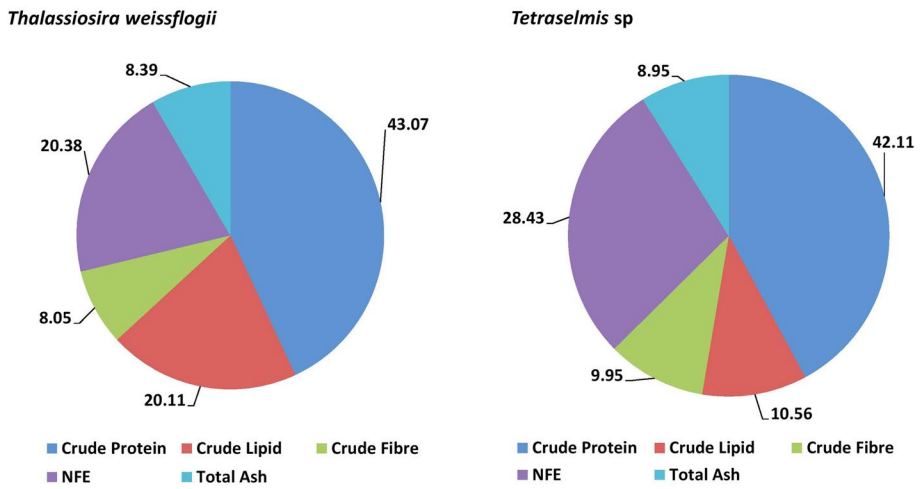

Fig. 3 Proximate composition of selected microalgae biomass (as % dry matter basis)

double the concentration of polyunsaturated fatty acids (PUFA) (Table 2) compared to *T. weissflogii* (56.32% vs 25.24%; $p < 0.05$). On the contrary, saturated fatty acid was significantly higher in *T. weisssflogii* (34.56%) than in *Tetraselmis* sp. (25.73%). The higher percentage of PUFA in *Tetraselmis* sp. is mainly attributed to the presence of linoleic and alpha-linolenic acids. In *T. weissflogii*, the PUFA percentage is mainly contributed by EPA presence.

Antioxidant properties of microalgae

The TPC of *T. weissflogii* was significantly higher than that of *Tetraselmis* sp. (45.83 ± 1.17 mg GAE g^{-1} vs. 36.18 ± 2.91 mg GAE g^{-1}; $p < 0.05$) (Fig. 4). However, no significant difference was found in the total flavonoid content (TFC) of *T. weissflogii* (6.56 ± 0.62 mg RE g^{-1}) and *Tetraselmis* sp. (7.26 ± 0.51 mg RE g^{-1}). In the case of total antioxidant activity, the values of *T. weissflogii* (4.9 ± 0.09 mg RE g^{-1}) are significantly higher than that of *Tetraselmis* sp. (3.39 ± 0.99 mg RE g^{-1}).

Anti-Vibrio properties of microalgae extracts

Methanolic extracts of *T. weissflogii* and *Tetraselmis* sp. showed antimicrobial activity against three pathogenic bacteria: *V. harveyi*, *V. parahaemolyticus*, and *V. campbellii* on the disc-diffusion assay (Table 3). The *Tetraselmis* extract showed a significantly ($p < 0.05$) higher level of antimicrobial activity against *V. parahaemolyticus* (16.94 ± 1.05 mm) compared to that of *T. weissflogii* against the same pathogen (13.59 ± 0.3). The *Tetraselmis* extract showed better antimicrobial activity against

Table 2 Fatty acid profiles of selected microalgae (as the percentage of total fatty acids)

