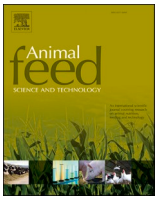
Animal Feed Science and Technology 296 (2023) 115549

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Evaluation of single cell protein on the growth performance, digestibility and immune gene expression of Pacific white shrimp, *Penaeus vannamei*

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ARTICLE INFO

Keywords:
Penaeus vannamei
Single cell protein
Methylococcus capsulatus
Growth
Digestibility

ABSTRACT

Growth and digestibility trials were undertaken to evaluate a single cell protein (SCP) produced from methane-oxidising bacteria *Methylococcus capsulatus* (String Bio, Bengaluru, India) as a replacement for fishmeal in the diets of Pacific white shrimp, *Penaeus vannamei*. Five iso-nitrogenous and isoenergetic diets were formulated to contain different levels of SCP: Control (Fishmeal, no SCP), SCP inclusion at 50 g/kg (S-5), 100 g/kg (S-10), 200 g/kg (S-20), 250 g/kg (S-25). The final body weight of the shrimp increased with increasing levels of SCP up to 200 g/kg (S-20) and recorded the best feed efficiency when compared to other treatments. Nitrogen (N) and phosphous (P) gain and retentions were improved in shrimp fed diets containing upto 200 g/kg SCP. There was no significant difference in the whole-body chemical and amino acid composition of shrimp. Apparent protein digestibility of the SCP was greater than 0.90. The expression levels of immune-relevant genes (lysozyme, Toll-like receptor and immune deficiency (IMD)) were up-regulated in the shrimp fed diet S-20. Results of this work demonstrate that SCP from *M. capsulatus* can be efficiently used in the diet of *P. vannamei* up to 200 g/kg (two-thirds of fishmeal replacement) with significant beneficial effects on growth, feed and nutrient utilisation.

1. Introduction

Global aquaculture production has dramatically increased in the past few years and in contrast, the capture fisheries have remained almost static (FAO, 2020). As the aquaculture sector continues to grow, there is an increasing demand for formulated feeds and protein (Hua et al., 2019). Feed based farmed fish and shrimp have been the largest consumers of capture fishery derived feedstuffs in the form of fishmeal (FM) and fish oil (Tacon and Metian, 2015). Although the use of fishmeal in aquatic animal feeds has many benefits, its inclusion level is reducing due to fluctuations in global availability, price instabilities, quality criteria and also in response to sustainability issues (Tacon and Metian, 2015; Aas et al., 2019; Hua et al., 2019; FAO, 2020; Tacon et al., 2022). Replacing fish meal in the feeds using various conventional and non-conventional protein sources has been the core subject of several studies (Amaya et al., 2007; Bu et al., 2018; Xie et al., 2016, 2018; Cummins et al., 2017; Shao et al., 2019; McLean et al., 2020). While replacing this protein-rich

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https://doi.org/10.1016/j.anifeedsci.2022.115549
Received 9 July 2022; Received in revised form 8 December 2022; Accepted 9 December 2022
Available online 15 December 2022

ingredient with various alternatives, a few issues like sustainability, climatic conditions and resource challenges should be considered and the alternatives should be less dependent on the natural ecosystem. One such alternative is the Single Cell Proteins (SCP), as they can be sourced from agricultural and industrial wastes which would be of public concern due to their large quantities of production and they do not rely on finite natural sources (Ritala et al., 2017; Hamidoghli et al., 2019; Hulsena et al., 2019; Zha et al., 2021; Nyyssölä et al., 2022).

SCP belong to a broad class of constituents that includes bacterial, yeast and microalgal derivative products (Glencross et al., 2020; Jones et al., 2020; Agboola et al., 2021). Although bacteria, yeast and microalgae are potential microbial sources of protein in fish feed, the most studied bacterial SCP for livestock rearing and aquaculture are those derived from Methylotrophs (Øverland et al., 2010; Strong et al., 2016; Kuźniar et al., 2019). The single cell proteins of bacterial origin are considered to be non-pathogenic and do not contain any anti-nutritional factors or toxins and as sustainable and renewable resources (Øverland et al., 2010). Bacterial SCPs have a high crude protein content of 500–800 g/kg with a good essential amino acid profile, along with other nutrients of importance. Production of bacterial SCP can be based on a variety of renewable sources such as CO_2, H_2, syngas, methane, methanol, *etc.* which are some of the primary carbon sources that facilitates cell growth and multiplication (Hulsena et al., 2019; Jones et al., 2020).

The potential of SCPs as a protein source and as a valid substitute for fishmeal in fish feeds has been recognised since long (Beck et al., 1978; Bergstrom, 1978; Kaushik and Luquet, 1980). A bacterial SCP named "Pruteen" derived from methanophilic bacteria (*Methylophilus methylotrophus*) was found to be a potential alternative to FM in the diets of Atlantic salmon, *Salmo salar* (Bergstrom, 1978) and could replace almost 800 g/kg of FM in the diets of rainbow trout (Kaushik and Luquet, 1980). Studies with different finfish species have shown the use of different SCPs at variable dietary incorporation levels: 100 g/kg in the diet of tilapia (Davies and Wareham, 1988); 85 g/kg in genetically improved farmed tilapia (GIFT) diet (Chama et al., 2021); between 100 and 270 g/kg in the diet of rainbow trout, *Oncorhynchus mykiss* (Kiessling and Askbrandt, 1993; Aas et al., 2006b; Hardy et al., 2018; Lee et al., 2020; Rajesh et al., 2022); 50–100 g/kg in the diet of Atlantic salmon (Storebakken et al., 2004; Berge et al., 2005; Tlusty et al., 2017); 200 g/kg in the Japanese yellowtail, *Seriola quinqueradiata* (Biswas et al., 2020); 100 g/kg in yellowtail kingfish, *Seriola lalandi* (Pilmer et al., 2022) or even upto 300 g/kg in Barramundi / Asian seabass, *Lates calcarifer* (Woolley et al., 2023). As regards shrimp, a few studies have dealt with the use of SCPs as a partial replacement of FM in the diets of Pacific white shrimp, *Penaeus vannamei* again with variable results (Tlusty et al., 2017; Hamidoghli et al., 2019; Chen et al., 2021). Tlusty et al. (2017) observed that inclusion of a SCP of bacterial origin (*Methylobacterium extorquens*) even at 63 g/kg of feed led to reduced growth in shrimp. The study undertaken by Hamidogli et al. (2019) with Pacific white shrimp fed diets contatining a SCP of another bacterial origin, *Corynebacterium ammoniagenes*, led to reduced growth and poor feed or protein utilisation beyond a dietary inclusion level of 20%. Chen et al. (2021) observed that inclusion of yet another source of SCP at levels upto 10.5% of the diet did not lead to any specific effects on growth and feed utilisation of shrimp, but found that dietary SCP had beneficial effects on response to a vibrio (*Vibrio parahaemolyticus*) challenge. Jintasataporn et al. (2021) fed graded levels (0%, 5%, 10% and 15%) of SCP replacing equivalent amounts of FM and observed very similar growth rates and feed utilisation in the Pacific white shrimp, but when challenged to *V. parahaemolyticus*, there was decreased mortality in shrimp fed diets containing SCP.

