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EFFECTS OF DIETARY ALGAE MEAL SUPPLEMENTATION ON GROWTH, WHOLE-BODY COMPOSITION, HISTOLOGICAL AND IMMUNE RESPONSES OF PACIFIC WHITE SHRIMP, *PENAEUS VANNAMEI*

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Abstract

Algae meal is an emerging shrimp feed additive due to its essential nutrient composition, such as fatty acids, amino acids, vitamins, and minerals. A 60-day feeding trial examined the effects of algae meal as an additive on growth, whole-body composition, and histological and immune responses of Pacific white shrimp, *Penaeus vannamei*. Six iso-protein and iso-lipid treatment diets were formulated and prepared using Best Mix feed formulation software and pelletizer, respectively. The diet without supplementation of algae meal was used as a control. Two treatment diets included algae meal at 0.5% (T1-AM) and 1% (T2-AM). After pelletization, another three treatment diets were vacuum coated with algae meal at 0.5% (T3-AM), 1% (T4-AM), and a combination of 0.5% algae meal and 0.5% probiotic (T5-AMP). A total of 900 juvenile shrimp, with an initial average weight of 3.12±0.07 g, were evenly distributed into 18 rectangular cages in three indoor cement tanks (5.0 m × 3.0 m × 1.2 m). Each diet was randomly allotted to triplicate groups of 50 shrimps (average weight of 3.12 ± 0.07 g) per treatment and fed thrice daily (9:00, 13:00, and 17:00). The results showed that significantly higher water stability of feed, water absorption (%), and dry matter retention were observed in the T5-AMP diet ($P<0.05$). Similarly, higher weight gain, protein efficiency ratio, specific growth rate, and better feed conversion ratio were observed in the shrimp-fed T5-AMP diet ($P<0.05$). The whole-body proximate and amino acid composition of shrimp were not affected by the inclusion and coating of algae meal and probiotic diets ($P>0.05$). Moreover, no significant abnormalities were found in the histology analysis of the hepatopancreas and intestine of shrimp-fed experimental diets. The relative mRNA expression of immune-related genes was significantly upregulated in shrimp fed a T5-AMP diet ($P<0.05$). In conclusion, this study indicated that 0.5% algae meal blended with probiotics through vacuum coating could improve the feed quality, nutrient utilization, growth performance, and immune responses of *P. vannamei*. A blend of algae meal and probiotics could help to develop nutritious immune-boosting shrimp feed and ensure sustainable shrimp farming practices.

Key words: ***Arthrospira platensis*, feed additive, feed utilization, lysozyme, probiotics, vacuum coating**

Shrimp have emerged as the leading and fastest-growing species in the crustacean aquaculture industry due to their high protein and mineral content. Over the last three decades, commercial shrimp farming has experienced consistent global expansion, with an 86% increase in global production, amounting to over 6.5 million tonnes and approximately 40 billion US dollars in 2019 (Amaya et al., 2007; Eissa et al., 2022). In 2022, world shrimp production reached 9.4 million tonnes (FAO, 2024). The Pacific white shrimp, *Penaeus vannamei*, holds the most significant economic importance among shrimp species, dominating the shellfish aquaculture market and accounting for 52% of the total production, with a global supply of 6.3 million tonnes (FAO, 2023). Compared with other shrimp species, *P. vannamei* accounts for primary production at 83.1% (Li et al., 2018; Lukwambe et al., 2019). This rapid expansion has underscored the need to develop effective management strategies to ensure sustainable shrimp production, focusing on novel and effective additives for shrimp feed to stimulate different phases of shrimp feeding behavior, such as detection, searching, and ingestion (Andersen et al., 2005; Sharawy et al., 2020).

Feed intake is crucial in farmed shrimp's growth and feed conversion efficiency. Fish and shrimp can adapt their feeding behavior and intake based on their dietary needs and nutritional properties, demonstrating "nutritional wisdom". However, slow feeding behavior in penaeid shrimp can lead to nutrient loss, particularly amino acids, when exposed to water for extended periods. Therefore, their water stability stresses shrimp

feed quality more; adding feed additives can enhance feed water stability, palatability, attractability, and cost efficiency (Sharawy et al., 2022). Commercial shrimp farming has tested numerous feed additives to achieve chemically attractive diets with specific cues that quickly trigger shrimp feed intake (Sanchez et al., 2007). These additives include primary metabolites (e.g., amino acids, fatty acids, phospholipids, cholesterol), chemical compounds (e.g., dimethyl sulfide, trimethylamine oxide), terrestrial components (such as yeast extract, feather meal, blood meal, soybean poultry by-product), and marine components (such as fish, squid, and krill meal) (Suresh and Nates, 2011; Bardera et al., 2020). Still, more research is required to determine the appropriate feed additives, their optimal inclusion levels, and their most effective application in feed production to enhance feed quality, feed intake, and their utilization to achieve growth and immune-promoting effect in farmed shrimp.

Algae biomass contains essential biochemical metabolites, including lipids, proteins, carbohydrates, and pigments (Apandi et al., 2019). In their natural habitat, microalgae serve as a primary food source for fish, making them suitable as either ingredients or additives for aquaculture feed production. They offer essential nutrients such as polyunsaturated fatty acids (PUFA), amino acids, vitamins, minerals, and active components (Carneiro et al., 2020). Furthermore, microalgae are a potential source of food and energy due to their efficient photosynthesis and high nutritional value (Ju et al., 2012). In shrimp experimentation, many types of microalgae have been found to increase growth (protein accretion), feed utilization, physiological activity, stress response, starvation tolerance, disease resistance, and carcass quality (Ashour et al., 2019; Mansour et al., 2021). Ju et al. (2012) reported that pure algae meal could partially replace fish meal in the diet of *P. vannamei*.