Fatty acids	*Thalassiosira weissflogii*	*Tetraselmis* sp.
14:0	7.62	1.08
15:0	3.9	1.42
16:0	14.8	15.15
17:0	1.36	0.6
18:0	3.5	1.46
20:0	1.95	2.15
22:00	0.57	3.31
24:00	0.86	0.56
∑SFA	34.56	25.73
14:1n7	1.55	0.22
16:1n7	22.5	2.24
17:01	2.52	0.56
18:1n9	12.39	3.36
20:1n11	–	1.21
24:1n	0.48	–
∑MUFA	39.44	7.59
18:2n6	0.66	15.67
18:3n6	1.53	2.58
18:3n3	0.82	15.24
20:2n6	0.75	11.9
20:3n6	0.53	0.3
20:4n6	0.15	0.3
20:5n3	17.77	8.39
22:5.n3	0.78	0.39
22:6n3	2.25	1.55
∑PUFA	25.24	56.32
n3	21.62	25.57
n6	3.62	30.75
n3/n6	5.97	0.832

14:0 Myristic acid; 15:0 Pentadecylic acid; 16:0 Palmitic acid; 17:0 Heptadecanoic acid; 18:0 Octadecanoic acid; 20:0 Arachidic acid; 22:00 Behenic acid; 24:00 Lignoceric acid; SFA: Saturated Fatty Acids; 14:1n7 Tetradecenoic acid; 16:1n7 Hexadecenoic acid; 17:01 Heptadecenoic acid; 18:1n9 Oleic acid; 20:1n11 Eecosenoic acid; 24:1n9 Nervonic acid; MUFA: Mono Unsaturated Fatty Acids; 18:2n6 Linoleic acid; 18:3n6 gamma-Linolenic acid; 18:3n3 alpha-Linolenic acid; 20:2n6 Eicosadienoic acid; 20:3n6 dihomo-gamma-Linolenic acid; 20:4n6 Arachidonic acid; 20:5n3 Eicosapentaenoic acid; 22:5.n3 Docosapentaenoic acid; 22:6n3 Dococsahexaenoic acid; PUFA: Poly Unsaturated Fatty Acid; n3: Omega 3 fatty acids; n6: Omega 6 fatty acids

V. harveyi also (16.58 ± 0.59 mm) as compared to that of *T. weissflogii* extract against same pathogen (15.66 ± 0.62 mm); however, the difference was nonsignificant (Table 3). Interestingly, *T. weissflogii* extract showed higher antimicrobial activity against *V. campbellii* (14.47 ± 1.19 mm) than *Tetraselmis* extract (13.4 ± 0.48 mm).

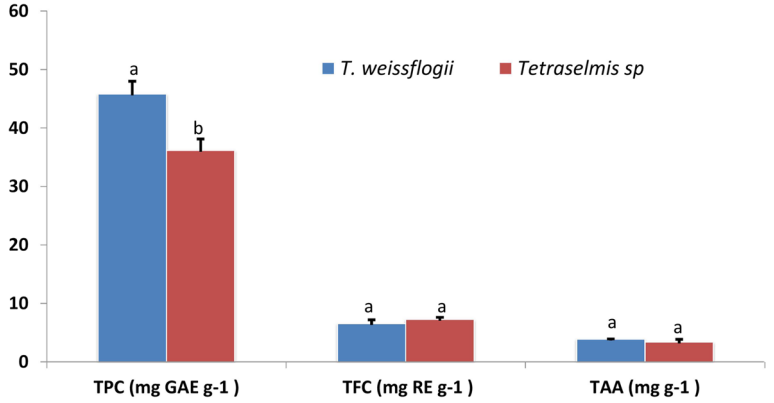

Fig. 4 Antioxidant properties of *T. weissflogii* and *Tetraselmis* sp. Data are shown as mean ± SD ($n = 3$); mean values with different alphabets differ significantly ($p < 0.05$); TPC, total phenolic content; TFC, total flavonoid content; TAA, total antioxidant activity