Over the years, advanced research in the area of the methanotrophs have led to the discovery and demonstration of their significant potential in feed industries as a protein source besides their potential for bioremediation of pollutants *via.* co-metabolism and assimilation of methane to mitigate greenhouse gas effects (Ritala et al., 2017; Kuźniar et al., 2019; Nyyssölä et al., 2022). The single cell protein used in the present study was derived from *Methylococcus capsulatus* utilising methane *via.* a fermentation process (String Bio Private Ltd, Bengaluru, India). This SCP has a high protein content with a very good essential amino acid (EAA) profile similar to that of fishmeal; with consistent availability and traceable production, this SCP also holds a high sustainability index.

Pacific white shrimp is the most important farmed crustacean species in the world and its production has increased rapidly especially in India over the recent years (MPEDA, 2018; Salunke et al., 2020). This has led to a consistent increase in demand for the production of shrimp feeds relying less on FM as the primary protein source using suitable and sustainable alternative protein sources. The present study was designed to evaluate the SCP on growth performance, feed utilization, digestibility, physiological and immune responses of Pacific white shrimp, *P. vannamei,* using diets containing graded levels of SCP.

2. Materials and methods

2.1. Ethical statement

The experiment was conducted following the procedures of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt. of India, on the care and use of animals in scientific research. This study was approved by the ethical committee of Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam, Tamil Nadu, India.

2.2. Methodology of production of SCP

The single cell protein ingredient was procured from String Bio Pvt. Ltd., Bengaluru, India. The production process comprises the steps of fermentation, separation and purification carried out in a centralized facility with large scale fermentation for the conversion of gaseous substrates into value-added products. SCP was produced by continuous aerobic fermentation of *Methylococcus capsulatus* using String Integrated Methane Platform (SIMP™) (Subbian et al., 2017). The harvested biomass was subjected to further downstream processing including cell separation and drying.

Table 1
Proximate and amino acid composition of fishmeal and SCP (*g/kg DM*).

	Fishmeal	SCP
Dry matter	892.0	940.0
Crude Protein	640.7	638.3
Crude lipid	58.7	62.9
Ash	140.8	92.7
Calcium	25.1	4.0
Phosphorus	16.3	15.8
Crude Fibre	< 10.0	< 10.0
Gross Energy (MJ/kg)	18.4	20.1
Essential amino acids		
Arginine	38.3	46.9
Histidine	17.3	20.6
Isoleucine	28.4	38.5
Leucine	48.1	54.6
Lysine	51.6	45.3
Methionine	17.8	12.8
Phenylalanine	26.2	31.0
Tyrosine	20.9	33.6
Threonine	27.6	30.8
Tryptophan	13.9	15.8
Valine	32.9	56.4
Nonessential amino acids		
Alanine	43.6	47.3
Aspartic acid	62.1	58.4
Glutamic acid	81.5	75.3
Glycine	50.4	36.9
Serine	25.1	20.4

2.3. Experimental design and diet preparation

Growth and digestibility trials were undertaken simultaneously to evaluate the single cell protein (SCP) as a replacement for fishmeal and to determine the digestibility of the SCP. The proximate and amino acid compositions of tuna fishmeal and SCP used for the study are presented in Table 1. For the growth trial, five isonitrogenous (380 g/kg protein) and isoenergetic diets (17.5 MJ/kg) were formulated (Table 2). The control diet was formulated to contain 300 g/kg of locally available tuna fishmeal without SCP. The diets S-5 to S-25 were formulated to contain SCP by altering the levels of fishmeal at graded inclusion of 50, 100, 200 and 250 g/kg respectively (16.7%, 33.3%, 66.7% and 83.3% fishmeal replacement). Data on amino acid composition of the diets are reported in Table 3. All the diets were formulated to meet the essential amino acid (EAA) requirements of shrimp, as per NRC (2011) for *Peneaus monodon* (tiger shrimp) as also summarised by Li et al. (2017) and Glencross (2021). For the digestibility trial, the reference diet (RF) was formulated with tuna fishmeal and yttrium oxide (Y_2O_3, 2.0 g/kg) as an inert marker and the SCP test diet was formulated by replacing 300 g/kg of the basal mixture (RF) with SCP protein, based on the 70:30 ratio approach for the ingredient evaluation (Cho and Slinger, 1979; Bureau et al., 1999). The feed formulation and their proximate composition are presented in Table 4. The diets were prepared in the Aquafeed mill, Directorate of Incubation and Vocational training in Aquaculture (DIVA), Tamil Nadu Dr. J. Jayalalithaa Fisheries University (TNJFU), Chennai, India. Dietary ingredients were finely ground (100-micron size), thoroughly mixed in a vertical ingredient mixer (Jinan Sunpring Machinery Co. Ltd.) at 960 rpm with the required level of water and 1 mm pellets were prepared in a pelletizer (Unique Engineering, Chennai, India) with steam at 5 bars to maintain the pelleting temperature at 70–80 °C. The pellets were then air-dried to get the desired moisture level of 100 g/kg. The feed formulation and proximate composition of the diets are reported in Table 2.

2.4. Shrimp and experimental conditions

Five thousand Pacific white shrimp postlarvae (PL 10) were obtained from Raj shrimp hatchery, Kovalam, Tamil Nadu, India. The growth and digestibility studies were undertaken in the experimental facilities of Directorate of Incubation and Vocational training in Aquaculture (DIVA), Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Chennai, India. Nursery rearing of post-larvae was carried out in two concrete tanks (16 m^3) with a density of 2500 in each tank for 30 days to attain an initial size of 1 g. During the nursery rearing period, proper aeration was given round the clock and the shrimp post-larvae were fed with a commercial diet (Royal Dragon DT311, Sheng long Biotech International Co., Ltd.; 360 g/kg protein, 50 g/kg lipid) thrice a day (9.00, 13.00 and 17.00 h). A 90-day growth trial was carried out in twenty experimental cages of size 1 × 1×1.5 m in four replicates. Each replicate cage was stocked with sixty shrimp (n = 60) with an average initial weight of 1.6 ± 0.08 g. Feeding was done *ad libitum* thrice a day (9.00, 13.00 and 17.00 h) exclusively in feeding trays (0.2 m^2) at one unit per cage.

