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PHYSIOLOGICAL, IMMUNOLOGICAL AND NUTRITIONAL ASSESSMENT OF *PENAEUS VANNAMEI* FED WITH DIFFERENT COMBINATIONS OF NOVEL FEED INGREDIENTS

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Abstract

An eight-week feeding trial was carried out to examine the effects of diets formulated with different combinations of novel feed ingredients as substitutes for fishmeal (FM). The research focused on evaluating their influence on fatty acid and amino acid profiles, immunological and plasma biochemical analysis, immune related gene expression and histological analysis of Pacific white shrimp, *Penaeus vannamei*. Five diets were developed for *Penaeus vannamei,* all of which were isonitrogenous (36%) and isolipidic (6%). Diet 1 (control): primary source of protein was fishmeal (FM); diet 2: replaced FM with a 1:1 ratio of poultry by-product meal (PBM) and single-cell protein (SCP); diet 3: replaced FM with a 1:1:1 ratio of insect meal (IM), rapeseed meal (RM), and SCP; diet 4 replaced FM with fish waste (FW), peanut meal (PM), and SCP in a 1:1:1 ratio; diet 5 replaced FM with PBM, SCP, IM, FW, PM, and RM in equal amounts (1:1:1:1:1:1). Nursery reared juvenile shrimps (1.05± 0.03 g) were stocked in triplicates in the experimental tanks at the rate of 35 shrimp per tank. The plasma biochemical responses and amino acid profile did not differ significantly (P>0.05) between the dietary groups. EPA was significantly higher in diets 1 and 5 which was not different from diet 4 and DHA was significantly higher in diet 1 which was not different from diet 5. Prophenoloxidase activity (proPO) was significantly higher (P<0.05) in diets 1 and 5, and respiratory burst activity was significantly (P<0.05) higher in diets 1 and 5 which was not different from diet 3. The immune gene expression (*LYZ*) was upregulated in diets 1 and 5. More B cells and few R cells were observed in diets 1 and 5 compared to other diets. Thus, the present study concluded that a mixture of PBM, SCP, IM, RM, PM and FW (1:1:1:1:1:1) serves as a good combination diet to totally replace FM in shrimp feed without compromising the fatty acid and amino acid profile and health of Pacific white shrimp (*P. vannamei*).

Key words: amino acid, fatty acid, fishmeal, novel feed, prophenoloxidase activity

The global shrimp farming industry has expanded rapidly in the last few decades, with a focus on farming Pacific white shrimp (*Penaeus vannamei*) due to its fast growth, high yield, and adaptability (Amelia et al., 2021; Karthik et al., 2016; Liao and Chien, 2011). In 2022, global aquaculture production of key species, including penaeid shrimp, was estimated at approximately 7.93 million tonnes, with whiteleg shrimp (*P. vannamei*) being the leading species, accounting for 6.8 million tonnes (FAO, 2024). Major producers include Ecuador, China, Vietnam, India, and Indonesia (Davis et al., 2022; Geetha et al., 2020; Kakoolaki et al., 2020). Fishmeal (FM) is a key protein source in shrimp feed. Fishmeal and fish oil (FO), traditionally used in shrimp feeds for their high-quality protein, essential fatty acids, vitamins, and minerals, also improve diet palatability (Rice, 2009; Khanjani et al., 2023 a). However, growing demand for these ingredients raises concerns about overfishing, sustainability (Tacon and Metian, 2008; Tacon et al., 2022), high costs, potential contamination, and environmental impacts like habitat destruction (Al Eissa et al., 2022; Chen et al., 2021 b). But its limited availability and high cost, challenge the sustainability of shrimp farming. As a result, using alternative proteins to reduce fishmeal reliance has become a major shrimp industry trend (Cai et al., 2022). Several different protein sources have been suggested for shrimp diets in order to meet sustainability requirements. Plant-based proteins, such as advanced vegetable proteins, have demonstrated efficacy in promoting the shrimp growth and health (Manikandan and Felix, 2020; Novriadi et al., 2022, 2023; Shao et al., 2019; Yao et al., 2020). Microbial proteins, like single-cell proteins from yeast, bacteria and biofloc also exhibit potential as long-term substitutes for fishmeal (Eroldoğan et al., 2023; Khanjani et al., 2023 b). And in the case of animal-based proteins, for example, insect-based proteins, such as black soldier fly (BSF) meal, are another option, providing excellent nutrition and sustainability by using organic waste as feedstock (Chen et al., 2021 a; Mousavi et al., 2020; Surendra et al., 2020). Fewer studies have looked into combining several protein sources as fishmeal substitutes (Ye et al., 2011; Yang et al., 2021) than the majority, which has concentrated on replacing a single protein source for fishmeal. The price of fishmeal

has increased due to rising demand, which has led to the quest for alternative protein sources for shrimp diets. The nutritional advantages of novel feed ingredients such as rapeseed meal (RM), peanut meal (PM)), single-cell protein (SCP), insect meal (IM), poultry by-product meal (PBM), and fish waste (FW) have been assessed specifically.

Insect meal is environmentally sustainable and an incredible replacement for fishmeal in aquafeed because it is high in protein (34–74% dry matter) including essential amino acids, vitamins (B_{12}) and minerals (iron, zinc) (Li et al., 2019; Gasco et al., 2020; Quang Tran et al., 2022; van Huis, 2022). Insects are efficient converter of organic waste into biomass rich in protein (van Huis, 2022; de Carvalho et al., 2020). Poultry by-product meal is a viable substitute for fishmeal and an excellent source of protein that is nutritious, easily absorbed, and provides important fatty acids, vitamins, and minerals (Cruz-Suárez et al., 2007; NRC, 2011; Cheng et al., 2002). Peanut meal made after the extraction of oil is a good source of protein (41–450% dry matter) (Neto et al., 2015). Rapeseed meal (RM) is an inexpensive protein source (32 to 45% dry matter), readily available, with balanced amino acid profile (Sallam et al., 2021). In aquaculture feeds, single-cell proteins (SCPs) show enormous potential as a fishmeal alternative (Guo et al., 2019; Jones et al., 2020; Thiviya et al., 2022). Fish waste biomass consists of more than half of processed fish and it contains high proteins, omega-3 fatty acids, bioactive peptides, and enzymes (Mo et al., 2018; Saleh et al., 2022; Khiari, 2022).