Growth of P. vannamei fed with microalgae incorporated diet

Post larvae (PL 18) fed with microalgae incorporated diet $THA_{1.0}$ showed significantly higher survival rates compared to other treatments (Fig. 5(d)). Results revealed significantly higher ($p < 0.05$) ABW in treatments fed with $TET_{0.5}$, $TET_{1.0}$, $TET_{1.5}$, $THA_{1.0}$, and $THA_{1.5}$ compared to control (Fig. 5(a)). The highest ABW was noticed in animals fed with $TET_{1.0}$ (0.96 ± 0.02 g), followed by $TET_{1.5}$ (0.89 ± 0.03 g), which was significantly higher than the rest. Significantly higher ($p < 0.05$) WG was observed in $TET_{1.0}$ (0.94 ± 0.02 g) compared to all other treatments, including the control (Fig. 5(b)). The WG % also followed the pattern of WG showing significantly higher value in $TET_{1.0}$ (4919.87 ± 473.36), than the rest of the treatments (Fig. 5(c)).

Significantly higher ($p < 0.05$) ADG was observed in $TET_{1.0}$ (22.48 ± 0.55 mg day^{-1}) compared to that of all other treatments, including control followed by $TET_{1.5}$ (20.82 ± 0.59 mg day^{-1}), $THA_{1.0}$ (19.69 ± 0.59 mg day^{-1}), and $THA_{1.5}$ (19.42 ± 0.44 mg day^{-1}). Lower ADG was observed in $THA_{0.5}$ and control (Fig. 5(e)). SGR of $TET_{1.0}$ was significantly higher ($p < 0.05$) than that of other treatments (9.32 ± 0.23). The lowest SGR was noticed in $THA_{0.5}$ (8.39 ± 0.17); however, the difference was non-significant with that of the control (Fig. 5(f)).

Table 3 Zone of inhibition shown by microalgae extracts against pathogenic bacteria in millimeter

Treatments	*Vibrio harveyi*	*Vibrio campbellii*	*Vibrio parahaemolyticus*
	Diameter of inhibition zone (mm)		
Positive control (streptomycin)	22.64 ± 1.75^{a}	20.16 ± 1.26^{a}	23.66 ± 1.3^{a}
Negative control (solvent)	NAD	NAD	NAD
Thalassiosira weissflogii	15.66 ± 0.62^{bc}	14.47 ± 1.19^{b}	13.59 ± 0.3^{c}
Tetraselmis sp	16.58 ± 0.59^{b}	13.4 ± 0.48^{b}	16.94 ± 1.05^{b}

Data are shown as mean ± SD ($n = 3$); mean values with different alphabets in different column differ significantly ($p < 0.05$); *NAD* no activity detected.

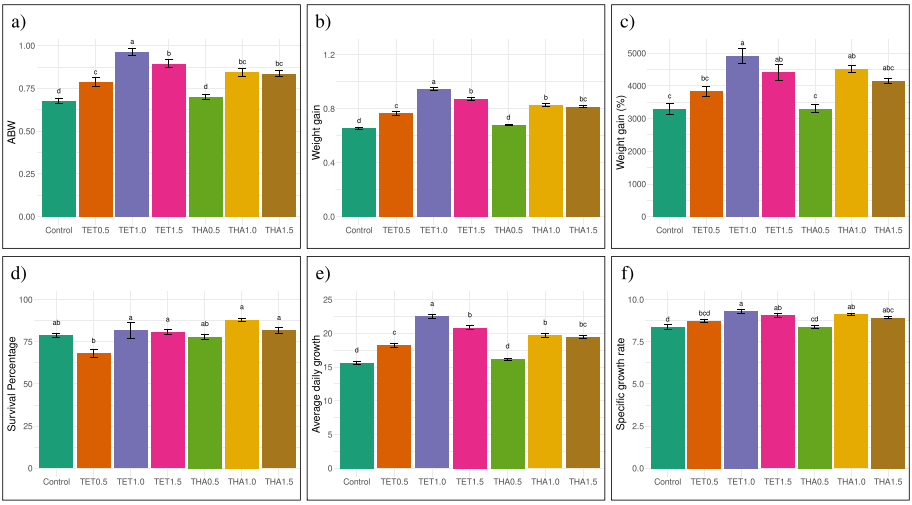

Fig. 5 Growth parameters of *P. vannamei* fed with microalgae incorporated diet (a: average body weight; b: weight gain; c: weight gain (%); d: survival (%); e: average daily gain; f: specific growth rate). Data are shown as mean ± SD ($n = 3$); mean values with different alphabets differ significantly ($p < 0.05$); ABW, average body weight; WG, weight gain; ADG, average daily gain; SGR, specific growth rate