The digestibility trial was carried out simultaneously along with the growth trial for 90-days in circular experimental troughs of 50-litre water volume. 80 shrimp (1.6 ± 0.1 g) were randomly distributed among eight experimental troughs with each replicate trough consisting of 10 shrimp and closed with a net to prevent leaping out of shrimp from the tanks, which were then allocated to two

Table 2
Formulation and proximate composition of experimental diets (*g/kg of diet*).

Ingredients	Dietary inclusion level				
	Control	**S-5**	**S-10**	**S-20**	**S-25**
Fishmeal[a]	300.0	250.0	200.0	100.0	50.0
SCP[b]	–	50.0	100.0	200.0	250.0
Soybean meal[a]	230.0	230.0	230.0	230.0	230.0
Shrimp head meal[c]	50.0	50.0	50.0	50.0	50.0
Wheat flour[a]	146.0	146.0	146.0	146.0	146.0
Corn flour[a]	100.0	100.0	100.0	100.0	100.0
Broken rice[a]	140.0	140.0	140.0	140.0	140.0
Fish oil	15.0	15.0	15.0	15.0	15.0
Soy lecithin	5.0	5.0	5.0	5.0	5.0
L-lysine[d]	2.0	2.0	2.0	2.0	2.0
DL-methionine[e]	3.0	3.0	3.0	3.0	3.0
Vitamin premix[f]	2.0	2.0	2.0	2.0	2.0
Ascorbyl monophosphate	0.2	0.2	0.2	0.2	0.2
Mineral premix[g]	2.0	2.0	2.0	2.0	2.0
Binder[h]	3.8	3.8	3.8	3.8	3.8
Monocalcium phosphate	1.0	1.0	1.0	1.0	1.0
Proximate composition of the diets (g/kg dry matter)					
Dry matter	899.7	898.7	900.9	903.6	907.3
Crude Protein	378.1	375.9	380.4	377.3	376.1
Crude lipid	44.0	44.8	42.5	48.0	46.9
Crude Fibre	21.9	20.3	19.9	20.8	19.9
Ash	76.6	75.8	69.1	70.2	64.9
Calcium	9.0	8.0	8.0	9.0	8.0
Phosphorus	6.7	7.1	8.5	8.1	7.8
Gross Energy (MJ/kg)	17.4	17.4	17.5	17.6	17.8

[a] National Cooperative Consumers' Federation of India (NCCF), Chennai, India.
[b] String Bio Private Ltd., Bengaluru, India.
[c] Aswinth Traders, Chennai, India.
[d] Ajinomoto Heartland, Inc., Chicago
[e] Evonik AG, Germany
[f] Composition of vitamin premix (quantity/kg of premix): Biotin 0.5 g; Folic acid 5 g; Inositol 70 g, Niacin 50 g; Pantothenic acid 40 g; Vitamin A 3.5 MIU; Vitamin B1 12.5 g; Vitamin B12 0.01 g; Vitamin B2 10 g; Vitamin B6 12.5 g; Vitamin C 75 g; Vitamin D3 1.5 MIU; Vitamin E 75 g; Vitamin K3, 15 g (From Anicare Industries, Chennai, India)
[g] Composition of mineral premix (g/kg of premix): $CaCO_3$, 200; $MgSO_4$, 20; $Ca(H_2PO_4)_2$, 100; KCl, 80; NaCl, 20; KI, 0.1; $CuSO_4$, 25; $FeSO_4$, 25; $MnSO_4$, 30; Na_2SeO_3, 1.5, $ZnSO_4$, 60; $CoCO_3$, 0.25 (From Anicare Industries, Chennai, India).
[h] Innobind, Natural Remedies, Bengaluru, India.

Table 3
Amino acid composition of the experimental diets (*g/kg of diet*).

	Diets						**Requirement**
	Control	**S-5**	**S-10**	**S-20**	**S-25**	NRC (2011)	Li et al. (2017)
Essential amino acids							
Arginine	26.6	26.5	26.3	27.1	28.3	19.0	23.0
Histidine	12.8	11.6	10.6	8.7	8.5	8.0	–
Isoleucine	20.1	20.1	19.9	20.0	20.7	10.0	16.0
Leucine	27.9	28.3	27.8	27.6	28.7	17.0	24.0
Lysine	28.0	28.1	25.8	24.0	21.8	21.0	16.0 – 21.0
Methionine	7.9	7.7	7.6	7.3	7.1	7.0	7.0 – 9.0
Phenylalanine	16.3	16.5	16.4	16.3	17.3	14.0	
Tyrosine	12.0	11.8	11.7	11.5	11.9		
Threonine	13.7	13.6	13.3	13.0	13.6	14.0	12.0 – 14.0
Tryptophan	3.5	3.7	4.1	4.0	4.4	2.0	3.6
Valine	28.6	28.8	28.6	28.7	30.0	–	14.0
EAAI*	**91**	**91**	**89**	**88**	**87**		
Nonessential amino acids							
Alanine	19.7	20.0	19.9	20.2	21.7		
Aspartic acid	31.5	30.8	31.9	31.5	29.9		
Glutamic acid	56.5	55.3	52.7	53.4	53.1		
Glycine	17.9	18.0	17.7	17.8	18.6		
Proline	19.5	18.7	20.8	21.8	22.6		
Serine	14.1	14.0	13.6	13.4	13.8		

EAAI = essential amino acid index.

Table 4
Reference and test diet formulations for determination of apparent digestibility coefficients (ADC) in Pacific white shrimp, *Penaeus vannamei* (*g/kg* of diet).

Ingredients	Dietary inclusion level	
	Control	**SCP**
Fishmeal	300.0	210.0
Soybean meal	230.0	161.0
Shrimp head meal	50.0	35.0
Wheat flour	146.0	102.2
Corn flour	100.0	70.0
Broken rice	138.0	96.6
Fish oil	15.0	10.5
Soy lecithin	5.0	3.5
L-lysine	2.0	1.4
DL-methionine	3.0	2.1
Vitamin premix	2.0	1.4
Vitamin C	0.2	0.1
Mineral premix	2.0	1.4
Pellet binder	3.8	2.7
Monocalcium phosphate	1.0	0.7
Yttrium oxide	2.0	1.4
SCP	–	300.0
Proximate composition of the diets (g/kg dry matter)		
Dry matter	899.7	900.2
Crude Protein	378.1	494.1
Crude lipid	44.0	52.8
Crude Fibre	21.9	19.3
Ash	76.6	82.0
Calcium	9.0	8.0
Phosphorus	6.7	9.7
Gross Energy (MJ/kg)	18.1	19.0

treatment diets (Reference and test diet) in four replicates.

