Evaluating the toxicity and immunomodulatory activity of a probiotic feed on *Litopenaeus vannamei*

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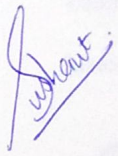
DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Evaluating the toxicity and immunomodulatory activity of a probiotic feed on *Litopenaeus vannamei***" is based on the results of investigations carried out by me in the Discipline Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Samantha Fernandes D'Mello and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.



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COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Evaluating the toxicity and immunomodulatory activity of a probiotic feed on *Litopenaeus vannamei***" is a bonafide work carried out by Mr. Shushant Shashikant Majalikar under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The study of use probiotics and the immune response of aquatic organisms has emerged as an important area of research in aquaculture science. Understanding how probiotics influence immune parameters in aquatic species such as shrimp (*Litopenaeus vannamei*) holds significant value for enhancing disease resistance and overall health in aquaculture systems. This study aims to investigate the impact of probiotic SKSF4, administered via newly formulated feed supplementation, on key immune parameters of *L. vannamei*, with a particular focus on haemocyte count, total plasma protein concentration, respiratory burst activity, phagocytosis, and Phenoloxidase enzyme activity. Also, an initiative to establish a stable and immortal hepatopancreatic cells and haemocyte cell lines is carried out. By determining the immunomodulatory effects of probiotics *in L. vannamei*, this research tends to contribute to the development of sustainable aquaculture practices aimed at improving shrimp health and productivity.

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Abbreviations

- ℃ - Degree Celsius
- ml – Milli litre
- µL – Micro litre
- mg – Milli gram
- g- Gram
- % - Percentage
- Min – Minutes
- Abs – Absorbance
- DPPH – 2,2-diphenyl-1-picrylhydrazyl
- DNSA – Dinitro salicylic acid
- CAC- sodium cacodylate buffer
- NBT- Nitro blue tetrazolium
- FBS -Fetal Bovine Serum
- L-15 – Leibovitz's L-15 Medium
- MTT - 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- RT – room temperature
- CFU – colony forming unit

- NA - nutrient agar
- NB – nutrient broth
- F+P: Feed supplemented with probiotics
- F-P: feed without probiotic

ABSTRACT

Aquaculture of shrimps worldwide is facing an economical loss due to high price of the available feed and the various infections and diseases. To overcome this, antibiotics were used which led to increase in environmental hazards and accumulation of antibiotics in the shrimp tissue. This study mainly focuses on the use of economically feasible feed along with probiotics SKSF4 as an immunomodulatory effect to enhance the immunity of *L. vannamei* culture. The probiotic was added to the feed which showed significant rise in the total plasma protein, respiratory burst activity, Phenoloxidase concentration , phagocytic activity and rise in total hemocyte count as compared to the control feed . Cytotoxicity analysis revealed that the probiotic feed had no adverse effect on the shrimps when given at a highest dosage of 10^{11} CFU/mL. No stable and immortal shrimp cell lines have been established till date which presents a great field for research. Primary cell culture of haemocytes and hepatopancreatic cells was carried out and various tests such as transfection of the cells with SV 40 vector, MTT assay and DAPI staining was conducted to enhance the cell stability.

CHAPTER - I

1. INTRODUCTION

1.1 BACKGROUND

Aquaculture is the practice of cultivating and farming aquatic organisms, including fish, shellfish, and aquatic plants, in controlled environments such as ponds, tanks, or ocean enclosures (FAO, 2020). As the world's population continues to grow, there is a rising demand for aquatic food products. However, production from traditional capture fisheries has plateaued, with many key fishing areas reaching their maximum capacity. Relying solely on capture fisheries will not suffice meeting the increasing global demand for aquatic food. Therefore, aquaculture is being recognized as a crucial opportunity to address the gap between supply and demand for aquatic food in most regions worldwide (Subasinghe et al., 2009). For over 40% of the world's population, aquatic animals contribute 20% of the animal protein in their diet. Furthermore, the rate of consumption growth for aquatic animal protein exceeds that of all other sources of animal protein combined (*The State of World Fisheries and Aquaculture 2020*, 2020).