Based on the above backdrop, the present study was undertaken with an objective to evaluate the effects of different combinations of these novel feed ingredients on fatty acid and amino acid profiles, immunological and plasma biochemical analysis, immune related gene expression and histological analysis of *Penaeus vannamei*.

Material and methods

Shrimp and experimental conditions

A 32 m³ nursery tank with continuous aeration was used to rear 1,000 post-larvae (PL 12) of *P. vannamei* to the juvenile stage (1 g). The larvae were procured from Star Aqua Hatchery in Koovathur, Tamil Nadu, India. During their acclimation, the shrimp were fed a commercial diet (Royal Dragon DT311, Sheng Long Biotech) four times daily. At the Institute of Fisheries Post Graduate Studies, TNJFU, Vaniyanchavadi, an eight-week feeding trial was conducted in 150 L FRP tanks. Fifteen tanks, including one control and four treatment groups (each in triplicate), were stocked with thirty-five juveniles (1.05±0.03 g). The treatment groups were fed prepared diets to apparent satiation four times a day at 09:00, 10:00, 14:00, and 18:00 hours. Weekly assessments were conducted on shrimp weight, survivability, and health, with feed rations adjusted as needed. Water was exchanged every third day using brackish water (15±1 ppt). Daily water quality monitoring revealed average values of 28±1°C for temperature, 8.0±0.2 for pH, 6.1±0.5 ppm for dissolved oxygen, and 580±16 ppm for salinity.

Diet preparation

The formulation and nutrient composition of the experimental diets are shown in Table 1. Five diets were developed for *Penaeus vannamei,* all of which were isonitrogenous (36%) and isolipidic (6%). Diet 1 (control): primary source of protein was fishmeal (FM); diet 2: replaced FM with a 1:1 ratio of poultry by-product meal (PBM) and single-cell protein (SCP); diet 3: replaced FM with a 1:1:1 ratio of insect meal (IM), rapeseed meal (RM), and SCP; diet 4 replaced FM with fish waste (FW), peanut meal (PM), and SCP in a 1:1:1 ratio; diet 5 replaced FM with PBM, SCP, IM, FW, PM, and RM in equal amounts (1:1:1:1:1:1). A 180-micron mesh screen filtered out any remaining dry ingredient particles before thorough mixing. After adding water, additives, and oil sources (fish oil and soy lecithin), the mixture was blended for 15 minutes to ensure homogenization. It was then cooked for 15 minutes at 80°C, pelletized with a 1.6 mm die, and air-dried at 45°C for 12 hours. The dried pellets were stored in sealed containers at 4°C until use.

Fatty acid analysis

A gas chromatograph equipped with a flame ionization detector (FID) was used to ascertain the content of fatty acids. Following Folch et al. (1957) lipids were isolated. Twenty grams of samples were combined with methanol and chloroform, filtered, then treated with KCl to get rid of impurities. After collecting the lipids, they were evaporated and methylated using BF3 methanol. On a PerkinElmer, PE Clarus 580 gas chromatograph, fatty acids were separated using an Agilent Technologies DB 23 column. The temperatures of the injector and detector were adjusted to 250°C and 300°C, respectively. Nitrogen was employed as the carrier gas in the analysis, and utilizing Turbochrom software, fatty acids were detected and measured by comparing retention periods with real standards (Sigma Chemicals).

Amino acid analysis

Based on Ishida et al. (1981), ultra-pressure liquid chromatography (UPLC, model Waters ACQUITY UPLC, Waters, USA) was used to examine the amino acid profile of ingredients, experimental diets, and the whole body. A 50 mg sample was hydrolyzed in 6 N HCl, filtered, derivatized using the AccQ Tag Ultra kit, and separated using a 2.1 × 100 mm, 1.7 µm AccQ Tag Ultra C18 column on a Waters ACQUITY UPLC. Amino acid standard H (Waters Corporation) was used to calibrate the absorbance at 260 nm with a tunable UV detector, interpret the results with Empower 2, and determine the amino acids.

Table 1. Ingredient and nutrient composition (g/kg) of diets formulated with different combination of novel feed ingredients

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal[1]	200	0	0	0	0
PBM[2]+ SCP[4]	–	211.19	–	–	–
IM[5]+RM[3]+SCP[4]	–	–	274.3	–	–
FW[6]+PM[3]+SCP[4]	–	–	–	316.3	–
PBM[2]+IM[5]+FW[6]+RM[3]+PM[3]+SCP[4]	–	–	–	–	288.2
Wheat gluten[9]	50	50	50	50	50
Soybean meal[3]	240	240	240	240	240
Wheat flour[3]	180	180	180	180	180
Corn flour[3]	169	165.1	95.7	60.7	89.8
Acetes meal[6]	50	50	50	50	50
Brewers yeast[7]	30	30	30	30	30
Fish oil[1]	20	20	20	20	20
Palm oil[3]	10	2	9	2	1
Soy lecithin[8]	10	10	10	10	10
Dicalcium phosphate[9]	10	10	10	10	10
Vitamin mix[10]	5	5	5	5	5
Mineral mix[11]	5	5	5	5	5
DL-Methionine[12]	2	2	2	2	2
L-Lysine[13]	2	2	2	2	2
Vitamin C[9]	2	2	2	2	2
Pellet binder[14]	10	10	10	10	10
Chromic oxide[15]	5	5	5	5	5
Nutrient composition (%)					
dry matter	904.1	903.7	899.5	900.8	902.2
crude protein	356.3	359.1	358.4	357.9	358.6
crude lipid	58.5	53.8	58.4	58.6	57.0
crude fiber	16.1	14.0	30.9	20.0	24.4
total ash	102.8	89.4	101.3	115.7	106.9
gross energy (MJ/Kg)	16.35	16.60	16.93	16.28	16.63

PBM, poultry by-product meal; SCP, single-cell protein; IM, insect meal; RM, rapeseed meal; FW, fish waste; PM, peanut meal. (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM, and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SCP).