The source of water for the growth study was from the nearby backwater with an average salinity of 30 ± 1 ppt. The water was initially pumped to a reservoir pond, treated and disinfected with bleaching powder. After dechlorination, the water was aerated vigorously to remove residual chlorine and pumped into the cement tanks. Water exchange was done every week during the 90-day growth trial to ensure optimum water quality. Water quality parameters like temperature (28 ± 1^0 C), pH (8.0 ± 0.2), dissolved oxygen (6.1 ± 0.5 ppm) and salinity (30 ± 1 ppt) were measured daily, while ammonia–N (0.02 ± 0.001 ppm), nitrite-N (0.05 ± 0.01 ppm), nitrate-N (11 ± 0.1 ppm), hardness (580 ± 16 ppm), alkalinity (160 ± 8 ppm) were measured once a week and maintained within the optimal range throughout the growth trial.

2.5. Faeces collection technique

The shrimp were manually fed the reference and test diets until satiation three times a day (9:00, 13:00 and 17:00 h). The collection of faecal samples was done after a week of feeding the experimental diets (acclimation period) following the methods of Lin et al. (2004). After an hour of each feeding, the troughs with uneaten feed and faeces were siphoned out and discarded. Faecal samples were collected three times a day from each replicate trough at 12:00, 16:00 and 20:00 h. The collected faeces were gently rinsed with distilled water, dried on filter paper and frozen immediately at − 18°C. Daily faeces samples were pooled and stored for chemical analyses.

2.6. Sampling and chemical analyses

Feed intake and mortality, if any, were monitored daily to determine survival, feed intake and feed efficiency. At the start (day 1) and at the end of the 90-day growth trial, shrimp were bulk-weighed from each replicate cage and survival numbers were counted to estimate the growth performances. Shrimp were also weighed every 30 days until the completion of the growth trial to obtain growth curves. The following growth parameters were calculated from the data obtained:

Mean weight gain = Final wt. (g) – Initial wt. (g)
Survival (%) = [Final no. of shrimp/Initial no. of shrimp] X 100
Feed gain ratio (FGR) = Total dry feed consumed (g) / Total wet weight gain (g)
Feed Efficiency (FE) = Wet weight gain / Dry feed intake
Protein efficiency ratio (PER) = Weight gain / total crude protein intake
Specific growth rate (SGR, % day^{-1}) = [ln Final wt. – ln Initial wt.]/duration in days X 100
Nutrient gain (mg nutrient/ shrimp) = (Final weight X Final body nutrient content) – (Initial weight X Initial body nutrient content)

Table 5
Primers used for qRT-PCR analysis of selected genes of Pacific white shrimp, fed feeds with graded levels of SCP.

Gene name	GenBank number	Primer sequence (5′−3′)
Lysozyme	AY170126	Forward: CGACCTCGATCAGTACATGG Reverse: GTAACCCTGGTGACAAGCCT
Toll-like receptor	DQ923424	Forward: TGGTGCTTTCGTCAAACTTC Reverse: AACCTGGCCATACACAATGA
IMD	FJ592176	Forward: ATCGAGGAACGAGACAAGGT Reverse: CGTACACTCGGTCGACATTC
β-actin	AF300705	Forward: CGCGACCTCACAGACTACCT Reverse: CTCGTAGGACTTCTCCAGCG

Nutrient retention (% intake) = Nutrient gain / Nutrient intake X 100

The following analyses were perfomed on the diets, whole-body and faeces by following the standard protocols of AOAC (2010): Dry matter, Crude protein, Ether extract, Ash, Fibre, Calcium and Phosphorus (procedure numbers 934.01, 984.13, 2003.06, 942.05, 962.09, 927.02, and 965.17 respectively) in Animal Feed Analytical and Quality Assurance Laboratory (AFAQAL), Veterinary College and Research Institute, TANUVAS, Namakkal, India. An initial sample of 100 shrimp (1.6 ± 0.08 g) was used for the initial whole-body composition analysis and at the end of the growth trial, 10 shrimp from each cage (40 shrimp per treatment) were randomly sampled for the final whole-body chemical composition analysis.

Yttrium oxide in the feed and faeces were analysed using Inductively coupled plasma mass spectrometry (iCAP RQ; Thermo Fisher Scientific, Germany). Briefly, Sample predigestion was carried out in a digestion vessel (MARSXpress™ Vessels; CEM Corporation, Matthews) by adding 2.5 ml of 65% nitric acid and allowed to stand for 1 h at room temperature in fume hood. Then, the sample was digested in a microwave digester (MARS 6 Microwave Digestion System; CEM Corporation) by adding 7.5 mlMilliQ water following a gradient temperature program with temperature varying from ambience to 160 °C, increasing at the rate of 20 °C min^{-1} at 1000 W, followed by a final hold at 160^0 C for 20 min. The temperature was brought down to 90 °C and the digested sample was used to determine the concentration in ICP-MS. Multielement standard (CRM-ICP multi-element standard XVI; HC60097287; Merck, Germany) was used for the analysis. The instrument was calibrated using serially diluted standard from 0.1 to 25 μg/l taken from 100 mg/l stock metal solution.

Amino acid composition analysis was undertaken by String Bio Pvt. Ltd., Bengaluru, India. The amino acid profile of the samples of diets and whole shrimp were separated and quantified using High-Performance Liquid Chromatography (Model: 1260 Infinity II with a quaternary pump, Agilent Technologies) equipped with Advance Bio AAA column (4.6 × 100 mm, 2.7 μm, Agilent Technologies). 100 mg of sample was hydrolysed with 10 ml of 6 N HCl at 110 °C for 24 h in a hot air oven and filtered using 0.22 μm PES filters and diluted with 0.1 N HCl before analysis in HPLC. Norvaline (2 mg) was used as an internal standard to calculate the recovery. Analysis of tryptophan involved prior hydrolysis with 5 ml of 4 M NaOH at 110 °C for 16 h in a hot air oven, neutralized and filtered with a 0.22 μm PES filter. Samples were pre-column derivatized using ortho-phthalaldehyde (OPA) and fluorenylmethoxy chloroformate (FMOC). Mobile phase for HPLC consisted of a mixture of A (10 mM dibasic sodium phosphate and 10 mM sodium borate, pH 8.2) and B (Methanol: Acetonitrile: Water, 45:45:10 (v:v:v)) with 1.0 ml/min flow rate. Tryptophan analysis was done using Poroshell 120 EC C18 column (4.6 × 250 mm, 4 μm, Agilent Technologies). The mobile phase for HPLC consisted of a mixture of 0.91 A (25 mM Sodium acetate, pH 7) and 0.09 B (Acetonitrile) with a 0.9 ml/min flow rate. The samples were monitored at 338 nm or 262 nm. Peak areas obtained from standards and samples were used to quantify the amino acids and expressed as relative content in percentage.