Haematological parameters

The THC was significantly higher ($p < 0.05$) in shrimps fed with $THA_{1.0}$ ($7.40 \pm 0.54 \times 10^7$) than in the rest of the treatments (Supplementary Fig. 1a). THCs of animals in $TET_{0.5}$, $TET_{1.0}$, $THA_{1.0}$, and $THA_{1.5}$ were significantly ($p < 0.05$) higher than those of the control ($4.30 \pm 0.10 \times 10^7$). The SNGHs were observed in animals fed with $TET_{1.0}$ followed by animals fed with $THA_{1.0}$ (Supplementary Fig. 1b). Higher levels of LNGHs were observed in animals fed with $TET_{1.0}$ and $THA_{1.0}$ (Supplementary Fig. 1c); however, there was no significant difference among the treatments. The SGH showed a significant increase ($p < 0.05$) in shrimps fed with $TET_{1.0}$ and $THA_{1.0}$ compared to other treatments (Supplementary Fig. 1d). The highest SGH was observed in animals fed with $THA_{1.0}$ ($4.30 \pm 0.57 \times 10^7$). There was a significant increase ($p < 0.05$) in LGHs in all treatments compared to the control (Supplementary Fig. 1e). Higher LGH was observed in $THA_{1.0}$ ($0.96 \pm 0.33 \times 10^7$) and $THA_{1.5}$ ($0.96 \pm 0.84 \times 10^7$). The lowest LGH value was observed in the control group.

There was no significant difference among control, $TET_{0.5}$, $TET_{1.0}$, and $THA_{1.5}$ in the percentage occurrence of SNGH (Supplementary Fig. 1f). The lowest percentage occurrence of SNGH was observed in $THA_{1.0}$ followed by $TET_{1.5}$. The highest percentage occurrence of LNGH was observed in $TET_{1.0}$ (Supplementary Fig. 1g). However, the differences were not significant between the treatments. The percentage occurrence of SGH was significantly lower in $THA_{1.5}$ compared to that of all other treatments (Supplementary Fig. 1h). The % of LGH was significantly higher ($p < 0.05$) in shrimps fed with $TET_{1.5}$ and $THA_{1.0}$ compared to other treatments (Supplementary Fig. 1i). The lowest percentage occurrence of LGH was observed in control.

Gut microbiology

An increase in the total microbial count was observed in treatments with microalgal supplementation compared to control except in $THA_{1.5}$ (Supplementary Fig. 2a). The highest level of microbial counts were observed in $THA_{1.0}$ which was significantly higher ($p < 0.05$) compared to control, $TET_{0.5}$, $TET_{1.0}$, and $THA_{1.5}$. The lowest gut bacterial count was observed in $THA_{1.5}$, which was non-significant that of control. The increasing inclusion levels of *Tetraselmis* and *T. weissfloggi* (THA) were found to decrease the level of *Vibrios* in the gut. Significant reduction ($p < 0.05$) in gut *Vibrio* count was noticed in animals fed with $TET_{1.0}$, $TET_{1.5}$, $THA_{1.0}$, and $THA_{1.5}$ as compared to control, $TET_{0.5}$, and $THA_{0.5}$ (Supplementary Fig. 2b). The lowest *Vibrio* count was observed in $THA_{1.5}$, followed by $TET_{1.5}$ and $TET_{1.0}$. There was no significant difference between the gut *Vibrio* counts of control, $TET_{0.5}$, and $THA_{0.5}$. In the case of the proteolytic bacterial count, there was a significant increase ($p < 0.05$) in $TET_{1.0}$, $TET_{1.5}$, and $THA_{1.0}$ compared to the control (Supplementary Fig. 2c). The lowest gut proteolytic count was noticed in control. However, there was no significant difference among the treatments that received microalgal supplementation. The amylolytic counts showed a reduction in $TET_{0.5}$, $TET_{1.0}$, and $TET_{1.5}$ compared to the control; however, there was no significant difference between the treatments (Supplementary Fig. 2d). Compared to the control, a significant reduction ($p < 0.05$) of gut amylolytic count was noticed in $THA_{0.5}$ and $THA_{1.0}$. The lowest gut amylolytic count was observed in $THA_{0.5}$, whereas the highest gut amylolytic count was observed in control.