Arthrospira platensis, commonly known as spirulina, is a filamentous cyanobacterium with biomass rich in proteins (up to 67%), crude lipids (7–8%), unsaturated fatty acids, antioxidant pigments (such as carotenoids), vitamins (particularly vitamin B_{12} and pro-vitamin A, or β-carotene), and essential minerals like iron. It also contains compounds that enhance the attractability and palatability of fish and shrimp feed. These components play vital biological roles and are highly valued in various industrial applications (Elabd et al., 2020; Zaki et al., 2021). It has proven to be an excellent substitute for fish meal in Siberian sturgeon, *Acipenser baerii* (Palmegiano et al., 2005). However, substantial studies have studied the effect of algae meal as a functional feed additive. Dietary supplementation of *A. platensis* in free-lipid biomass form enhanced growth performance and immune responses in *P. vannamei* (Ashour et al., 2024). Additionally, using *A. platensis* nanoparticles as a feed additive improved growth and feed utilization in both *P. vannamei* (Sharawy et al., 2022) and Nile tilapia, *Oreochromis niloticus* (Mabrouk et al., 2022). The above beneficial effects of algae made them ideal for cultivation due to their gaining interest among fish farmers. To date, it has garnered elevating attention in shrimp nutrition. However, there are challenges associated with algae production, including the risk of contamination by other species, pathogens, and grazers (Benemann, 2013), the high production cost, and the need to identify suitable algae species. As earlier said, shrimp culture with *P. vannamei* in the tropic region is one of the most significant and well-invested aquaculture activities; any improvements to *P. vannamei* feed will positively impact this industry. In line with this strategy, the present study evaluates the effectiveness of supplementing algae meal on feed quality, feed utilization, growth performances, and immunological responses of *P. vannamei*.

Material and methods

Experimental shrimp larval rearing

Five thousand 10-day post larvae (PL_{10}) of *P. vannamei* were procured from Star Aqua hatchery (Koovathur, Tamil Nadu, India). Upon arrival, in the post-larval nursery unit of the Directorate of Incubation and Vocational Training in Aquaculture, Chennai, India, the PL was acclimatized in brackish water with 25 ppt salinity. During the acclimatization period, shrimp PL were initially fed with commercial feed (Royal Dragon DT311, Shenglong Biotech International Co., Ltd.) that contained 35% crude protein, 5% crude fat, 4% fiber, and 12% moisture. During the nursery rearing period, proper aeration was given around the clock. PL were fed an 8% feeding rate thrice daily (9:00, 13.00 and 17.00) (Felix et al., 2023). After nursery rearing, 45-day-old shrimp juveniles with an average initial size of 3 gram $shrimp^{-1}$ were utilized for this feeding experiment.

Experimental design and feed assembling

The algae meal (*A. platensis*), commercial probiotic, and binding gel were acquired from M/s. Algatomic Pvt Ltd., Namakkal, India, and M/s. Raalis India Ltd, respectively. Six iso-protein (360 g kg^{-1}) and iso-lipid (60 g kg^{-1}) treatment diets were formulated and prepared. The diet without supplementation of algae meal was used as a control. Two treatment diets were included with algae meal at 0.5% (T1-AM) and 1% (T2-AM). After pelletization, three treatment diets were vacuum coated with algae meal at 0.5% (T3-AM), 1% (T4-AM), and a combination of 0.5% algae meal and 0.5% probiotic (T5-AMP) (Table 1). The experimental feeds were prepared in an aqua feed mill at the Directorate of Incubation and Vocational Training in Aquaculture (DIVA), TNJFU, Chennai, Tamil Nadu, India.

Table 1. Composition and proximate analysis of experimental diets fed to *P. vannamei*

Ingredient (%)	Control	T1-AM	T2-AM	T3-AM	T4-AM	T5-AMP
Tuna fish meal	20	20	20	20	20	20
Hipro soy[1]	25	25	25	25	25	25
Corn gluten meal	4	4	4	4	4	4
Shrimp (*Acetes*) meal	5	5	5	5	5	5
Fish oil	2	2	2	2	2	2
Soy lecithin	2	2	2	2	2	2
Wheat flour	22	22	22	22	22	22
Broken rice	11.6	11.1	10.6	11.1	10.6	10.6
Phyco gel	1	1	1	1	1	1
Vitamin premix[2]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[3]	0.5	0.5	0.5	0.5	0.5	0.5
Dicalcium phosphate	1	1	1	1	1	1
Dl-methionine[4]	0.2	0.2	0.2	0.2	0.2	0.2
L-lysine[5]	0.2	0.2	0.2	0.2	0.2	0.2
Algae meal[6]	–	0.5	1	0.5	1	0.5
Probiotics[7]	–	–	–	–	–	0.5
Analyzed nutrient composition (% dry matter basis)						
Moisture (%)	8.97	9.59	9.63	9.48	9.65	9.23
Crude protein (%)	36.89	37.06	37.53	36.82	37.33	37.55
Crude fat (%)	6.38	6.48	6.29	6.19	6.35	6.30
Crude fiber (%)	4.24	4.54	4.27	4.54	4.29	4.71
Ash (%)	13.13	12.98	13.85	13.22	13.53	13.47
Gross energy (MJ g^{-1})	1.66	1.69	1.67	1.64	1.65	1.70

[1]Sakthi soya, Marchinaickenpalayam, Tamil Nadu, India.
[2]Vitamin premix: vit. A – 10,000,000 IU, vit. B_1 – 5,000 mg, vit. B_2 – 5000 mg, vit. B_3 – 6000 mg, vit. B_5 – 6,000 mg, vit. B_6 – 6,000 mg, vit. C – 60,000 mg, vit. D_3 – 2,000,000 IU, vit. E – 10,000 EU, vit. H – 200 mg.
[3]Mineral premix: magnesium – 2800 mg, iodine – 7.4 mg, iron – 7400 mg, copper – 1200 mg, manganese – 11600 mg, zinc – 9800 mg, chlorides cobalt – 4 mg, potassium – 100 mg, selenium – 4 mg, calcium carbonate – 27.25, aluminum – 1500 mg and choline chloride – 10000 mg.
[4]Evonik AG, Germany.
[5]Ajinomoto Heartland, Inc., Iowa, Chicago.
[6]Crude protein – 67%, crude fat – 7–8%.
[7]Light brown colored powder, sparingly soluble in water, moisture – 4.35%, *Bacillus subtilis* – 200 billion cfu/g.