[1]Bismi Fisheries Pvt. Ltd., Mayiladuthurai, Tamil Nadu, India.

[2]Pragathi broilers Pvt Ltd., Chennai, India.

[3]Mahindra feeds Pvt. Ltd., Namakkal, Tamil Nadu, India.

[4]Algavista Greentech Pvt Ltd., Pudukottai, Tamil Nadu, India.

[5]Zigma Global Environ Solutions Pvt Ltd, Erode, Tamil Nadu, India.

[6]K.S.S. Fish meal and dry fish, Pulicat, Ponneri, Tamil Nadu, India.

[7]Angel Yeast Co., Ltd., China.

[8]Otto chemie Pvt. Ltd., Mumbai, India.

[9]Jain industrial chemicals, Chennai, India.

[10]Anicare Pvt. Ltd., Chennai, Tamil Nadu, India. Composition of vitamin premix (quantity/kg): vit. A – 10,000,000 IU, vit. B_1 – 5000 mg, vit. B_2 – 5000 mg, vit. B_3 – 6000 mg, vit. B_5 – 6000 mg, vit. B_6 – 6000 mg, vit. C – 60,000 mg, vit. D_3 – 2,000,000 IU, vit. E – 10,000 IU, vit. H –200 mg.

[11]Anicare Pvt. Ltd., Chennai, Tamil Nadu, India. Composition of mineral premix (quantity/kg): magnesium – 2800 mg, iodine – 7.4 mg, iron – 7400 mg, copper – 1200 mg, manganese – 11,600 mg, zinc – 9800 mg, chlorides cobalt – 4 mg, potassium – 100 mg, selenium – 4 mg, calcium carbonate – 27.25%, phosphorus – 7.45 mg, sulfur – 0.7 mg, sodium – 6 mg, Calpan – 200 mg, aluminium – 1500 mg, choline chloride –10,000 mg.

[12]Evonik AG (DL-methionine: MetAMINO® – 99%).

[13]Ajinomoto Heartland, Inc., Chicago (L-Lysine HCL – 98.5%).

[14]PEGABIND®, Bentoli Agrinutrition India Pvt. Ltd., Chennai, India. Synergistic combination of modified urea-formaldehyde, catalyzing agents, free-flow anti-caking agents.

[15]Thermo Fisher Scientific India Pvt. Ltd., Maharastra.

Immunological analysis

To analyze the immunological parameters, the hemolymph samples were collected from three shrimps in each tank via the ventral sinus of the cephalothorax using a 2 mL syringe with a 26-gauge needle containing 50 µL of anticoagulant solution (100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 510 mM NaCl and 10 Mm EDTA.Na2: pH = 7.3) (Vargas-Albores et al., 1993).

Prophenoloxidase (proPO) activity

The prophenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-3, 4-dihydroxyphenylalanine (L-DOPA) (Hernández-López et al., 1996), where L-DOPA serves as a substrate and laminarin is used as an activator of proPO. Briefly, the hemolymph was centrifuged at 700 × g for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in 200 µL of cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.26 M magnesium chloride, 0.01 M calcium chloride, pH 7.0) called hemocyte lysate suspension (HLS). 100 µL of HLS was preincubated with 50 µL of laminarin (1 mg mL^{-1} of cacodylate buffer) for 10 min at 20°C before the addition of 50 µL of L-DOPA, followed by the addition of 800 µL of cacodylate buffer after 5 min. The optical density at 490 nm was then measured using a spectrophotometer (Hitachi U-2800). One unit of proPO activity was defined as an increase in OD at 490 nm of 0.001 per min under these conditions.

Respiratory burst activity (RBA)

In the hemolymph, respiratory burst activity was estimated by quantifying the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) produced by following previously described methods (Anderson and Siwicki, 1995). Briefly, 0.2% of NBT solution was added to 100 µL of hemolymph and incubated at room temperature for 30 min, after which 50 µL of the suspension was taken and added to the glass tubes containing 1 mL of N, *N*-Dimethyl formamide (DMF). The suspension was then centrifuged (Sigma, 3 K18, Rotor No. 12154) at 2292 × g for 5 min at 15°C. The supernatant was then separated and the absorbance was measured at 540 nm in an ELISA reader (Multiskan EX, Thermo Fisher Scientific, USA). The reduction in NBT was expressed as the respiratory burst per 10 µL of hemolymph.

Histological analysis

The hepatopancreas tissue which is fixed in Davidson's fixative was dissected and immersed in the fixative for 48 h. They were transferred to 70% ethanol and processed using routine histological techniques for shrimps (Bell and Lightner, 1988). Briefly, the tissues were dehydrated using a series of graded alcohols (70%, 90%, and 100%) for 60 min in each. After this, the tissue was treated twice with xylene for 60 min and embedded in paraffin wax using a tissue embedding system (HistoStar, Thermo Scientific, USA). The tissue Sect. (5 µm) was made using a microtome and further cleared with xylene, stained with hematoxylin, and counterstained with eosin. The stained sections were mounted with DPX mounting solution, dried, and observed under a phase contrast microscope (Nikon Eclipse-Ni, Tokyo, Japan).