Discussion

Microalgal concentrates have been proven to be a viable alternative for live microalgae in hatcheries to reduce the risk associated in maintaining mass culture like the sudden crash culture. However, due to its small size, harvesting microalgae is still a challenge in aquaculture production systems (Vandamme et al. 2010). In the present study, chemical flocculation using 10 mM NaOH was successfully used in concentrating cultures of *T. weissflogii* and *Tetraselmis* sp. Earlier researchers also reported the efficiency of strong alkali to flocculate microalgae by altering pH (Vandamme et al. 2012; Li et al. 2020).

The nutritional composition of microalgae largely depends on many factors like growth stage, culture conditions (nutrients, temperature, light, etc.), strain difference, and species. As far as aquaculture is concerned, the nutritional quality of microalgae biomass is an essential factor. The present study estimated the protein content in *T. weissflogii* as 43.07%. The superior nutritional quality of *T. weissflogii* is already reported (Kiatmetha et al. 2011; Sandeep et al. 2019). The protein content in *Tetraselmis* sp. was (42.11%) similar to the values reported in previous studies (Abiusi et al. 2014) and higher than the values reported by D'Souza and Kelly (2000). The lipid content of *T. weissflogii* (20.11%) was similar to the earlier reports (Sandeep et al. 2019). The levels of PUFA observed in *Tetraselmis* sp. (56.32%) in the present study are higher than the earlier reports (Guzmán et al. 2010). In the present study, fatty acid profiling of *T. weissflogii* revealed higher EPA content (17.77% of total fatty acids) which was in consonant with the observations recorded in the earlier studies on different species of *Thalassiosira* sp. (Thompson and Harrison 1992; Sandeep et al. 2019). In the present study, the EPA content of *Tetraselmis* sp. was 8.39% of total FA, which is higher than the values reported by some of the earlier studies in *Tetraselmis suecica* (D'Souza and Kelly 2000; Abiusi et al. 2014). The clear understanding

of nutritional composition of these microalgae helps in their inclusion in shrimp feed as additives.

Microalgae provide various health benefits due to their high levels of polyunsaturated fatty acids, phytosterols, sulfated polysaccharides, pigments, and phenolic compounds (Ghasemi et al. 2011). Earlier studies have described their polyphenolic and antioxidant components (Hajimahmoodi et al. 2010). The antioxidant properties are essential to understand the potential of a microalgae species to be used as a nutraceutical in aquaculture. Chemical properties like total phenolic, total flavonoid, and total antioxidant activity are essential to screen the potential microalgae for application in aquaculture as a nutraceutical. The methanolic microalgae extracts were used in the present study to screen the properties as per the earlier reports (Widowati et al. 2017). The present study recorded a higher total phenolic content from the methanolic extract of *T. weissflogii* (45.83 ± 1.17 mg GAE g^{-1}), which was higher than the earlier report (Bhattacharjya et al. 2020). However, there are reports on high total phenolic content in microalgae, *Scenedesmus rubescens*, up to 48.57 ± 3.99 mg GAE g^{-1} (Morowvat and Ghasemi 2016). The total phenolic content of *Tetraselmis* sp. in the present study was 36.18 ± 1.9 mg GAE g^{-1}, which is higher than the earlier report (Widowati et al. 2017). The total flavonoid content of the methanolic extract of *Tetraselmis* sp. in the present study was 7.26 ± 0.05 mg RE g^{-1} which is higher than the values reported earlier (Haoujar et al. 2019). The total flavonoid content of the methanolic extract of *T. weissflogii* in the present study was 6.56 ± 0.62 mg RE g^{-1}, which is higher than the values reported by Bhattacharjya et al. (2020). The reasons for higher phenolic and flavonoid may be indicated.