2.7. Digestibility estimations

The apparent digestibility coefficients (ADC) for dry matter and the nutrients were estimated using the following equation.

$$ADC_{diet} = 1 - [(Y_d / N_d) \text{ X } (N_f / Y_f)]$$

Where Y_d is the concentration of yttrium oxide in the diet, Y_f is the concentration of yttrium oxide in the shrimp faeces, N_d is the concentration of nutrients in the diet and N_f is the concentration of nutrients in the shrimp faeces.

The ADCs of nutrients in the test ingredient was calculated from their respective ADC of reference and test diet based on a 70:30 substitution of test ingredient in the RF diet (Cho and Slinger, 1979; Bureau and Hua, 2006).

$$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref\cdot diet}) \text{ X } (0{\cdot}7 \text{ X } D_{ref} / 0{\cdot}3 \text{ X } D_{ingr})]$$

Where, $ADC_{test\ diet}$ = Apparent digestibility coefficient of nutrient in test diet; $ADC_{ref.\ diet}$ = Apparent digestibility coefficient of nutrient in reference diet; D_{ref} = concentration of nutrient in reference diet mash; D_{ingr} = concentration of nutrient in test ingredient.

2.8. Quantitative real-time PCR (qRT-PCR)

The relative mRNA expressions of genes related to Toll-like receptor, immune deficiency (IMD), and lysozyme from gill tissues of *P. vannamei* were analysed at the end of the growth trial. All shrimp were fasted for 24 h before sample collection. The shrimp were

Table 6
Growth and feed utilisation in Pacific white shrimp fed diets containing different levels of SCP.

Parameters	Diets					
	Control	S-5	S-10	S-20	S-25	P value
Initial body weight (g)	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.680
Final body weight (g)	19.7^{c} ± 0.6	21.4^{b} ± 1.3	23.0^{a} ± 0.6	23.3^{a} ± 0.9	21.5^{b} ± 1.0	< 0.001
Weight gain (g)	18.1^{c} ± 0.3	19.7^{b} ± 0.6	21.4^{a} ± 0.3	21.7^{a} ± 0.4	19.8^{b} ± 0.5	< 0.001
Survival (%)	94.6 ± 2.8	95.4 ± 0.8	96.7 ± 1.4	94.2 ± 2.15	93.3 ± 3.5	0.528
Specific growth rate (%)	2.77^{b} ± 0.05	2.83^{b} ± 0.09	2.96^{a} ± 0.04	2.98^{a} ± 0.03	2.84^{b} ± 0.08	0.001
Feed intake (g/shrimp)	42.3^{a} ± 1.2	41.9^{a} ± 2.1	41.3^{ab} ± 2.0	38.6^{b} ± 1.3	39.8^{ab} ± 1.8	0.045
Feed gain ratio	2.34^{a} ± 0.07	2.13^{b} ± 0.06	1.93^{cd} ± 0.09	1.78^{d} ± 0.04	2.02^{bc} ± 0.18	< 0.001
Feed efficiency	0.43^{d} ± 0.01	0.47^{c} ± 0.01	0.52^{b} ± 0.03	0.56^{a} ± 0.01	0.50^{bc} ± 0.05	< 0.001
Protein efficiency ratio	1.15^{d} ± 0.03	1.25^{cd} ± 0.03	1.37^{b} ± 0.07	1.51^{a} ± 0.03	1.33^{bc} ± 0.12	< 0.001
N gain (mg N/shrimp)	487.0^{c} ± 17.9	525.8^{b} ± 20.9	576.6^{a} ± 20.2	567.1^{a} ± 21.4	531.2^{b} ± 23.9	< 0.001
N retention (% intake)	19.4^{d} ± 0.5	20.9^{cd} ± 0.3	23.0^{b} ± 1.0	24.7^{a} ± 1.1	22.3^{bc} ± 1.8	< 0.001
P gain (mg P/shrimp)	38.4^{b} ± 3.3	41.3^{b} ± 1.5	42.5^{b} ± 3.3	59.3^{a} ± 1.9	61.2^{a} ± 5.1	< 0.001
P retention (% intake)	13.0^{b} ± 1.0	$^{b}14.1$ ± 0.6	15.5^{b} ± 0.9	19.2^{a} ± 1.1	19.2^{a} ± 1.3	< 0.001

Note: Values expressed as means ± SD of four replicate cages per treatment (n = 4). Values within a row with different superscript values indicate significant difference *($p < 0.05$)* as determined by one way ANOVA followed by Duncan's multiple range test.

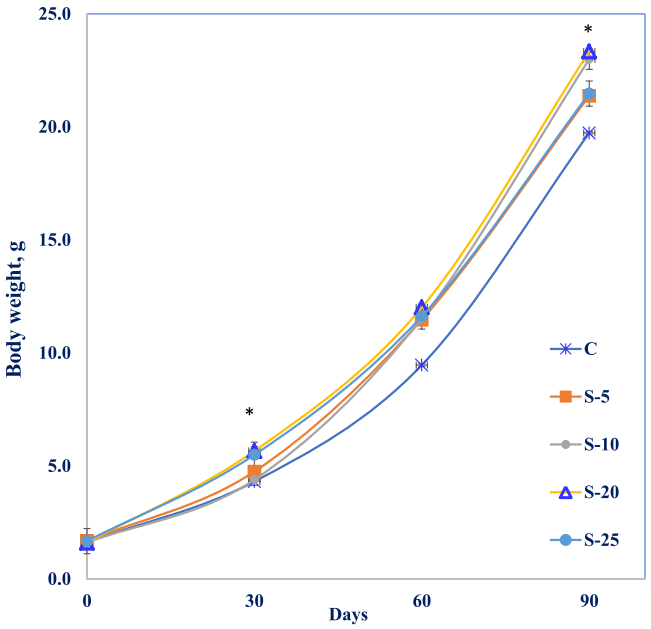

Fig. 1. Growth of shrimp fed the diets with different levels of SCP over the 90 days growth trial. Values are expressed as means ± SD (n = 3).

euthanized after an overdose of MS-222 and gill tissues of three shrimp per cage (replicate) were collected and the RNA was isolated using RNA iso-plus reagent (Takara Bio Inc., Otsu, Shiga, Japan) according to the RNA extraction protocol. The samples were kept at −18°C until analysis. cDNA was synthesized from total RNA (2 μL) using the first-strand cDNA synthesis kit (Thermo Scientific, Vilnius, Lithuania) by following the manufacturer's protocol. The primers and protocols of Yan et al. (2020) were followed for relative gene expression studies (Table 5). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed in triplicates in a C1000 Touch thermal cycler-CFX96 Real-time PCR (Bio-Rad, Hercules, CA) using SYBR@Premix ExTaq™ Kit (Takara Bio Inc., Otsu, Shiga, Japan). Amplification was performed in a total volume of 25 μL (12.5 μL SYBR Green, 1 μL of forward and reverse primers each, 2 μL cDNA, and 8.5 μL DEPC-H_2O). The cycling condition was programmed with an initial denaturation at 95 °C for 30 s, followed by 39 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s β-actin was amplified as an internal reference (housekeeping gene) and the relative mRNA expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

All statistical analyses were carried out with the statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA). Data were expressed as means ± standard deviation (SD) of four replicates. The means were subjected to one-way ANOVA and compared using

Table 7
Whole body chemical and amino acid composition (*g/kg dry weight basis*) of Pacific white shrimp fed diets containing different levels of SCP.