In the feed preparation process, all dry feed ingredients were initially crushed into powder form using a Mega Hammer mill pulverizer (Prater, Bolingbrook) and screened individually through an 80 µm mesh to obtain a sufficient particle size. The powdered ingredients were weighed according to the formulation and transferred into the vertical mixer for mixing. Then, the mixture was blended with the gradual inclusion of fish oil, soybean oil, soy lecithin, and an appropriate water level. Later, the homogenized ingredients were screw pressed through a pelletizer (Unique Engineering, Chennai) with a 1.2 mm die and pellet strand cut length of 3–4 mm to produce uniform-size sinking pellets. The pellets were coated with algae meal and probiotics using a vacuum coater (Forberg International AS, Norway). After coating, the coated pellets were air dried, then packed in an airtight container and stored at –20°C until use for the feeding trial. The proximate composition of experimental diets is shown in Table 1.

Determination of feed water stability

The stability of the prepared experimental feeds was assessed by measuring the dry matter retention (DMR) and water absorption % (Argüello-Guevara and Molina-Poveda 2013). For DMR, after immersing 3 g pellets from each diet in a shaking water bath for 2 hours, the pellets were then placed in labelled and tared polyvinyl chloride (PVC) tubes with 1 mm mesh bottom, shaken at 30 rpm at 28°C and 25 ppt salinity, drained for 20 minutes, and dried in an oven at 60°C until a constant weight was achieved. Water absorption % was measured by gravimetric difference. The formulae used were as follows:

$$\textit{Dry matter retention (DMR,\%)} = 100 - \frac{DW_{bi} - DW_{ai}}{DW_{bi}} \times 100$$

$$\textit{Water absorption \%} = \frac{\textit{Weight of wet pellets (g)} - \textit{Weight of dry pellets (g)}}{\textit{Weight of dry pellets (g)}} \times 100$$

DW_{bi}, dry weight of pellet before immersion; DW_{ai}, dry weight of pellet after immersion.

Experimental trial and feeding management

Prior to the start of the experiment, a total of 900 shrimps of similar size were graded from the nursery tank. After that, shrimp (with an initial average weight of 3.12±0.07 g) were distributed evenly into 18 cages (1 m × 1 m × 1.5 m) with a rearing density of 50 shrimp per cage. Six experimental diets were randomly assigned to each triplicate cage (n=3). Based on a completely randomized design, cages were allocated in three indoor cement tanks (4.0 m × 4.0 m × 1.5 m). Shrimp were fed three times per day at 9:00, 13:00, and 17:00 with a ratio of 6–8% of total live body weight, and the experimental trial lasted for 60 days. At intervals of two weeks, the total weight of the shrimp was measured in bulk, and 50% of the water was refreshed until the end of the experiment. Water quality indicators were maintained within the following ranges suitable for average shrimp growth: temperature = 30.0±1.0°C, pH = 8.5±0.5, salinity 25.0±1.0 ppt, hardness = 310±50 ppm, alkalinity = 120±10 ppm, and dissolved oxygen = 5.5±0.3 ppm. The water quality parameters were analyzed following the APHA protocols (APHA, 2012).

Sample collection

At the end of the feeding experiment, the shrimp were deprived of feed for 24 hours and euthanized with 100 mg/L of eugenol before sampling. All shrimp in each cage were counted and weighed to assess the basal growth indices. Fifteen shrimp per cage (45 shrimp for each treatment) were randomly selected for whole-body proximate and amino acid composition analysis. The gills of experimental shrimp were collected, and after being frozen in liquid nitrogen, samples were immediately put into 2 ml sterilized Eppendorf tubes containing RNA later and kept at –80°C for the analysis of immune-related gene expression. The intestine and hepatopancreas of the experimental shrimp were sampled separately and fixed in a 4% paraformaldehyde solution for histological analysis.

Nutrient utilization and basal growth indices

The indices of interest were calculated using the following formulas:

$$\text{Weight gain}\left(WG, \frac{g}{\text{shrimp}}\right) = W_f - W_i$$

$$\text{Specific growth rate (SGR,\%/d)} = 100 \times \frac{[Ln\ W_f - Ln\ W_i]}{d}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

$$\text{Feed efficiency ratio (FER)} = \frac{1}{FCR}$$ (Felix et al., 2023)

$$\text{Survival rate (SR,\%)} = 100 \times \frac{N_f}{N_i}$$

W_i, initial mean body weight (g); W_f, final mean body weight (g); N_i, initial number of shrimps; N_f, final number of shrimps; d, number of days of this experiment.

Sample analysis

Determination of nutrient composition

Before analysis, dried shrimp's whole-body carcass and feed samples were ground to powdered form separately. The moisture, crude protein, and crude fat contents of shrimp's whole-body and experimental feed samples were determined according to the method of the Association of Official Analytical Chemists (Cunniff, 1995). The moisture content was determined by drying a given weight of samples in a hot air oven at 105°C for six hours to constant weight. The crude protein (N × 6.25) and crude fat contents were determined by following Kjeldahl (Kelplus-Distyl Em Ba, Pelican equipment, Chennai, Tamil Nadu, India) and Soxhlet (Socsplus – SCS 04 AS DLSTS, Pelican equipment, Chennai, Tamil Nadu, India) extraction method, respectively. Ash content was determined based on weight difference after incineration in a muffle furnace at 550°C for six hours. Gross energy was determined using the IKA C 3000 oxygen bomb calorimeter (India Pvt Ltd, Bengaluru).

Determination of amino acid composition

The amino acid composition of whole-body carcass samples was determined by the method of Ishida et al. (1981). An ultra-pressure liquid chromatography (UPLC; Model-Waters ACQUITY-UPLC, Waters) was used for the identification and quantification of amino acids in the samples at the State referral laboratory, TNJFU – Institute of Fisheries Post Graduate Studies, Chennai, India. Powdered samples were dried in a hot air oven at 105°C for 12 hours to remove the moisture content. A dry sample of 0.2 g was weighed and hydrolyzed with 10 mL of 6N HCL in a 25 ml hydrolysis tube. The tube was placed under high-purity N_2 and then put at constant temperature (110°C) for 24 hours for digestion and to determine the overall amino acids present. The hydrolysate of the sample was homogenized with 5 ml of 0.1% formic acid and 20% methanol. After centrifugation at 10,000 g for 15 min, the solution was filtered through a 0.2 µm ultrafiltration membrane. Subsequently, 20 µl of sample solution was injected into UPLC-MS/MS system for analysis.