In 2016, Asian production dominated the global aquaculture landscape, contributing 89% of the total production of 80 million metric tons (mmt) followed by, 2.9 mmt, South America with 2.3 mmt, Africa with 2.0 mmt, North America with 1.0 mmt, and Oceania with 0.2 mmt. India along with China and Indonesia are recognized as the leading aquaculture producers (Garlock et al., 2020).

Shrimp stands out as a highly popular and crucial aquaculture commodity, playing a significant role in the global seafood industry. The aquaculture of shrimp has rapidly emerged as one of the fastest-growing sectors worldwide. India, notably, holds a prominent position as one of the leading exporters of shrimp (FAO, 2018). In the realm of shrimp aquaculture, the worldwide production of white Leg shrimp (*L. vannamei*) and black tiger prawn (*Penaeus monodon*) demonstrated elevated production rates. These particular shrimp species were predominantly chosen for cultivation due to the

substantial yields they provided (Tacon, 2017). The white leg shrimp, which is indigenous to tropical marine environments in Asia South America, Peru, Mexico, and Central America, prefers constant temperatures of 20 °C or above. The high market demand for these species has led to a substantial expansion in shrimp cultivation in India in recent decades. Approximately 176,000 hectares of land are dedicated to the cultivation of shrimp in the nation; 91% of this land is grown for *Litopenaeus vannamei*, 8% for *Penaeus monodon*, and 1% for *Macrobrachium rosenberger* (Nisar et al., 2021).

In terms of value, the White leg shrimp *L. vannamei* emerged as the leading shrimp species and aquaculture species, totalling US$26.7 billion. Notably, the major farmed crustacean species commanded significantly higher unit values compared to most farmed fish species due to its wide range of salinity, SPF brood stock and unique taste (Tacon, 2020).

Commercial feeds designed for shrimp and prawns command higher prices compared to other feed options, leading to an elevated market cost for these seafood products. Despite the relatively expensive feeds, shrimp cultivation remains a profitable venture, primarily due to the high market value associated with these aquatic products. These feed mainly contain 40% proteins, 6% crude lipids followed by 3% of fibre (Cannon et al., 1975).

Antibiotics are widely employed in shrimp farming for disease prevention and treatment, but their use can have harmful effects on cultured organisms, coastal ecosystems, and contribute to antibiotic-resistant pathogens. Despite these concerns, there is limited information on appropriate antibiotic administration practices (Holmström et al., 2003a). As a result of the inclusion of antibiotics in feed, adverse effects have been observed, due to which, the utilization of probiotic bacteria has been proposed for incorporation into shrimp feed formulations (Farzanfar, 2006).

Alternatively probiotics consisting bacterial consortium was also found to be effective in improvising the immunity of shrimps by elevating innate and humoral immunity.(Hoseinifar et al., 2018). (Ringø & Song, 2016; Suva et al., 2017) reported that Numerous species of *Bacillus* probiotics are recognized for their ability to boost the host's innate and adaptive immunity through immunostimulatory effects and the promotion of beneficial gut microflora.

Establishing a stable shrimp cell line is crucial for diagnosing and preventing both present and emerging prawn viruses. Additionally, it is essential for studying the interactions between viruses and host cells, as well as understanding the mechanisms of viral infection (Assavalapsakul et al., 2003).

Successful subculture of primary cell cultures is essential for the development of a continuous cell line. However, the subculture of primary shrimp cell cultures presents challenges. Conventional methods such as trypsin solution or mechanical detachment are ineffective, as shrimp cells struggle to reattach and proliferate in vitro after being dislodged (Sloane, 1975).

Despite numerous attempts since the first attempt in 1986 (Chen et al., 1986), immortalized shrimp cell lines have not been successfully established to date (Han et al., 2013).