Plasma biochemical analysis

For biochemical analysis, a part of the hemolymph sample was immediately centrifuged at 3000 rpm for 20 min and the supernatant (plasma) was collected and stored at –20°C until analysis. The plasma biochemical parameters such as triglycerides (TG), cholesterol (CHO), total protein (TP), alanine aminotransferase (ALT) or serum glutamic pyruvic transaminase (SGPT) and aspartate aminotransferase (AST) or serum glutamic oxaloacetic transaminase (SGOT) were analyzed by the commercial kit (Pathozyme Diagnostics Pvt. Ltd., Maharashtra, India) using a semi-automatic biochemistry analyzer (Cytokine-SK3002B, Cytokine Healthcare Pvt Ltd., Chennai, Tamil Nadu, India).

Quantitative real time PCR (qRT-PCR)

The hepatopancreas (sample) tissue from each group was directly excised with fully sterile dissection tools under cold condition to analyze the gene-expression of lysozyme (*LYZ*) at the end of the experiment. The samples were kept at −80°C until gene-expression analysis. Total RNA was extracted from the shrimp abdominal portion using the TRIzol method (easy-RED, iNtRON Biotechnology) as directed by the manufacturer. The OD ratio at 260/280 nm of RNA purity was determined using a NanoDrop system (BioDrop), and the samples with the highest ratio (A260/A280 1.8) were used for cDNA synthesis (1 ng μL^{-1}) for each reaction. Total RNA was treated with DNase I (NEB, USA) as the template for the synthesis of first-strand cDNA using reverse transcriptase (RT-PCR beads, Enzynomics, Korea), and the reaction was carried out using PCR amplification (Applied Biosystems Veriti 96-Well Thermal Cycler, USA) under the manufacturer's conditions. The following cDNA was used in the Real-Time PCR reaction (Bico, Thermo-Fisher): initial denaturation at 95°C for 15 min, 40 cycles with the following parameters (95°C, 10 s; 62°C, 20 s; and 72°C, 30 s), unique and specific products were seen as a melting curve at the end of the last cycle when the temperature increased from 62 to 95°C in increments of 0.5°C. Table 2 lists the primers used in this gene-expression analysis, and the housekeeping gene (β-actin) was used to measure gene expression or fold shift of the target genes. The values give out in an n-fold difference relative to the calibrator (control) when the $2^{-\Delta\Delta Ct}$ method is applied in the normalized critical threshold (Ct) quantities of target genes with quantities of β-actin (Livak and Schmittgen, 2001).

Table 2. Primer sequences for real-time PCR used for gene-expression analysis

Gene	Primer sequence (5'-3')	Size (bp)
Lysozyme (lzm)	F: 5'-GCAAGAACGTCTGCAAAATCC-3' R:5'-CCAGCACTCTGCCATGTACTG-3'	190
β-actin	F: 5'-GCCCATCTACGAGGGATA-3' R: 5'-GGTGGTCGTGAAGGTGTAA-3'	121

Statistical analysis

The statistical software program SPSS 20.0 (SPSS, Chicago, IL, USA) was used to conduct the statistical analyses. The means ± standard deviation of the mean (SDM) was used to express the data from three replicates. One-way ANOVA was used to compare the means of the five dietary groups, and Duncan's multiple-range test was employed for further analysis. At $P<0.05$, the changes between the treatments were determined to be statistically significant.

Table 3. Whole-body fatty acid profile (% total fatty acid) of *P. vannamei* fed diets formulated with different combination of novel feed ingredients

Fatty acids (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	P value
Myristic acid (C14:0)	0.077±0.003	0.082±0.004	0.082±0.003	0.085±0.003	0.082±0.003	0.156
Palmitic acid (C16:0)	2.51±0.041 ab	2.57±0.060 a	2.53±0.073 a	2.38±0.025 b	2.43±0.045 ab	0.008
Stearic acid (C18:0)	9.01±0.081	8.99±0.098	8.95±0.010	8.85±0.065	8.96±0.065	0.139
Arachidic acid (C20:0)	0.63±0.037 ab	0.55±0.047 ab	0.54±0.056 b	0.66±0.047 a	0.58±0.025 ab	0.03
∑SFA	12.23±0.11	12.20±0.17	12.10±0.10	11.98±0.069	12.06±0.10	0.132
Palmitoleic acid (C16:1)	0.96±0.09 ab	0.92±0.05 ab	1.01±0.04 a	0.83±0.045 b	0.84±0.01 b	0.009
Oleic acid (C18:1 n-9)	28.00±0.12 ab	27.88±0.07 b	28.12±0.03 a	27.95±0.06 ab	27.87±0.10 b	0.031
Gondoic acid (C20:1 n-9)	1.02±0.03	1.09±0.03	1.04±0.06	1.07±0.01	1.03±0.03	0.257
∑MUFA	30.0±0.20 ab	29.9±0.10 ab	30.18±0.05 a	29.86±0.11 ab	29.75±0.13 b	0.022
Linoleic acid (C18:2 n-6)	11.61±0.12 a	11.31±0.06 bc	11.36±0.07 abc	11.09±0.15 c	11.43±0.05 ab	0.001
Arachidonic acid (C20:4 n-6)	1.32±0.03	1.28±0.03	1.32±0.03	1.28±0.03	1.36±0.03	0.077
∑PUFA n-6	13.22±0.05 a	12.83±0.10 bc	12.92±0.05 b	12.62±0.18 c	13.08±0.04 b	<0.001
Linolenic acid (C18:3 n-3)	2.72±0.15 b	3.12±0.08 a	2.95±0.14 ab	3.09±0.13 a	3.08±0.12 a	0.018
Eicosapentaenoic acid (C20:5 n-3)	12.26±0.08 a	11.85±0.07 b	11.92±0.10 b	11.96±0.17 ab	12.26±0.13 a	0.004
Docosahexaenoic acid (C22:6 n-3)	10.94±0.07 a	10.62±0.06 b	10.67±0.09 b	10.67±0.12 b	10.85±0.09 ab	0.009
∑PUFA n-3	25.92±0.07 ab	25.60±0.14 ab	25.55±0.16 b	25.90±0.02 ab	26.02±0.31 a	0.025
∑PUFAs	38.48±0.02 a	38.44±0.25 b	38.48±0.11 b	38.52±0.17 b	39.10±0.28 a	0.002
n-3/n-6 PUFAs	1.96±0.01 b	1.99±0.05 ab	1.97±0.02 b	2.05±0.03 a	1.98±0.02 b	0.004

Data represented as mean ± SD (n = 3) with different letters are significantly different among treatments according to ANOVA test (P<0.05). (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SCP).