Preparation of histological sections

The fixed hepatopancreas and intestine samples were dehydrated in a series of gradient ethanol concentrations and embedded in paraffin. Then, it was placed on a slicer and sectioned into slices with a thickness of 7 µm. The section samples were placed flat on a glass slide and then stained with hematoxylin/eosin for examination. The sections were washed with phosphate-buffered saline (PBS) until they turned blue, dried, and then enclosed with glycerin gelatin for microscopy purposes.

Table 2. Primers used for relative mRNA expression of immune-related genes in *P. vannamei* fed different experimental feeds (Felix et al., 2023)

Sl. No	Gene name	GenBank number	Primer sequence (5′- 3′)
1.	Lysozyme	AY170126	Forward: CGACCTCGATCAGTACATGG Reverse: GTAACCCTGGTGACAAGCCT
2.	Toll-like receptor (TLR)	DQ923424	Forward: TGGTGCTTTCGTCAAACTTC Reverse: AACCTGGCCATACACAATGA
3.	Immune deficiency (IMD)	FJ592176	Forward: ATCGAGGAACGAGACAAGGT Reverse: CGTACACTCGGTCGACATTC
4.	β-actin	AF300705	Forward: CGCGACCTCACAGACTACCT Reverse: CTCGTAGGACTTCTCCAGCG

Relative mRNA expression of immune-related genes

RNA stabilized gill tissue samples were analyzed to quantify the relative expressions of immune-related genes, such as lysozyme, Toll-like receptor, and immune deficiency (IMD). These gene primers are shown in Table 2. According to the RNA extraction protocol, the samples' total RNA was extracted using RNA iso-Plus (Takara Bio Inc., Otsu, Shiga, Japan). The quantity and purity of the RNA sample were determined by a Nanodrop spectrophotometer (Thermo Scientific, USA) based on the ratio of absorbance at 260 and 280 nm (ranging from 1.9 to 2.1) and electrophoresed on a 1% agarose gel, respectively. Then, two microliters of total RNA from the sample were reverse transcribed into first-strand complementary DNA (cDNA) using a first-strand cDNA synthesis kit (Thermo Scientific, Vilnius, Lithuania) following the manufacturer's protocols. Real-time quantitative PCR (qRT-PCR) was performed using a SYBR@Premix ExTaq™ Kit (Takara, Japan) in the CFX96™ real-time system (Bio-Rad, Hercules, CA). The amplification was carried out in a 25 µL total volume, including 12.5 µL SYBR Green Premix ExTaq, one µL forward primer, one µL reverse primer, two µL cDNA, and 8.5 µL DEPC-H_2O. The PCR program consisted of denaturation at 95°C for 30 s, followed by 40 cycles of PCR amplification at 95°C for 5 s and 60°C for 30 s. This was succeeded by a melting curve analysis at 95°C for 10 s, 60°C for 60 s, 95°C for 1 s, and finally cooling to 4°C. The β-actin housekeeping gene was amplified as an internal reference, and the experiment was repeated three times. The Auto CT method was used to determine the gene threshold, and the $2^{-\Delta\Delta CT}$ method was employed to estimate the relative gene expression. Housekeeping genes (HKGs) are essential for normalizing RT-qPCR data, and their selection should consider their stability and expression levels. Using housekeeping genes as internal references is crucial for accurate and reliable gene expression analysis in real-time PCR experiments (Livak and Schmittgen, 2001).

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) to determine significant differences between the means of each variable. All the data were analyzed using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA). After the one-way analysis of variance (ANOVA), Tukey's multiple comparison test was used to determine whether the differences among groups were significant ($P<0.05$). The values were expressed as mean values ± standard deviation (SD) of all three replicates (n=3) per treatment.

Results

Water stability of feed

Among the experimental feeds, the T5-AMP resulted in significantly higher water absorption % and DMR (Table 3, $P<0.05$) than other treatment diets. The control feed had a significantly lower water absorption % and DMR than other treatment diets ($P<0.05$).

Nutrient utilization and basal growth indices

Among the experimental feeds, shrimp fed T5-AMP diets resulted in significantly higher weight gain (Table 4, $P<0.05$) compared to other groups, including the control diet. This feed also led to the significantly highest average daily growth and the best FCR and FER ($P<0.05$). It also showed a significantly higher PER and SGR than other groups, including the control diet. The control diet showed lower weight gain, FCR, FER, PER, and SGR performance than other experimental diets ($P<0.05$). The survival of shrimps fed the experimental feed did not show a significant difference and ranged from 97% to 98% ($P>0.05$).

Whole-body proximate and amino acid composition

The whole-body proximate composition and amino acid profile of the shrimp fed experimental diets are given in Table 5. Different experimental diets did not significantly affect shrimp's whole-body protein, lipid, and ash contents. Similarly, the treatment diets did not significantly influence the experimental shrimps' whole-body essential and non-essential amino acid profile.

Table 3. Water stability of the prepared experimental feeds

Parameters	Control	T1-AM	T2-AM	T3-AM	T4-AM	T5-AMP	P value
DMR	93.25±1.22 B	94.27±0.47 B	93.61±0.82 B	97.13±0.84 A	96.58±0.74 A	97.47±0.56 A	<0.001
Water absorption %	165.79±0.90 C	171.11±1.50 B	171.45±2.42 B	177.84±1.24 A	177.92±0.95 A	181.16±1.88 A	<0.001

DMR, dry matter retention.

Values are presented as mean ± standard deviation of triplicate samples. Values within the same row denoted by distinct letters indicate significant differences (P<0.05).