This study is carried out to analyze the immunostimulant activity of probiotic feed consisting of bacterial consortium SKSF4 on the *L. vannamei* culture from post-larval to adult stage and to establish an immortal and stable cell line of the haemocyte and hepatopancreatic cells of *L. vannamei.*

1.2 AIM AND OBJECTIVES

Aim:

To study the effect of a formulated economical feed with probiotic consortium SKSF4 on *L. vannamei* culture from post-larvae to adult stage.

Objectives:

1. To mass produce a formulated probiotic feed and assess its stability in-vitro.
2. Assessing the impact of the prepared feed on the growth and immunity of *Litopenaeus vannamei* (post larval to adult stage).
3. In-vitro and in-vivo toxicity evaluation of the prepared feed.
4. Primary cell culture of haemocytes and hepatopancreatic cells.

1.3 RESEARCH HYPOTHESIS

In this study, it is hypothesize that the administration of probiotics SKSF4, along with formulated feed supplements, will elicit significant alterations in key immune parameters of *L. vannamei*. Specifically, we aim to understand that a probiotic treated shrimp would exhibit an increase in haemocyte count, total plasma protein concentration, increased respiratory burst activity, increased Phenoloxidase enzyme activity and phagocytotic activity compared to control groups. We also intend to understand the safety of the formulated feed on shrimps from post-larval stage to adult stage. Along with immune parameters, a study to establish a stable cell line of hepatopancreatic cells will be carried out.

1.4 SCOPE

This study focuses on investigating the immunomodulatory effects of a newly formulated economical feed along with probiotic SKSF4 (comprising *Bacillus licheniformis* TSK71, *Bacillus amyloliquefaciens* SK27, *Bacillus subtilis* SK07, *Pseudomonas* sp. ABSK55) on *L. vannamei* (post larvae to adult phase). The experimental design will involve controlled laboratory experiments, where shrimp will be exposed to probiotics under standardized conditions. Key immune parameters, including hemocyte count, total plasma protein concentration, respiratory burst activity, and Phenoloxidase enzyme activity, will be assessed at specific time points to evaluate the immunomodulatory effects of probiotics. Also a study is conducted to standardize and establish a stable and immortal primary cell lines of haemocytes and hepatopancreatic cells. This study also aims at providing insights into the potential use of probiotics as a strategy for enhancing immune function and disease resistance in *L. vannamei*, thus contributing to the advancement of sustainable aquaculture practices.

CHAPTER - II

2. LITERATURE REVIEW

2.1 Trends in aquaculture

Aquaculture meets global seafood demand sustainably with diverse species farmed in controlled environments with the help of new Innovations that enhance efficiency and sustainability (Soto & Food and Agriculture Organization of the United Nations, 2009). It offers economic opportunities and tackles food production challenges. As a rapidly growing sector, aquaculture offers economic opportunities while addressing key challenges in food production and conservation (FAO, 2022).

Over the last 50 years, global aquaculture has grown significantly, reaching 52.5 million tonnes valued at US$98.5 billion in 2008. Asia, led by China and India, dominates production, accounting for 89% by volume and 79% by value. This expansion is driven by factors like existing aquaculture practices, population and economic growth, relaxed regulations, and rising export opportunities (Bostock et al., 2010).

2.2 Shrimp aquaculture

India has appeared to be one of the world leader in production and export of shrimp in recent years (Felix et al., 2021). However the land used for cultivation of shrimp is much less in India (160,000 Ha) as compared to other neighbouring countries like Bangladesh (216,000 Ha) (Jamal, 2023). This achievement was mainly due to replacing the low yield species i.e. black tiger shrimp with *L. vannamei*. Since commercial production of *L. vannamei* in India started in 2010 by Indian government permitting large scale cultivation along with trials and risk analysis, yet in 2019, India led the global shrimp export market, generating USD 4.7 billion, representing 24% of the total share (FAO, 2022). Vietnam, Ecuador, and Indonesia also achieved significant export success, with earnings of USD 1.98 billion, USD 2.92 billion, and USD 1.34 billion, respectively (FAO, 2022).