		Diets					
	Initial	**Control**	**S-5**	**S-10**	**S-20**	**S-25**	**P value**
Proximate							
Moisture (as is)	734.0	755.3 ± 6.1	766.2 ± 7.5	761.8 ± 5.9	767.6 ± 3.1	763.7 ± 6.4	0.154
Crude Protein	611.2	684.7 ± 13.8	711.0 ± 9.3	710.4 ± 9.6	696.2 ± 12.4	708.6 ± 10.8	0.066
Crude lipid	51.5	57.0 ± 3.3	62.0 ± 3.2	56.9 ± 2.1	54.0 ± 2.84	55.8 ± 3.1	0.068
Ash	164.2	123.4 ± 2.8	117.4 ± 7.2	120.9 ± 4.9	122.5 ± 3.4	125.2 ± 4.9	0.385
Essential amino acids							
Arginine	66.9	66.6 ± 1.0	68.3 ± 1.0	65.5 ± 5.0	67.7 ± 6.0	69.0 ± 2.0	0.754
Histidine	11.6	12.6 ± 1.0	13.1 ± 1.0	13.5 ± 1.0	13.9 ± 2.0	13.0 ± 1.0	0.697
Isoleucine	34.3	34.3 ± 0.4	34.9 ± 2.0	35.1 ± 1.0	36.4 ± 3.0	33.8 ± 0.4	0.385
Leucine	48.2	48.8 ± 2.0	48.5 ± 1.0	50.6 ± 0.3	49.8 ± 1.0	49.1 ± 1.0	0.362
Lysine	49.3	53.2 ± 1.0	51.2 ± 0.4	53.5 ± 1.0	53.6 ± 2.0	52.2 ± 1.0	0.261
Methionine	15.0	15.0 ± 2.0	15.7 ± 1.0	15.4 ± 1.0	15.0 ± 1.0	15.9 ± 1.0	0.895
Phenylalanine	29.3	29.4 ± 1.0	30.0 ± 1.0	31.2 ± 1.0	31.5 ± 1.0	30.5 ± 1.0	0.189
Tyrosine	24.6	26.2 ± 1.0	27.2 ± 2.0	27.1 ± 1.0	26.5 ± 1.0	26.5 ± 1.0	0.779
Threonine	19.5	23.2 ± 1.0	23.5 ± 3.0	24.9 ± 0.3	25.7 ± 1.0	24.4 ± 1.0	0.355
Tryptophan	7.0	6.7 ± 1.0	7.2 ± 0.2	5.8 ± 0.3	6.6 ± 0.3	5.6 ± 0.4	0.146
Valine	32.1	32.7 ± 1.0	33.8 ± 3.0	35.1 ± 1.0	33.7 ± 0.3	33.3 ± 1.0	0.371
Non-essential amino acids							
Alanine	43.6	45.9 ± 2.0	45.9 ± 2.0	45.4 ± 1.0	45.4 ± 1.0	43.4 ± 1.0	0.288
Aspartic acid	54.7	56.8 ± 1.0	55.1 ± 1.0	60.6 ± 1.0	61.0 ± 1.0	58.9 ± 2.0	0.282
Glutamic acid	95.5	98.0 ± 2.0	96.3 ± 3.0	99.7 ± 6.0	100.2 ± 3.0	97.3 ± 1.0	0.609
Glycine	55.0	52.5 ± 1.0	53.7 ± 3.0	52.4 ± 4.0	54.7 ± 1.0	52.1 ± 1.0	0.608
Proline	43.5	43.2 ± 0.3	43.2 ± 1.0	39.6 ± 1.0	40.1 ± 1.0	42.5 ± 1.0	0.763
Serine	21.7	23.9 ± 2.0	23.3 ± 3.0	24.5 ± 1.0	24.2 ± 1.0	23.8 ± 2.0	0.901

Note: Values are expressed as means ± SD of four replicate cage per treatment (n = 4).

Duncan's multiple range test. The differences among the treatments were considered statistically significant at $p < 0.05$.

3. Results

3.1. Growth performances and feed utilization

All the diets were formulated to meet the EAA requirements of shrimp, based on literature data available (NRC, 2011; Li et al., 2017; Glencross, 2021; Mai et al., 2021). The essential amino acid index (EAAI) of the diets was calculated as per Oser (1959) in comparison to data on EAA requirements of shrimp as mentioned above and found that all diets had an EAAI above 87. There was a slight decrease in EAAI as well as that of histidine content with increasing dietary SCP levels (Table 3), but the levels were above the recommended EAA rewuirements of shrimps. Data on growth performances of shrimp fed the different diets over the 90 days growth trial are provided in Table 6. The growth curves of the different groups over the trial period are presented in Fig. 1. Feed intake by shrimp was slightly redued but not significantly *($p > 0.05$)* in shrimp fed diets S-20 and S-25. Shrimp fed diet S-20 exhibited the highest performance in terms of final weight, weight gain and feed efficiency. The body weight significantly *($p < 0.05$)* increased in shrimp fed diet with S-10 than in the control, but there was no significant *($p > 0.05$)* increase when compared with shrimp fed diet S-20 even though the average final body weight of S-20 was numerically higher. On further replacement of fishmeal with the SCP (S-25), the average final body weight of shrimp was found to be significantly *($p < 0.05$)* lower compared to that of the shrimp fed diet S-20 but was significantly *($p < 0.05$)* higher than that of shrimp fed the control diet. There were no siginificant variations in the survival of shrimp fed the different diets. Feed efficiency was the highest in the group fed diet S-20 (0.56 ± 0.01), which was significantly *($p < 0.05$)* higher than in the other groups except S-10 (0.52 ± 0.03). Nitrogen gain and nitrogen retention (% of intake) were significantly higher in shrimp fed diets containing SCP upto a level of 200 g/kg in the diets than in the control group. Phosphorus gain and phosphorus retention (% of intake) were also significantly *($p < 0.05$)* higher even up to the SCP inclusion level of 250 g/kg in shrimp diets than in control.