Table 4. Nutrient utilization and basal growth indices of *P. vannamei* fed different experimental feeds

Parameters	Control	T1-AM	T2-AM	T3-AM	T4-AM	T5-AMP	P value
IBW[1] (g)	3.13±0.04	3.16±0.02	3.11±0.02	3.10±0.07	3.11±0.03	3.14±0.02	0.042
FBW[2] (g)	15.09±0.17 B	14.54±0.14 B	16.13±3.19 B	16.64±0.14 B	16.23±0.66 B	19.43±0.50 A	0.012
WG[3] (%)	381.5±9.17 BC	346.3±16.42 C	418.8±100.67 BC	436.9±2.72 B	431.5±17.01 B	529.4±6.04 A	0.004
ADG[4] (g)	0.20±0.003 B	0.19±0.003 B	0.22±0.053 B	0.23±0.003 B	0.22±0.011 B	0.27±0.008 A	0.010
FCR[5]	2.14±0.04 BC	2.26±0.03 C	1.97±0.42 BC	1.84±0.02 B	1.89±0.009 B	1.47±0.04 A	0.003
FER[6]	0.47±0.009 B	0.44±0.008 C	0.52±0.128 B	0.54±0.007 B	0.53±0.026 B	0.68±0.020 A	0.003
Survival (%)	98.00±2.00	97.33±3.05	98.00±2.00	97.33±3.05	98.0±2.00	98.0±2.00	0.997
PER[7]	1.30±0.026 B	1.23±0.021 B	1.46±0.357 B	1.51±0.020 B	1.47±0.073 B	1.88±0.054 A	0.003
SGR[8]	2.62±0.034 BC	2.49±0.061 C	2.72±0.309 BC	2.820±0.40 B	2.78±0.054 B	3.07±0.016 A	0.004

[1]IBW, initial body weight; [2]FBW, final body weight; [3]WG, weight gain; [4]ADG, average daily growth; [5]FCR, feed conversion ratio; [6]FER, feed efficiency ratio; [7]PER, protein efficiency ratio; [8]SGR, specific growth rate.

Values are presented as mean ± standard deviation of triplicate samples. Values within the same row denoted by distinct letters indicate significant differences (P<0.05).

Table 5. Whole-body proximate composition of *P. vannamei* fed different experimental feeds

Nutrients	Control	T1-AM	T2-AM	T3-AM	T4-AM	T5-AMP	P value
Crude protein	68.19±0.33	68.64±0.33	68.95±0.60	68.36±0.57	68.85±0.38	68.32±0.03	0.394
Crude lipid	4.29±0.44	4.36±0.36	4.27±0.29	3.90±0.20	4.161±0.35	4.53±0.17	0.335
Ash	15.03±0.20	14.89±0.32	14.65±0.37	14.86±0.45	14.70±0.49	15.06±0.43	0.678
Amino acid profile of *P. vannamei* fed different experimental feeds							
Arginine	3.57±0.36	3.56±0.32	3.66±0.27	3.61±0.36	3.57±0.36	3.61±0.15	0.52
Histidine	1.41±0.22	1.57±0.11	1.57±0.27	1.37±0.10	1.43±0.205	1.59±0.37	0.56
Isoleucine	2.37±0.14	2.52±0.125	2.44±0.130	2.50±0.13	2.57±0.90	2.48±0.26	0.25
Leucine	4.59±0.55	4.56±0.10	4.54±0.11	4.55±0.13	4.49±0.11	4.40±0.73	0.754
Lysine	3.57±0.144	3.53±0.33	3.86±0.12	3.78±0.19	3.61±0.22	3.79±0.24	0.423
Methionine	2.69±0.23	2.80±0.11	2.79±0.20	2.60±0.22	2.74±0.18	2.75±0.20	0.894
Phenylalanine	2.55±0.22	2.37±0.25	2.76±0.11	2.69±0.14	2.62±0.15	2.59±0.25	0.637
Threonine	2.44±0.18	2.53±0.14	2.67±0.26	2.74±0.198	2.72±0.17	2.45±0.132	0.598
Valine	3.52±0.07	3.63±0.15	3.67±0.17	3.46±0.27	3.63±0.11	3.77±0.10	0.156
Alanine	6.50±0.32	6.79±0.14	6.57±0.241	6.54±0.19	6.67±0.25	6.74±0.60	0.210
Aspartic acid	5.81±0.156	5.47±0.205	5.54±0.146	5.76±0.184	5.46±0.23	5.66±0.17	0.929
Cysteine	0.92±0.35	0.80±0.15	0.78±0.20	0.67±0.21	0.69±0.11	0.67±0.90	0.410
Glutamic acid	8.60±0.18	8.56±0.100	8.65±0.91	8.61±0.64	8.76±0.62	8.64±0.16	0.458
Glycine	9.35±0.13	9.49±0.23	9.50±0.15	9.72±0.12	9.55±0.11	9.53±0.36	0.122
Proline	3.35±0.127	3.66±0.80	3.34±0.21	3.55±0.13	3.62±0.12	3.67±0.26	0.225

Values are presented as mean ± standard deviation of triplicate samples.

Histological analysis

As shown in Figure 1, no changes were observed in the hepatopancreas of shrimp fed different experimental diets. However, shrimp fed a T3-AM diet exhibited a slight increase in B cell activity and mild degeneration of hepatopancreatic tubules (Figure 1 D). Shrimp fed the T5-AMP diet showed mild dilatation of the tubules and multifocal mild degeneration (Figure 1 F). As shown in Figure 2, no significant abnormalities were observed in the intestine of shrimp fed experimental diets.