Many microalgae have antibacterial properties besides their rich nutritional composition. Microalgae produce a wide range of bioactive secondary metabolites, either stored in the cell or excreted in the surrounding environment after the exponential and stationary growth phase ends (Bhuvaneswari et al. 2013; Tavakoli et al. 2021). Research is being done to uncover natural molecules to help in limiting the misuse of commercial antibiotics because extended use may lead to the development of resistant bacterial strains coupled with antibiotic residues in the final produce. The extract of *Thalassiosira* sp. showed inhibition zones of 12 mm, 13 mm, and 17 mm for *E. coli*, *S. aureus*, and *B. subtilis*, respectively (Bhattacharjya et al. 2020). Similarly, in the present study, the methanolic extract of *T. weissflogii* showed a zone of inhibition against *V. harveyi* (15.66 ± 0.18 mm), *V. campbellii* (14.47 ± 0.78 mm), and *V. parahaemolyticus* (14.59 ± 0.29 mm). Moreover, the study by Jusidin et al. (2022) revealed that the hydrophilic chemicals in *T. weissflogii* extract have antibiotic activity against the highly virulent *V. harveyi*. The antibacterial properties of *Tetraselmis* sp. were studied against different pathogenic bacteria (Guzmán et al. 2019; Widowati et al. 2021). In the present study, the zone of inhibition shown by methanolic extract of *Tetraselmis* sp. was 13.58 ± 0.26 mm against *V. harveyi*, 12.40 ± 0.67 mm against *V. campbellii*, and 12.94 ± 0.73 mm against *V. parahaemolyticus*. Due to the presence of phenolic and flavonoid contents, bioactive compounds in microalgae are effective scavengers of intracellular free radicals. Earlier studies showed potent antibacterial activity of methanolic extract of *Chlorella vulgaris*, whereas the extracts were rich in phenolic and flavonoid contents (Pradhan et al. 2021). The present study clearly showed the better antibacterial properties of *Tetraselmis* sp. against *V. parahaemolyticus* and *V. harveyi* compared to *T. weissflogii*. The results suggest the benefits of the incorporation of *Tetraselmis* sp. and *T. weissflogii* in *P. vannamei* nursery diet.

The present study demonstrated that animals fed with the concentrate of 1% *Tetraselmis* sp. attained the highest body weight (Table 3). Furthermore, all the growth characteristics were significantly higher in the treatment fed with *Tetraselmis* sp. incorporated diet. Similarly, Sharawy et al. (2020) showed that including *Tetraselmis suecica* (7.5 g kg^{-1}) improved the growth and nutritional quality of *P. vannamei*. This may be due to the various properties

attributed to *Tetraselmis* sp., such as high protein, lipid, PUFA, antioxidant properties, and anti-vibrio properties (Widowati et al. 2017; Guzmán et al. 2019). The addition of *Thalassiosira weissflogii* biomass or concentrate to the diet of *P. vannamei* has not been documented in any published literature. The most important hurdle in utilising *Thalassiosira* biomass is the constraints in harvesting, and this was addressed in the present study by concentrating the microalgae biomass by flocculation. The results of the present study revealed the potential of dietary microalgae concentrates in growth of the *P. vannamei* post larvae and would open the avenues for next-generation nursery diets with these algae as functional ingredients for growth.