Table 4. Whole-body essential amino acid profile (% of wet weight) of *P. vannamei* fed diets formulated with different combination of novel feed ingredients

Amino acids (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	P value
Essential amino acids						
Arginine	3.23±0.16	3.64±0.28	3.39±0.10	3.47±0.27	0.36±0.19	0.315
Histidine	1.16±0.42	1.32±0.15	1.37±0.21	1.34±0.12	1.28±1.75	0.171
Isoleucine	2.15±0.24	2.23±0.12	2.34±0.16	2.41±0.17	2.26±0.14	0.698
leucine	4.29±0.20	4.42±0.03	4.29±0.25	4.28±0.16	4.33±0.12	0.219
Lysine	3.55±0.21	3.56±0.39	3.48±0.36	3.54±0.16	3.59±0.17	0.189
Methionine	2.72±0.11	2.71±0.20	2.52±0.22	2.66±0.18	2.67±0.20	0.809
Phenylalanine	2.29±0.25 b	2.68±0.11 a	2.61±0.14 ab	2.54±0.15 ab	2.51±0.25 ab	0.603
Threonine	2.45±0.14	2.59±0.26	2.66±0.19	2.64±0.17	2.37±0.13	0.532
Valine	3.44±0.07	3.55±0.15	3.59±0.17	3.38±0.27	3.55±0.11	0.160

Values were presented as mean ±SD of three tanks per treatment (n=3). (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SCP).

Table 5. Whole-body non-essential amino acid profile (% of wet weight) of *P. vannamei* fed diets formulated with different combination of novel feed ingredients

Amino acids (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	P value
Non-essential amino acids						
Alanine	6.42±0.32	6.71±0.14	6.49±0.24	6.46±0.19	6.59±0.25	0.520
Aspartic acid	5.73±0.15	5.39±0.20	5.46±0.14	5.68±0.18	5.38±0.23	0.870
Cystine	0.84±0.03	0.72±0.15	0.70±0.20	0.59±0.21	0.61±0.11	0.410
Glutamic acid	8.25±0.18	8.48±0.10	8.57±0.09	8.53±0.06	8.68±0.06	0.460
Glycine	9.27±0.13	9.41±0.23	9.42±0.15	9.47±0.11	9.64±0.12	0.601
Proline	3.27±0.12	3.58±0.08	3.26±0.21	3.47±0.13	3.54±0.12	0.430
Serine	3.27±0.22	3.24±0.07	3.39±0.07	3.43±0.20	3.38±0.29	0.302
Tyrosine	1.57±0.15	1.46±0.04	1.56±0.34	1.70±0.30	1.82±0.28	0.242

Values were presented as mean ±SD of three tanks per treatment (n=3). (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SC

Results

Fatty acid profile

The whole-body fatty acid profile (% total fatty acid) of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients is shown in Table 3. Saturated fatty acids such as myristic acid, palmitic acid, stearic acid, arachidic acid; monounsaturated fatty acids such as palmitoleic acid, oleic acid, gondoic acid; n-6 PUFA such as linoleic acid, arachidonic acid; and n-3 PUFA such as linolenic acid, EPA and DHA were analyzed. Palmitic acid was significantly higher in shrimp fed with diets 2 and 3 which was not different from diets 1 and 5. Arachidic acid was significantly higher in shrimp fed with diet 4 which was not different from diets 1, 2 and 5. Palmitoleic acid was significantly higher in shrimp fed with diet 3 which was not different from diets 1 and 2. Oleic acid was significantly higher in shrimp fed with diet 3 which was not different from diets 1 and 4. Linoleic acid was significantly higher in shrimp fed with diet 1 which was not different from diets 3 and 5. Linolenic acid was significantly lower in shrimp fed with diet 1 which was not different from diet 3. EPA was significantly higher in shrimp fed with diets 1 and 5 which was not different from diet 4 and DHA was significantly higher in diet 1 which was not different from diet 5. Fatty acids such as myristic acid, stearic acid, gondoic acid and arachidonic acid showed no significant difference in shrimp ($P>0.05$) fed diets formulated with different combination of novel feed ingredients.

Amino acid profile

The whole-body essential and non-essential amino acid profile (% of wet weight) of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients is shown in Tables 4 and 5, respectively. The amino acids were classified into two types as essential amino acids such as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine and non-essential amino acids such as alanine, aspartic acid, cystine, glutamic acid, glycine, proline, serine and tyrosine. No significant differences ($P>0.05$) were observed among essential and non-essential amino acids of *Penaeus vannamei* which was fed diets formulated with different combination of novel feed ingredients.