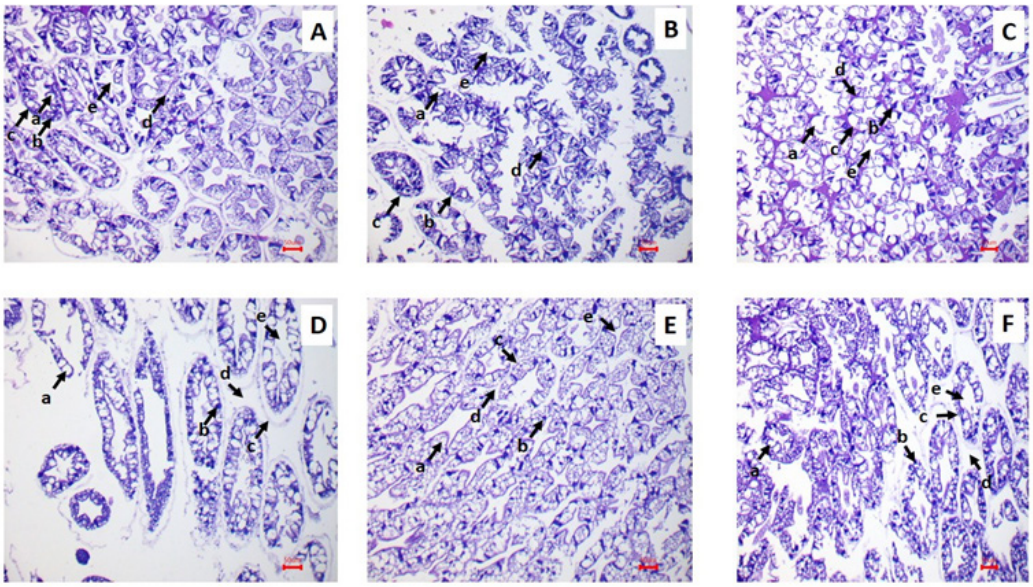


Figure 1. Hepatopancreatic histology (A: Control; B: T1-AM; C: T2-AM; D: T3-AM; E: T4-AM; F: T5-AMP) of *P. vannamei* fed experimental diets (a: B-cells secretory cell, b: E-cells embryonic cell, c: hepatopancreatic tubule, d: basal membrane, e: lumen of hepatopancreatic tubule)

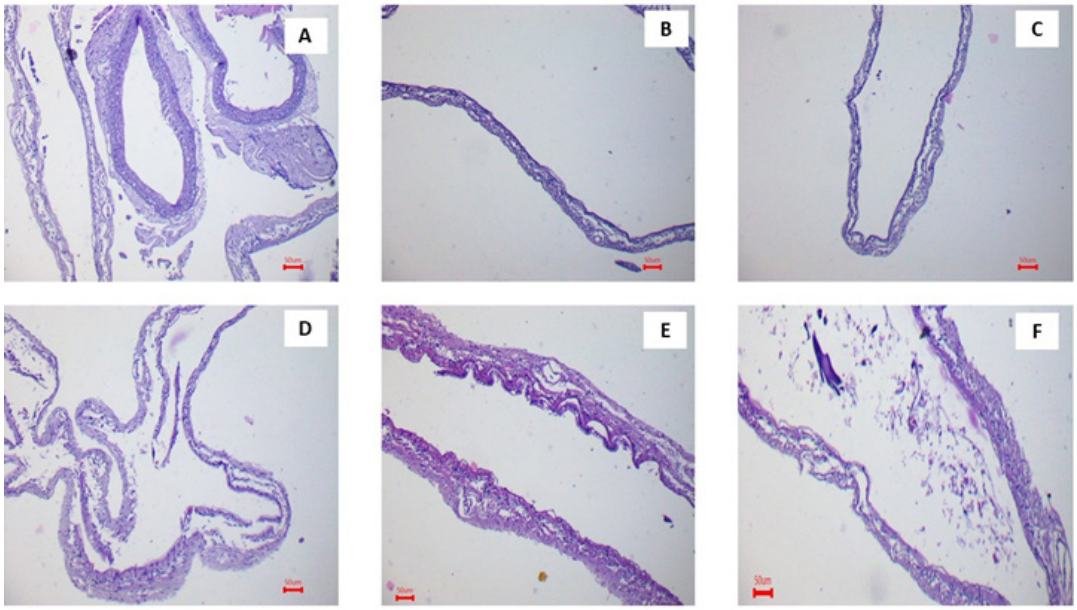


Figure 2. Intestinal histology (A: Control; B: T1-AM; C: T2-AM; D: T3-AM; E: T4-AM; F: T5-AMP) of *P. vannamei* fed experimental diets

Immune-related gene expression

The relative mRNA expression levels of immune-related genes (lysozyme, Toll-like receptor, and IMD) in gill tissues of shrimp fed different experimental diets are shown in Figure 3. The relative expression of lysozyme was significantly upregulated in shrimp fed T5-AMP diet compared to other diets, while downregulated lysozyme expression was observed in control groups. The relative expression of the Toll-like receptor was upregulated in shrimp fed T5-AMP diet, while the relative expression of the Toll-like receptor was downregulated in shrimp fed control diet. Similarly, downregulated relative mRNA expression of IMD was observed in shrimp fed the control diet compared to other treatment diets, and upregulated levels were found in the shrimp fed T5-AMP diet.

Discussion

There is a tremendous use of feed additives in the aquaculture sector to maximize the quality of feed, minimize the use of antibiotics, and enhance the growth and health of cultured animals (El-Kassas et al., 2020; Hassona et al., 2020; Mohamed et al., 2021). Algae meal has

been studied extensively as a functional feed additive and a novel protein source in aquafeed. Over the past few years, many studies have mainly focused on a substitute for fishmeal protein sources (Gong et al., 2019; Rosas et al., 2019; Valente et al., 2019). Algae meal supplementation (up to 1% in feed) has a more beneficial effect on aquatic animals' feed utilization, growth, and immune functions. However, substantial studies have studied the effect of algae meal as a functional feed additive. To date, it has garnered elevating attention in shrimp nutrition. Our study elucidates those different ways of supplementing algae meal as a functional feed additive that significantly influences feed quality, growth performance, and immunological responses of *P. vannamei.*

Regarding feed quality, significant improvements in water absorption % and DMR were observed among the pellets of algae meal vacuum-coated feeds (T3-AM, T4-AM and T5-AMP). According to Cuzon et al. (1994), shrimp feed with good water stability must maintain a minimum of 90% DMR after a 1-hour immersion in water. In the present study, all experimental feeds have better values of water absorption % (95.38±1.89) and DMR (174.21±5.53); comparatively, vacuum-coated feeds have the higher values among them. The previous studies reported the advantages of vacuum coating in preparation of higher fat diets (>40%) for salmonid and trout (Cho and Bureau, 1997; Chaabani et al., 2020). In the present study, even at low levels of 3–6% fat addition, vacuum coating can aid in filling the feed pores with additives – algae meal and probiotic.