3.2. Whole-body composition and amino acid profile

The whole-body chemical and amino acid compositions of shrimp fed the different diets are presented in Table 7. There were no significant ($p > 0.05$) differences in the protein, lipid and ash contents among the different groups of *P. vannamei*. No significant ($p > 0.05$) differences were observed in the whole-body amino acid profiles of shrimp fed diets containing graded levels of SCP.

3.3. Apparent Digestibility Coefficients

Data on the apparent digestibility coefficients (ADC) for the reference diet, test diet and the test ingredient are reported in Table 8.

Table 8
Nutrient digestibility of the reference diet, test diet and of the SCP tested in Pacific white shrimp.

	Reference diet	Test diet	SCP Tested
Dry matter	0.67 ± 0.009	0.70 ± 0.007	0.80 ± 0.044
Protein	0.75 ± 0.005	0.82 ± 0.004	0.91 ± 0.003
Lipids	0.70 ± 0.007	0.78 ± 0.006	0.92 ± 0.004
Calcium	0.57 ± 0.003	0.58 ± 0.002	0.60 ± 0.003
Phosphorus	0.35 ± 0.005	0.51 ± 0.010	0.70 ± 0.021

Note: Values expressed as means ± SD of three replicates (n = 3).

The SCP tested was found to be highly digestible with the digestibility coefficients of 0.91, 0.92, 0.60 and 0.70 for protein, lipid, calcium and phosphorus respectively. The digestible protein and lipid content of SCP were calculated to be 577.7 g/kg and 57.9 g/kg respectively.

3.4. Immune-related gene expression

Data on the relative levels of expression of immune responsive genes (lysozyme, Toll-like receptor and immune deficiency (IMD)) in shrimp fed the different diets are presented in Fig. 2(a to c). The relative gene expression of lysozyme, Toll-like receptor and immune deficiency (IMD) were all significantly ($p < 0.05$) up-regulated in shrimp fed diet S-20 as compared to shrimp fed the control diet or other SCP diets.

4. Discussion

The present study was undertaken in experimental facilities simulating practical shrimp farming conditions in the backwaters. The results of the present study show that it is possible to include upto 200 g/kg of SCP derived from *M. capsulatus* (replacing up to two thirds of fishmeal) in the diets of Pacific white shrimp still maintaining good survival, growth and feed efficiency. In fact, at the dietary SCP inclusion level of 200 g/kg, despite lower feed intakes, the mean weight gain and feed efficiency were better compared to those of shrimp fed the control diet. It is indeed promising to see that shrimp performed well at SCP inclusion level of 200 g/kg, better than those reported by Chen et al. (2021) in Pacific white shrimp where they found that another SCP can replace 45% of dietary FM (105 g SCP/kg diet). In finfish, studies on the use of SCP from *M. capsulatus* with rainbow trout have shown good growth at dietary inclusion levels of 270 g/kg (Aas et al., 2006b) and 125 g/kg (Rajesh et al., 2022), while it was possible to replace 85 g/kg in the diet of tilapia (complete FM replacement; Chama et al., 2021). The variations and adverse effects observed on the growth in some of the studies on FM replacement with SCP derived from *M. capsulatus* might be related to the source and production process, target species, inclusion levels in the diets, preparation of diets, nucleic acid content and the different experimental conditions. There was no significant difference observed in the survival of shrimp among the different treatment groups, but shrimp fed diets with 50 and 100 g/kg of SCP exhibited higher survival than the control group. Hamidoghli et al. (2019) reported an increasing trend, albeit not significant, in the survival of shrimp on the addition of a SCP obtained from a bacteria (*Corynebacterium ammoniagenes*) and stated that the increase might be due to the increase of non-specific immune responses by the dietary inclusion of SCP. Similarly, Chen et al. (2021) observed that survival of shrimp increased with the addition of SCP derived from *M. capsulatus* in the diet although they did not find statistically significant differences among the treatments.

N gain and N retention of SCP included diets were higher than that of the control diet. This is in line with the higher growth of shrimp on SCP inclusion with better feed efficiencies. The nucleic acids in SCP can also have N-sparing effect leading to increased protein efficiency ratio, N gain and retention (Aas et al., 2006a). In rainbow trout, Rajesh et al. (2022), found no differences in N gain or retention when the dietary SCP inclusion level was about 125 g/kg. The increased phosphorus gain and retention in shrimp fed diets with SCP can be attributed to the higher digestibility of phosphorus in the SCP and as the phosphorus content in SCP is attributed to the levels of nucleic acids and phospholipids (Øverland et al., 2010), while phosphorus in fishmeal would be mostly in the form of a complex with calcium (calcium hydroxyapatite complex, Rajesh et al., 2022).

Whole-body chemical composition and amino acid profile of shrimp did not vary significantly ($p > 0.05$) with the increasing levels of SCP. This is in conformity with the general observations of a relatively constant protein-bound amino acid composition of shrimp irrespective of dietary treatments (Peñaflorida, 1989; Alam et al., 2002; Xie et al., 2018). Similar to our observations, Hamidoghli et al. (2019) found no significant difference in the whole body chemical composition of white shrimp fed diets containing different levels of another protein of bacterial origin (*C. ammoniagenes*); they found however an increase in lipid level at a dietary SCP inclusion level of 80 g/kg, which they attributed to a possible imbalance in the amino acid composition of the SCP they used. Strangely enough, these authors also observed that the whole body EAA levels were lower in the SCP fed shrimp, again incriminating a possible EAA imbalance in the SCP and diets they used. In the present study, the amino acid profile of SCP was similar to that of the FM used and all the diets were formulated to have an equal proportion of amino acids, also considering the basic requirement of each amino acid.

Of practical importance to the feed formulators is knowledge on digestibility of nutrients and energy from specific ingredients rather than on whole diets (Kaushik, 1990; Glencross et al., 2020). The digestibility values obtained in the present study were higher than those of earlier reports with other SCPs measured in finfish (Skrede et al., 1998; Storebakken et al., 2004; Øverland et al., 2010; Tlusty et al., 2017) and in Pacific white shrimp (Hamidoghli et al., 2019). The low digestibility values of some bacterial proteins in fish