The present study showed that haematological parameters such as THC, SNGH, and LNGH were significantly higher in shrimps fed with microalgal concentrates of *Thalassiosira* and *Tetraselmis* compared to the control. In crustaceans, particularly in shrimps, the haemocytes perform many vital functions related to the growth and well-being of the animals, and largely the levels are attributed to the physiological condition of the shrimps (Song and Hsieh 1994; Cheng and Chen 2001). Hence, in the present study, the supplementation of microalgae concentrates in the diet might have enhanced the health condition of shrimps due to the presence of bioactive compounds present and the nutritional quality of the microalgae. Many earlier researchers reported an increase in haematological characteristics. Raji et al. (2018) showed an increase in haematological parameters (WBC and RBC) in African catfish (*Clarias gariepinus*) fed with a diet supplemented with *Arthrospira platensis* and *Chlorella vulgaris*. Prabhu et al. (2018) also reported an increase in total haemocyte count in *Penaeus vannamei* fed with *Syzygium cumini* leaf powder. The haematological parameters indicated the beneficial effects on important haematological parameters and further supported the growth findings observed in this study.

The dietary application of microalgae in shrimps has shown positive effects on growth, gut microbiology, and disease resistance (Wang et al. 2017; Liu et al. 2022). Sharawy et al. (2020) showed that the total heterotrophic bacterial count was significantly enhanced in *P. vannamei* fed with feed containing dried *Tetraselmis suecica*. Gut microbiology affects aquatic organisms' health by influencing digestion, nutrient assimilation, immunity, biological antagonism, and anti-aging (Li et al. 2018). The present study showed a significant increase in total gut bacterial count in all treatments fed with microalgae supplemented diet except $THA_{1.5}$ compared to the control. Moreover, the present study showed a significant reduction in total *Vibrio* count in the animals fed with 1% and 1.5% microalgae (both *Tetraselmis* and *Thalassiosira*) compared to other treatments. Previous studies documented the anti-*Vibrio* properties of these microalgae (Regunathan and Wesley 2004; Quin et al. 2013; Dash et al. 2017; Widowati et al. 2021). The result on antibacterial assays of microalgae extracts against *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* in the present study supports the findings of gut microbiology. Hence, the dietary application of *Tetraselmis* and *Thalassiosira* might have helped to reduce the *Vibrio* load in the gut of *P. vannamei* in the present study. The results of this study clearly elucidated the antimicrobial property of these microalgae and this would be helpful to the commercial hatchery operators for maintaining the post larval and nursery stages using these potential antimicrobial agents present in these microalgae.

Conclusion

The results of the present study give the baseline information about the benefits of including microalgae concentrates in shrimp nursery feed in terms of growth and health. The study also revealed the various properties of *T. weissflogii* and *Tetraselmis* sp. to use as

feed additives in the penaeid shrimp diet. As the nursery phase of *P. vannamei* is getting more important in the present farming regime, a nursery feed with nutraceutical properties would ensure a healthier and better crop for the farmer. The feed which can provide nutrition and health benefits in the early stages of shrimp can reduce the occurrence of disease in the initial months of the culture which will ensure an enhanced productivity and would pave the way for sustainability in the shrimp aquaculture. Moreover, the results of the study can be an opening for the new avenues of functional feeds in the early life stages of shrimp larval feeds containing the potential microalgae concentrates.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10499-023-01114-7.

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Author contribution Sandeep KP, Chadha NK, and Ambasankar K designed the study. Sandeep KP, Sivaramakrishnan T, and Sudhin S conducted the experiments and collected the data. Sandeep KP, Sudheer NS, Raymond JAJ, Syama Dayal J, and Balasubramanian CP analysed and interpreted the data, and Ambasankar K supervised the study. Aananda Raja R analysed the haematological parameters Sujeet K analysed the gut microbiology. Sandeep KP wrote the manuscript. Balasubramanian CP, Sivaramakrishnan T, Paramita BS, and Ambasankar K revised the manuscript. All authors edited and approved the final manuscript for submission to the journal.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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