In many tropical regions, shallow ponds are utilized for shrimp farming, with Pacific white Shrimp (*Litopenaeus vannamei*) and the giant tiger shrimp of Asia (*P. monodon*) accounting for 22% and 58% of production, respectively (Claude et al., 1998).

The past decade, marked by the remarkable growth of vannamei shrimp, can undoubtedly be termed as the 'shrimp revolution era'.

2.3 ***Litopenaeus vannamei***

The introduction of *Litopenaeus vannamei*, commonly referred to as white leg or vannamei shrimp, has transformed the global shrimp industry in the past decade. Its remarkable yield potential, improved disease resistance, shorter production cycles, cost-effectiveness, and rising international demand have been key factors in this transformation (FAO,2020; Menaga 2020).

2.4 Viral infections and antibiotics

Infectious agents such as viruses are the main cause of the diseases that affect cultivated penaeid shrimp throughout Asia, the Indo-Pacific region, and the Americas. In the penaeid shrimp industry, outbreaks of viruses like WSSV (White Spot) and TSV (Taura Syndrome), and to a lesser extent, IHHNV (Infectious Hypodermal and Hematopoietic Necrosis Virus) and YHV (Yellow Head), have caused billions of dollars worth of lost jobs and export earnings (Lakshmi et al., 2013).

Antibiotics are widely applied to shrimp farming to prevent and treat diseases. However, their extensive use can harm the cultured organisms, disrupt coastal ecosystems, and

contribute to the rise of antibiotic-resistant pathogens. Yet, there's limited guidance on the proper antibiotic administration practices (Holmström et al., 2003b).

2.5 Probiotics in enhancing immunity of *Litopenaeas vannamei*

Probiotics, are live microorganisms which whenincorporated as feed additives or included to aquaculture water, positively impact the host (Fernandes et al., 2021). Their use in aquaculture improves feed efficiency, balances gut flora, boosts immune functions, and lowers pathogen presence(Kumar et al., 2016). Probiotics frequently employed in aquaculture are derived from the intestinal flora of both aquatic and terrestrial animals (Van Hai & Fotedar, 2010). Probiotics are being considered for use as therapeutic agents rather than antibiotic treatment (H. Liu et al., 2014).Various probiotics, including *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Enterococcus, Vagococcus, Bacillus, Clostridium butyricum, Micrococcus, Rhodococcus, Brochothrix, Kocuria, Pseudomonas, Aeromonas, Shewanella, Enterobacter, Roseobacter, Vibrio, Zooshikella, Flavobacterium,* and certain yeasts, are commonly employed in aquaculture to manage infectious diseases and enhance overall health, contributing to the improved quality of aquaculture production (Hoseinifar et al., 2018). The halotolerant probiotic SFSK4 boosts immunity of *Litopenaeus vannamei* by increasing proteins related pattern recognition and cell mediated immunity. It is non-toxic and could serve as an eco-friendly water supplement, enhancing shrimp resistance to vibriosis (Fernandes et al., 2021b). Also algae produces bioactive compounds that enhance the immune response by stimulating the production of haemocytes in reaction to oxidative stress and combating infectious diseases like *V. parahaemolyticus* or WSSV (Anaya-Rosas et al., 2019). *Lactobacillus plantarum* at a dosage of 10^{10}CFU/Kg diet helped white shrimp resist

Vibrio alginolyticus and improve both innate and humoral immune responses. However, more investigation is needed to understand the humoral immune factors, including antimicrobial peptides, in shrimp that have been administered *Lac. Plantarum* (C.-H. Chiu et al., 2007). The application of probiotics in various shrimp species has enhanced innate immunity, also known as natural or non-specific immunity. Numerous studies have shown that the utilization of probiotics promotes the production of cellular components, including phagocytosis, encapsulation, formation of nodules, as well as humoral components such as anticoagulant proteins, agglutinins, and phenol oxidase enzyme (Song et al., 2014). The confirmation of increase in shrimp immunity against viral diseases through the use of probiotics was further validated through RNA interference (RNAi) assays (Kawai & Akira, 2006).