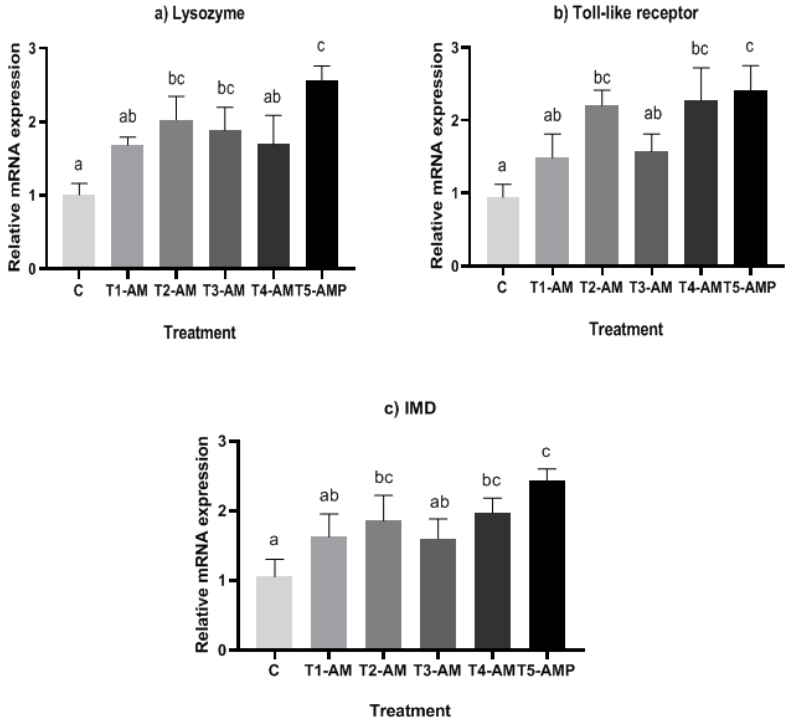
Moreover, it improves the water stability by reducing the chances of water penetrating the shrimp feed (Global Seafood Alliance, 2006). Otherwise, the slow feeding behavior of shrimp can lead to the pellets disintegrating in water before being ingested. In our study, vacuum coating can reduce the leaching of algae meal and other nutrient sources in the pellets into the rearing water. After ingestion, feed must undergo various physiological processes, including digestion, nutrient absorption, and excretion. The digestion and absorption of algae meal are crucial as they determine the nutritional bioavailability of feed. Increasing their digestion and nutritional bioavailability required a pre-treatment process such as ultrasonication, ball mills, bead beating, microwave treatment, and autoclaving to rupture their rigid cellulosic structure (Sarker et al., 2016; Tibbetts et al., 2017). Shrimp do not have an acidic stomach to digest complex forms of algae nutrients like cellulose (Katiyar and Arora, 2020). In this study, the algae meal was pre-treated through pelletization in the treatment diets (T1-AM and T2-AM). The pelletization process can disrupt rigid cell walls, allowing animals to utilize more algae nutrients (Venou et al., 2009; Shi et al., 2016; Maehre et al., 2016). However, the pre-treatment of algae meal is questionable in vacuum-coated feeds (T3-AM, T4-AM and T5-AMP), where algae meal was coated after pelletization. Herein, shrimp fed algal meal and probiotic coated (T5-AMP) diet had a higher growth rate than other treatment diets associated with better feed and nutrient utilization (i.e., FCR, FER, and PER) because of the promoting abilities of gut probiotics. Also, previous studies reported different gut probiotic strains on nutrient utilization in different culture fish species such as *O. niloticus*, rohu (*Labeo rohita*), striped catfish (*Pangasianodon hypophthalmus*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Abd El-Rahman et al., 2009; Nayak and Mukherjee, 2011; Boonanuntanasarn et al., 2019; Adeshina et al., 2020; Hooshyar et al., 2020). Similarly, in *P. vannamei*, the dietary inclusion of the gut probiotic *Lactobacillus pentosus* BD6 significantly improved feed efficiency (Liu et al., 2022). Besides, improved nutrient digestibility and utilization have been proven in *P. vannamei* when a diet includes a mixture of probiotics instead of a single probiotic strain (Wang et al., 2019). On the other side, earlier research on supplementing algae meal into the diet has demonstrated an enhancement in the FCR and growth rate of *P. vannamei* (Sharawy et al., 2020; Wang et al., 2017). It enhances diet utilization by promoting beneficial intestinal flora, stimulating the production of digestive enzymes, and increasing intestinal villi length and absorption area (James et al., 2006; Khalila et al., 2018). Lin et al. (2010) also found that immersing or injecting *P. vannamei* larvae with a hot-water extract of *A. platensis* improved their growth performance. The positive effect on growth is related to its rich nutritional profile and bioactive compounds, including gamma-linolenic acid, polysaccharides, and carotenoids (Mansour et al., 2021). Our study reveals that vacuum-coating of algae meal enhances the stability of feed and nutrient content. At the same time, adding gut probiotic synergistically improves algae meal nutrient digestibility and bioavailability for growth. Similarly, intestinal short-chain fatty acids (SCFAs) play a key role in maintaining intestinal health, closely tied to growth performance (Chen et al., 2020). SCFAs are the main metabolites produced by probiotic bacteria in the gut and are also formed through interactions between gut microbes and A. platensis polysaccharides. These SCFAs are absorbed by intestinal epithelial cells as an energy source, boosting nutrient absorption capacity (Koh et al., 2016; Higashi et al., 2020). Liu et al. (2022) reported that supplementation of *A. platensis* algae blended with the gut probiotic (*L. pentosus*) enhanced the growth performance of *P. vannamei*. However, pessimistic results were obtained from the shrimp's whole-body proximate composition and amino acid profile.