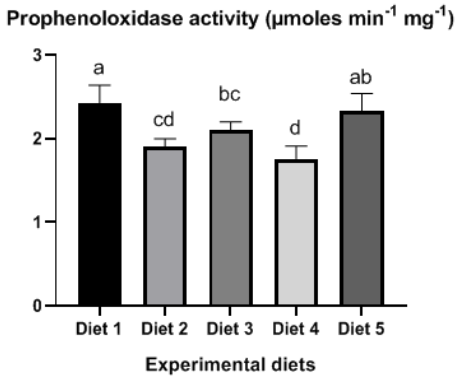

Figure 1. Prophenoloxidase activity in the hemolymph of *P. vannamei* fed diets formulated with different combination of novel feed ingredients. Bars with different letters are significantly different ($P<0.05$)

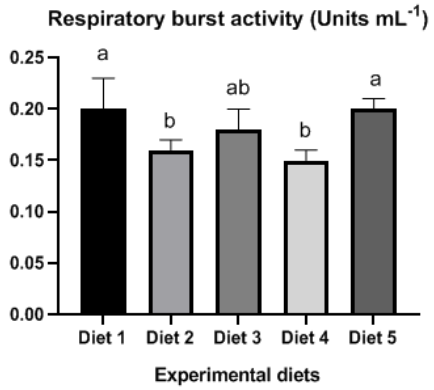

Figure 2. Respiratory burst activity in the hemolymph of *P. vannamei* fed diets formulated with different combination of novel feed ingredients. Bars with different letters are significantly different ($P<0.05$)

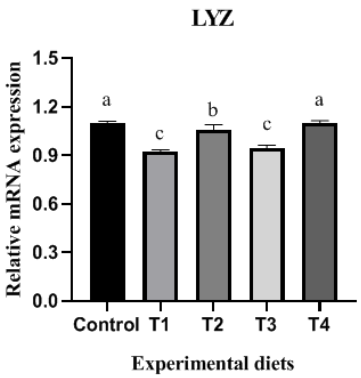

Figure 3. Immune gene expression (*LYZ*) in the hepatopancreas of *P. vannamei* fed diets formulated with different combination of novel feed ingredients. Bars with different letters are significantly different ($P<0.05$)

Table 6. Plasma biochemical responses of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients

Plasma biochemical responses	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	P value
TG (mg/dL)	68.94±3.52	64.59±4.06	64.96±1.13	64.14±2.20	65.39±0.95	0.263
CHO (mg/dL)	54.30±0.76	53.47±0.60	54.63±2.36	53.02±1.87	55.81±0.87	0.242
TP (g/dL)	3.05±0.25	2.87±0.08	2.90±0.06	2.72±0.11	3.02±0.22	0.169
ALB (g/dL)	1.85±0.04	1.81±0.03	1.76±0.05	1.89±0.05	1.86±0.08	0.126
ALT or SGPT(U/L)	127.11±2.23	121.87±6.42	121.07±1.67	124.33±3.76	125.08±3.39	0.367
AST or SGOT(U/L)	4.22±0.13	4.20±0.21	4.19±0.11	4.11±0.16	4.26±0.10	0.780

The data are represented as mean ± SD (n=3). (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SCP).

Abbreviations: TG, triglycerides; CHO, cholesterol; TP, total protein; ALB, albumin; ALT, alanine aminotransferase or SGPT, serum glutamic-pyruvic transaminase; AST, aspartate aminotransferase; SGOT, serum glutamic-oxaloacetic transaminase.

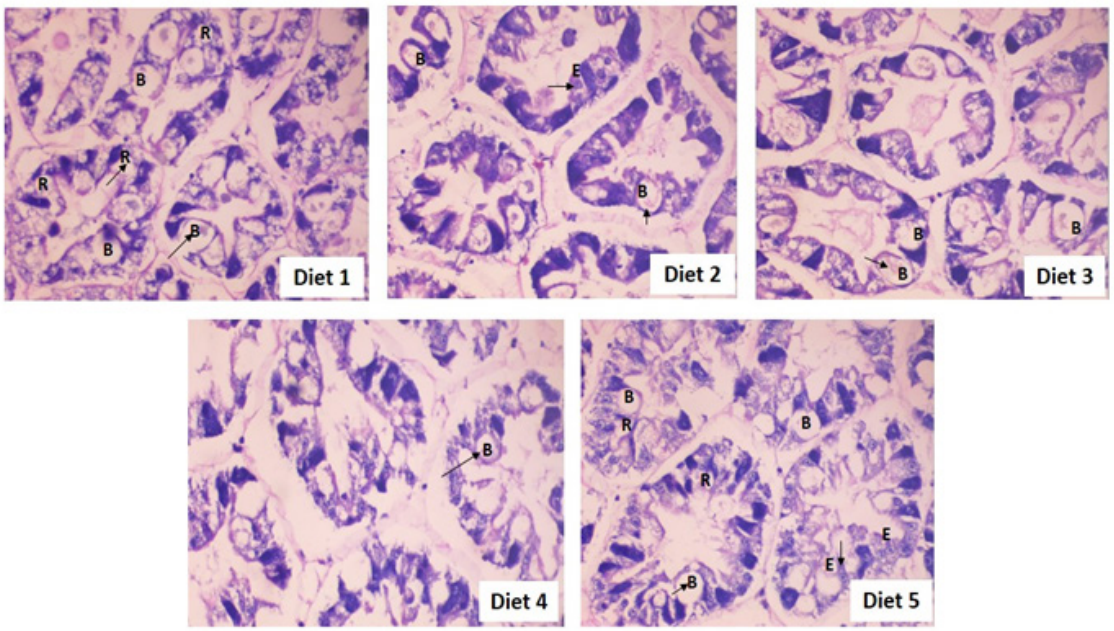

Figure 4. Transverse sections of the hepatopancreas from *P. vannamei* fed diets formulated with different combination of novel feed ingredients; The sections were stained in H & E to enhance the contrast (40x); (diet 1, 20% fishmeal (control); diet 2, combination of PBM and SCP; diet 3, combination of IM, RM and SCP; diet 4, combination of FW, PM and SCP; diet 5, combination of PBM, IM, FW, PM, RM and SCP) (B-cells secretory cell; R-cells resorptive/absorptive cell; E-cells embryonic cells)

Immunological responses

The immunological activities such as prophenoloxidase activity and respiratory burst activity of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients are shown in Figures 1 and 2, respectively. Prophenoloxidase activity was significantly higher ($P<0.05$) in shrimp fed with diets 1 and 5. Respiratory burst activity was significantly ($P<0.05$) higher in shrimp fed with diets 1 and 5 which was not different from diet 3.