The immune system of crustaceans primarily comprises haemocytes and active factors present in or released from haemocytes into the haemolymph. These elements collectively constitute a nonspecific immune system, acting as a defence mechanism against pathogen invasion (Liu et al., 2017). Key indicators such as THC (Total Haemocyte Count), lysozyme, phenol oxidase, superoxide dismutase, and antibiotic peptides play crucial roles in assessing a prawn's immunity and contribute significantly to the overall functionality of the immune system (Liu et al., 2017).

2.6 Cell culture of *Litopenaeus vannamei*

Numerous publications covering primary cell cultures from various organs across different shrimp species such as *L. vannamei, Penaeus monodon* utilizing various media in maintenance of these cells have already been reported in literature over the years (Kawai & Akira, 2006).

The scarcity of shrimp cell cultures and the absence of stable continuous cell lines have constrained research efforts. Therefore, the maintenance and standardization of primary cell cultures are crucial for advancing further studies. In comparison to other tissues and organs of penaeid shrimp, the lymphoid organ has been favoured by a majority of researchers due to its convenient in vitro performance. Optimal results in terms of cell survival and performance for primary cell cultures of the lymphoid organ were achieved using a double-strength L-15 medium supplemented with 20% FBS (Fan & Wang, 2002). L-glutathione has been proven to be helpful for haemocyte survival of *L. vannamei in vitro* which might be due to the strong anti-oxidant activity of GSH (Dantas-Lima et al., 2012). Reductions in the survival, growth, feed utilization and haemocyte populations of *L. vannamei* was observed when exposed to CO_2-driven acidified seawater by elevating levels of antioxidants, lipid peroxidation, and metabolic enzymes, indicating that the shrimps were experiencing both free radical stress and metabolic stress (Hsu et al., 1995).

CHAPTER –III

3. MATERIALS AND METHODOLOGY

MATERIALS REQUIRED

Chemicals Used

- Nutrient broth
- Sodium hydroxide
- Sodium chloride
- Glucose
- Bovine Serum Albumin
- Latex beads
- Heparin
- D, L- DOPA
- Nitro tetrazolium blue
- Sodium cacodylate buffer
- Ascorbic acid
- Bradford Reagent
- DPPH
- Bovine serum albumin
- DAPI
- MTT reagent
- Glutaraldehyde
- Trypsin-EDTA
- Phosphate buffered saline

Apparatus

- SORVALL ST 8R refrigerated bench-top centrifuge
- UV mini 1240 UV-Vis spectrophotometer
- Refractometer
- pH meter (pH 700, Eutech Instruments, Thermo Fisher Scientific, India)
- Autoclave
- Laminar Air Flow
- Mortar and pestle
- Hot air oven
- Refrigerator
- Coolsafe Lyophilizer
- Bio rad Elisa plate reader
- CO_2 incubator

3. METHODOLOGY

3.1. Preparation of formulated feed

3.1.1. Preparation of feed ingredients

a) Spirulina

Spirulina was cultured in 2L of Zarrouk's media and incubated in sunlight for 20 days. After 20 days spirulina biomass was harvested using a sieve of pore size 0.45 micron. The harvested sample was sundried to obtain dry powder.