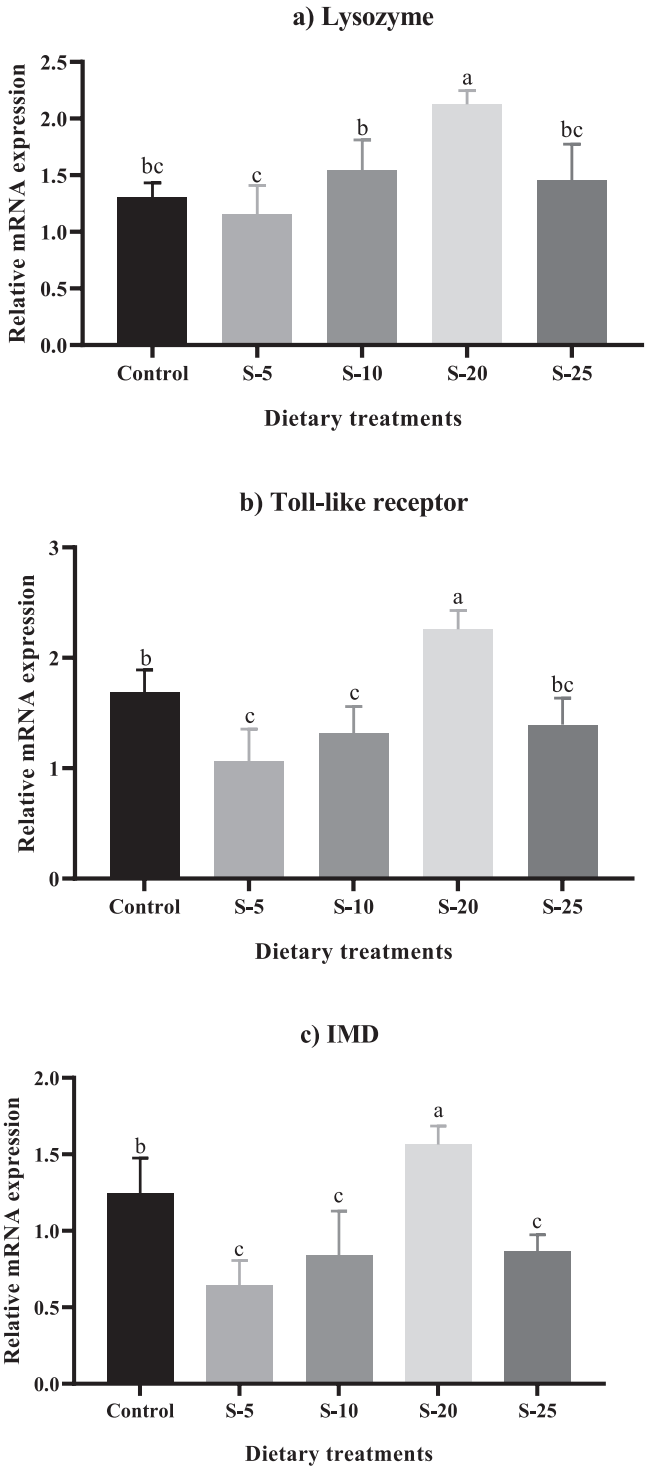

Fig. 2. : Relative mRNA expression of immune genes a) Lysozyme; b) Toll-like receptor; c) immune deficiency (IMD) in the gill tissues of *P. vannamei* fed diets containing different levels of SCP for 90 days. Values expressed as means ± SD represented by vertical bars and error indicator for each treatment (n = 4). Values with different superscripts indicate significant difference ($p < 0.05$) as determined by one way ANOVA followed by Duncan's multiple range test.

has been imputed to the poorly digestible cell wall and membranes of the microbial cells (Øverland et al., 2010; Rajesh et al., 2022). The cell rupture by downstream processing of the bacterial protein used here might be an important aspect to look into while increasing the digestibility of these protein sources. The plausible reason for the high digestibility in the present study might be attributed to the method of the downstream process carried out in the preparation of this SCP.

Some earlier studies with finfish have reported other beneficial effects of dietary inclusion of SCP at the digestive level. In the Atlantic salmon, Romarheim et al. (2011) reported that dietary bacterial SCP prevented the development of soybean meal-induced enteritis, an issue of concern. They also found that this was found to be dose dependant (Romarheim et al., 2013a) and mainly related to the cell wall fractions of the SCP (Romarheim et al., 2013b). In the rainbow trout, Rajesh et al. (2022) noted that dietary SCP inclusion levels of even upto 480 g/kg did not cause any abnormal changes in the stomach, pyloric caeca, posterior intestine, liver and kidney. Even in some animal and human models, the potential of SCPs to modulate immune response and attenuate epithelial injuries, colitis or dendritis by enhancing the digestive barrier function is receiving more attention (Klieveland et al., 2013; Indrelid et al., 2017; Aaen, 2019). Given that penaeid shrimp solely rely on their innate immune system to protect themselves against pathogens (Vazquez et al., 2009; Cerenius et al., 2010) and that lysozyme is considered one of the earliest known antibacterial proteins implicated in innate immune response in crustaceans (Sotelo-Mundo et al., 2003), its activity and expression are often used as a pertinent biomarkers of innate immunity in crustaceans (Zheng et al., 2017; Yan et al., 2020). The gradual increase in the transcript levels of lysozyme as observed here in shrimp fed diets with increasing levels of SCP upto 200 g/kg possibly reflect the immunostimulatory properties of the SCP used. The two major immune signalling pathways that are considered to be involved in the recognition of and response to pathogenic bacteria and viral infection in shrimp are Toll like receptors (TLRs) (Li et al., 2018) and IMD (Wang et al., 2020). The upregulation of TLR and IMD transcript levels in shrimp fed a diet with 200 g/kg SCP (Fig. 2), suggests the plausible role of dietary SCP in the innate immunity of shrimp to protect against pathogens, with the recognition and resistance against pathogens as was also suggested by Santos et al. (2019). The putative beneficial role of dietary nucleotides on immune response and growth in white shrimp (Andrino et al., 2012) is another point worth considering.

5. Conclusion

Given the good nutrient digestibility of the SCP, good feed intake, excellent growth, feed efficiency and good survival of shrimp observed over 90 days of the study under semi-practical farming conditions clearly suggest that the single cell protein, derived from methane utilising bacteria, holds much promise as a sustainable and reliable alternative protein source to replace a significant proportion of fishmeal in the diets of Pacific white shrimp. The responses in terms of biomarkers of the immune system of shrimp are of great interest warranting further dedicated studies.

CRediT authorship contribution statement

Nathan Felix: Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. **Kalidoss Manikandan:** Investigation, Formal analysis, Writing – original draft. **Arumugam Uma:** Resources. **Sadasivam J. Kaushik:** Conceptualization, Methodology, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The financial expenses of this study were partly met by String Bio Pvt. Ltd., Bengaluru, India through a collaborative industry-institute project between Tamil Nadu Dr. J. Jayalalithaa Fisheries University (TNJFU) - Directorate of Incubation and Vocational training in Aquaculture (DIVA), Tamil Nadu, India and String Bio Private Ltd., Bengaluru, India. The authors would like to express their gratitude and sincerely thank all staff of TNJFU - DIVA for their help during the study. The authors thank the Director, TNJFU - DIVA for providing the Indoor aquaculture facility to conduct the growth and digestibility trial. The authors would also like to thank the Professor and Head, TNJFU - State Referral Laboratory for Aquatic Animal Health (SRLAAH) for the assistance in the gene expression studies.

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