Histology

The hepatopancreas histology of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients is shown in Figure 3. Shrimp hepatopancreas are composed of many hepatopancreas tubules. Four kinds of cells dominate the hepatopancreas tubules, namely E ('embryonalzellen' or embryonic) cells, R ('restzellen') cells, F ('fibrillenzellen' or fibrous) cells and B ('blasenzellen') cells (Franceschini-Vicentini et al., 2009). More B cells and few R cells were observed in shrimp fed with diets 1 and 5 compared to other diets.

Plasma biochemical responses

The plasma biochemical responses of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients are shown in Table 6. No significant difference was found in plasma biochemical responses such as triglycerides, cholesterol, total protein, albumin (ALB), aspartate aminotransferase and alanine aminotransferase of shrimp fed diets formulated with different combination of novel feed ingredients ($P>0.05$).

Immune related gene expression

The relative mRNA expression of *LYZ* of *Penaeus vannamei* fed diets formulated with different combination of novel feed ingredients is shown in Figure 4. The mRNA expression of *LYZ* was upregulated in shrimp fed with diets 1 and 5.

Discussion

Fatty acid composition

Aquaculture species such as *P. vannamei* depend on fatty acids for their growth and health. They are integral to cell membranes and serve as key energy sources. For *P. vannamei*, maintaining an optimal balance of fatty acids in the diet is crucial for proper development, immune function, and stress resilience. Essential fatty acids, such as omega-3 and omega-6, cannot be synthesized by the shrimp and must be provided through their diet. In commercial shrimp farming, feeds with optimized fatty acid profiles lead to better growth performance and higher survival rates (Khanjani et al., 2023 b). Polyunsaturated fatty acids (PUFAs) are particularly important for the growth of most marine species. PUFAs in the diet are crucial for optimizing *P. vannamei* growth and feed efficiency, as multiple research studies have shown (González-Félix et al., 2002; Lim et al., 1997; Tan et al., 2005). Significant differences were observed in the levels of palmitic acid, arachidic acid, palmitoleic acid, oleic acid, linoleic acid, and linolenic acid in the present study. However, no significant differences were identified in myristic acid, stearic acid, gondoic acid, or arachidonic

acid. The efficient and highly needed EPA and DHA were similar in diets 1 and 5 (P>0.05) and this can be correlated with the immune related gene expression. In contrast, studies testing individual protein ingredients such as poultry by-product meal have shown reduced growth in *P. vannamei* due to a deficiency of dietary n-3 highly unsaturated fatty acids (HUFA) (Chi et al., 2009). Studies have also reported that incorporating insect meals into diets increases the concentration of monounsaturated fatty acids (MUFA) while decreasing levels of highly unsaturated fatty acids, such as DHA and EPA, in shrimp muscle (Panini et al., 2017) and fish (Sánchez-Muros et al., 2016). Chen et al. (2021 a) observed that black soldier fly larvae meal contains a high amount of crude lipids, and its substantial inclusion can alter the fatty acid composition of the feed, leading to an increase in lauric acid and a reduction in polyunsaturated fatty acids. However, the diet 5, with a combination of all ingredients, provided adequate levels of PUFAs, making it the best option for supporting the shrimp growth and health and it is equally effective as fishmeal-based diet for shrimp.

Amino acid composition

In aquaculture species like *P. vannamei*, amino acids are critical for growth and health. They serve as the building blocks of proteins, which are vital for immune system function, metabolism, and muscle growth. In order to get optimal growth rates, disease resistance, and overall productivity for *P. vannamei*, the feed must have a balanced amino acid profile. Since shrimp are unable to produce necessary amino acids on their own, commercial aquaculture must provide these through their food, which emphasizes the necessity for specifically developed feeds that fulfill their particular nutritional requirements. The present experiment showed no significant differences (P>0.05) in amino acid profiles among *P. vannamei* fed diets formulated with different combination of novel feed ingredients. In the present study, diet 5, which consists of all ingredient combination, satisfied the amino acid requirements for shrimp and demonstrated an amino acid profile that was either similar to or better than diet 1, which contained 20% fishmeal. Similarly, Yang et al. (2021) found that a blend of Atlantic krill meal (AKM) and porcine by-product meal (PBM) provides a balanced amino acid profile along with potentially beneficial micro molecules, offering a promising alternative to improve the efficiency of fishmeal replacement in diets. In contrast, amino acid imbalance is often highlighted as a key factor contributing to poor growth and low muscle protein content in diets containing rapeseed meal (Jiang et al., 2016). Rapeseed meal is typically deficient in essential amino acids (EAA), particularly lysine (Lys) and methionine (Met) (Hu et al., 2013), which can adversely affect nutrient absorption, synthesis, and metabolism (Luo et al., 2012; Men et al., 2014). Additionally, in this research, the findings of the immunological responses and EPA, DHA profile have indicated that diet 5 is performing on parallel with the control diet, indicating that the amino acid composition of diets 1 and 5 satisfies the shrimp's requirement for amino acids.

Immunological responses

Assessment of health status is essential when evaluating the effectiveness of alternative proteins (Daiyong et al., 2009). Antioxidant defense and immune regulation are key indicators of health, providing insights into the immune and physiological responses critical for assessing shrimp health (He et al., 2021; Khanjani et al., 2023 c). In addition to growth and nutrient absorption, it is important to examine factors influenced by novel feed ingredients, such as digestive status and improvements in immunity and pathogen resistance. Insects are rich in bioactive compounds like chitin, antimicrobial peptides, and specific fatty acids and plays important role in shrimp nutrition (Sharifinia et al., 2023 b). Penaeid shrimp primarily depend on their innate immune system for defense against pathogens (Cerenius et al., 2010; Vazquez et al., 2009). When testing single protein alternatives, Shin and Lee (2021) found that supplementing diets with insect meals enhanced the innate immune responses and antioxidant enzyme activities in *P. vannamei*. This improvement is likely due to the presence of antimicrobial peptides (AMPs) in insects, which possess health-promoting properties, including antibiotic activity (Yi et al., 2014). In the present experiment, prophenoloxidase activity and respiratory burst activity was similar in diets 1 and 5 (P>0.05). This can be corroborated with upregulation of immune gene expression (*LYZ*) which confirms that the immune response was better with the combination of ingredients similar to the fishmeal-based diet.