Determination of whole-body proximate composition and amino acid profile is a valuable indicator of shrimp physiology. In feed, nutrient supplementation can either positively or negatively impact the nutrient content of the shrimp's whole body (Xiong et al., 2018; Ayiku et al., 2020). However, in this present study, no significant changes were observed in the shrimp whole-body proximate composition and amino acid profile. These results suggest that the dietary supplementation of algae meal as a functional feed additive did not affect feed intake

and nutrient deposition in the shrimp body. In general, diets containing more than 15% algae meal inclusion significantly impact shrimp whole-body proximate composition and amino acid profile (Walker and Berlinsky, 2011; Gong et al., 2020). Previous studies have shown that algae meal from *A. platensis* can be supplemented to replace fish meal without altering the whole-body proximate composition in some fish and shellfish species, including A. baerii, white sturgeon (*Acipenser transmontanus*), giant freshwater prawn (*Macrobrachium rosenbergii*), and *P. vannamei* (Palmegiano et al., 2005, 2008; Nakagawa and Gomez-Diaz, 1995; Hanel et al., 2007; Pakravan et al., 2017). The nutrient and amino acid composition of algae meal is similar to that of fish meal. Therefore, the present study suggests that although algae meal has a well-balanced amino acid profile, the amount of algae meal included in the diet may not be sufficient to improve nutrient deposition in the body. This suggestion is consistent with previous studies, which also reported that feed intake and nutrient deposition in the body are proportional to the amount of algae meal in the feed (Abdelghany et al., 2020; Gong et al., 2020). Alternatively, this may be consistent with the overall trend of shrimp having a relatively stable composition of protein-bound amino acids, regardless of their diet (Alam et al., 2002; Xie et al., 2016).

Histology of hepatopancreas reveals crustaceans' nutritional and physiological status (Fernández Gimenez et al., 2004). The B cells, blister-like or Blastozellen, are present in the hepatopancreas and responsible for producing digestive enzymes and nutrient accumulation (Raja et al., 2017). Moreover, the tubular epithelium renewal is significantly influenced by E cells or embryonic cells of hepatopancreas (Sonakowska-Czajka et al., 2021; Ambasankar et al., 2022). The status of B, R, and E cells, tubular lumen, and rounded epithelial tubule of hepatopancreas exhibited the health conditions of shrimp (Raja et al., 2017). Likewise, the present findings displayed no significant abnormalities in *P. vannamei* hepatopancreas by supplementing algae meal. These results might be due to the immunomodulatory effects of *A. platensis* algal meal incorporation in diets of *P. vannamei* (Kizhakkekarammal Puthiyedathu et al., 2022).

In shrimp, the lysozyme gene is expressed in various tissues, such as the hepatopancreas, gills, hemocytes, and sometimes in the gut. The expression of this gene leads to the production of lysozyme, which can be secreted into the hemolymph as well as other tissue fluids (Ye et al., 2009). Lysozyme gene plays a crucial role in the immune regulation of shrimp by providing a mechanism to combat bacterial pathogens through direct lysis and by supporting the broader innate immune response (Hikima et al., 2003). Carotenoids, the dominant pigments in *A. platensis* algae, include β-carotene, zeaxanthin, myxoxanthophyll, and chlorophyll a. Other pigments, such as pheophytin-like compounds, siphonein, astaxanthin, and minor carotenoids, are also present in *A. platensis* (Mansour et al., 2021, 2022). In our current study, we observed an upregulation of relative mRNA expression of lysozyme was found in shrimp fed a diet containing algae meal combined with probiotics. We speculate that this may be attributed to the occurrence of astaxanthin, one of the main bioactive components in algae meal.



Values are presented as mean ± standard deviation of triplicate samples. Values within the exact figure denoted by distinct letters indicate significant differences (P<0.05).

Figure 3. Relative expression of immune related genes a) Lysozyme; b) Toll-like receptor; c) IMD in the gill tissues of *P. vannamei* fed experimental feeds

Toll-like receptors (TLRs) are integral to the immune system in many organisms, including vertebrates and invertebrates like shrimp. TLR genes in shrimp play a pivotal role in regulating immunity by recognizing pathogens, activating signaling pathways, and promoting the production of immune response proteins, contributing to the shrimp's ability to defend against infections and maintain health (Habib and Zhang, 2020). Furthermore, the IMD gene in shrimp is crucial for controlling their innate immune system. It primarily works by activating pathways that stimulate the production of antimicrobial peptides and other immune responses. The proper functioning of this gene is vital for protecting shrimp against bacterial infections and other harmful agents, which is why it has become a significant focus of research in shrimp health (Hoffmann and Reichhart, 2002). Supplementing the diet of *P. vannamei* with algae meal from *Chlorella vulgaris* can enhance their immunity (Qingman et al., 2019). This enhancement occurs through the Toll and IMD signal transduction pathways, activated by the chlorella polysaccharide bioactive compound obtained from *Chlorella vulgaris*. In this present study, we found that the mRNA expression of TLR and IMD genes was significantly upregulated and downregulated in shrimp fed T5-AMP and control diet. This change in gene expression was reflected in the growth performances and feed utilization of these groups. Thus, the results suggest that the active components present in the dietary algae meal and probiotic supplementation can activate the immune response and improve the health of the shrimp.

Conclusion

Based on the present study's findings, supplementing 0.5% algae meal and a probiotic-coated diet through vacuum coating could positively impact the feed quality, growth, and immunity of *P. vannamei*. Moreover, notable enhancements were observed in the expression of immune genes, such as lysozyme, TLRs, and IMD. In addition, the active components in algae meal were found to be potent immunostimulants. Therefore, vacuum coating is highly recommended while supplementing functional feed additives in shrimp feed to achieve higher growth performance and nutrient utilization.

Author contribution

Elangovan Prabu: supervision, methodology, visualization, original draft; Nathan Felix: investigation, conceptualization, methodology, validation. Sundaram Sivasankar: funding acquisition, resource; Arangannal Thirumalai: funding acquisition, resource; Arumugam Uma: gene expression and data analysis; Kalidoss Manikandan: histology and data analysis; Govindharaj Sathishkumar: formal analysis, data curation, writing – review and editing; Thangaraju Thiruvasagam: data curation, writing – review and editing.

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Ethics approval

The experiment was carried out under the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Government of India, on the care and use of animals in scientific research. Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam, Tamil Nadu, India, granted ethical approval.

Competing interests

The authors declare no competing interests.

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