Histology of hepatopancreas

Histological analysis of the hepatopancreas is a key method for evaluating shrimp health (Sun et al., 2015; Wu et al., 2008). Research has shown that the hepatopancreas undergoes structural changes in response to different diets. This organ is composed of various tubules and four main types of cells: E ('embryonalzellen' or embryonic) cells, R ('restzellen' or resorptive/absorptive) cells, F ('fibrillenzellen' or fibrous) cells, and B ('blasenzellen' or blister like) cells (Franceschini-Vicentini et al., 2009). Our findings revealed a significant increase in the prevalence of B-cells in shrimp fed diets 5 and 1 compared to other groups. B-cells are responsible for synthesizing digestive enzymes (Pourmozaffar et al., 2019). Additionally, a higher prevalence of R-cells was observed in shrimp fed the control diet and diet 5, which suggests improved hepatopancreatic health due to lipid storage in R-cells and their roles in immune response, nutrient storage, and metabolism. In context with these findings, Sharifinia et al. (2023 a) reported that fishmeal can be replaced with mealworm larvae (*T. molitor*) in diets for *L. vannamei* juveniles without adversely affecting growth performance or hepatopancreatic biochemical indices. Conversely, defatted black soldier fly larvae meal in shrimp feed at an

inclusion level of 234.9 g/kg was able to replace up to 60% of fishmeal without hindering shrimp growth (Wang et al., 2021).

Plasma biochemical responses

Plasma biochemical responses are essential indicators for evaluating the health, nutritional status, and physiological condition of aquatic organisms in aquaculture (Khanjani et al., 2023 c). Despite the lack of standardized values and their limited use, these metrics provide important information about the physiological status and health of aquatic organisms under various nutritional conditions (Kader et al., 2010; Mastoraki et al., 2020; Roque et al., 2010). Hematological indexes such as total protein (TP), alkaline phosphatase (AKP), acid phosphatase (ACP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) are commonly used to evaluate the health status of fish (Khanjani and Sharifinia, 2024; Velisek et al., 2005; Zhou et al., 2015). AST and ALT activities are particularly important as biomarkers for liver function and can indicate liver damage (Gholipour Kanani et al., 2014; Khanjani and Sharifinia, 2024; Sheikhzadeh et al., 2012). Wu et al. (2021) reported that increasing dietary levels of rapeseed meal led to elevated serum triglyceride levels, indicating potential negative effects on liver function in GIFT fish. In the present study, no significant differences ($P>0.05$) were observed in the plasma biochemical responses, indicating that the shrimp were unaffected by any of the health defects when fed diets formulated with different combination of novel feed ingredients. On the other hand, Sharifinia et al. (2023 b) found that increasing the replacement of fishmeal with mealworm in the diet led to reduced levels of cholesterol, triglycerides, and glucose in the hemolymph of *P. vannamei*. In conclusion, no negative health effects were observed in *P. vannamei*. The stable plasma biochemical responses, which indicate overall health, suggest that diets formulated with different combinations of novel feed ingredients could be beneficial for enhancing the health status of *P. vannamei*.

Immune gene expression

The antibacterial protein lysozyme has been identified as a crucial component of the innate immune system in invertebrates (Kaizu et al., 2011) because of its modest molecular weight and bacteriolytic action. The *LYZ* gene is therefore essential for innate immunity. In this experiment, the expression levels of immune-related gene *LYZ* was significantly upregulated ($P<0.05$) in shrimp fed diets 1 and 5 compared to other diets. This suggests a positive correlation between immune gene expression and immune response. Similarly, *P. vannamei* fed single-cell protein observed a significant ($P<0.05$) upregulation in the relative gene expression of lysozyme in shrimp (Felix et al., 2023). Ayiku et al. (2020) also found that including 1%–2% brewer's yeast in shrimp feed, as a replacement for fishmeal or soybean meal, enhanced the expression of immune-related genes and improved disease resistance. The results indicate that *LYZ* gene expression was also upregulated in diets 1 and 5, likely due to its crucial role in pathogen defense and maintaining shrimp health. This finding is supported by the improved immune response and plasma biochemical responses observed in diet 5 (a combination of ingredients).

Conclusion

The results of this study indicated that a combination of ingredients in a 1:1:1:1:1:1 ratio, consisting of poultry by-product meal (PBM), insect meal (IM), rapeseed meal (RM), peanut meal (PM), single-cell protein (SCP), and fish waste (FW) is an effective combination to replace fishmeal in shrimp feeds without compromising physiological, immunological and overall health of *Penaeus vannamei*.

Ethical statement

The Government of India's Ministry of Environment and Forests required the experiment to comply with CPCSEA guidelines for animal care and use in scientific research. The Institutional Animal Ethics Committee (IAEC) of Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam, Tamil Nadu, India, has approved it (3/1128/IAEC/TNJFU/IFPGS).

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Author contribution

Kalaivanan Rajalakshmi: investigation, formal analysis, writing – original draft. Nathan Felix: supervision, conceptualization, methodology, investigation, resources, writing – review & editing. Amit Ranjan: supervision, conceptualization, methodology, visualization, writing – review & editing. Arumugam Uma: supervision and visualization. Govindharaj Sathishkumar: sampling and formal analysis.

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Data availability

All data of the research were provided within this manuscript.

Competing interests

The authors declare no competing interests.

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