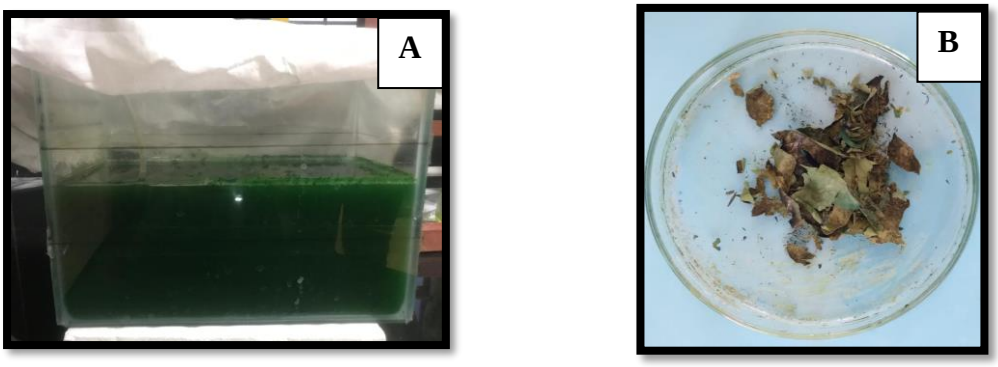


Fig 01 : Production of Spirulina A) Spirulina cultured in Zarrouk's media B) Sundried spirulina powder

b) Artemia

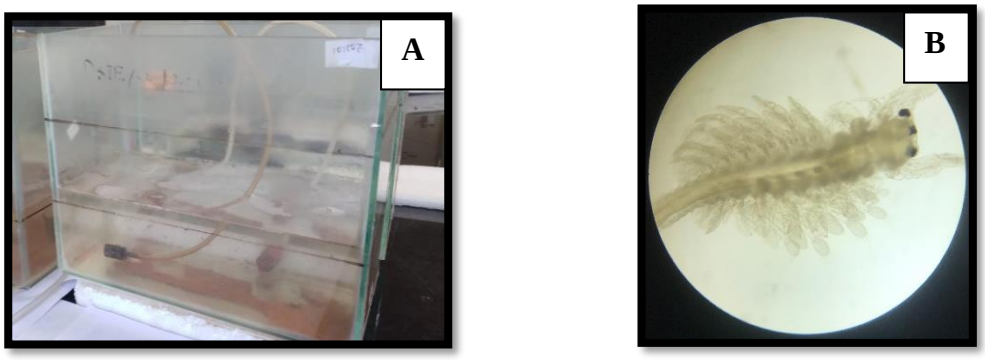


Fig 02 : Artemia culture A) Hatching and maintenance of artemia B) Artemia under 400x magnification

Artemia cysts were purchased from a retail outlet and inoculated in a tank containing 4 litres of seawater (30 ppt salinity) and an aeration supply. The growth was observed for a week. Once the artemia reached the juvenile phase, tank water was filtered using a vacuum filter (0.45 µm membrane) and artemia biomass was sun dried and stored.

c) <u>Shrimp waste</u>

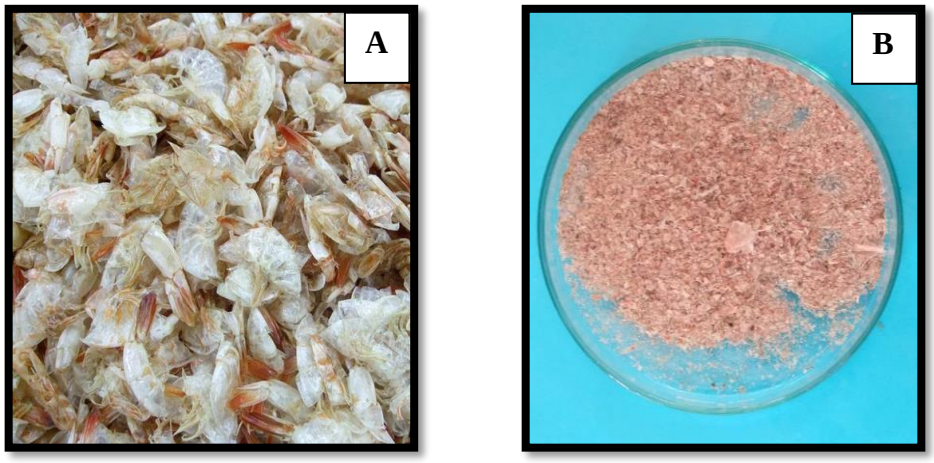


Fig 03 : Shrimp shell A) dried head and shells of shrimp B) shrimp shells ground into powder

Shrimp shells were collected from a local supermarket and sundried for a week. Once dried the shrimp waste was ground in a mixer and the crude waste was sieved to obtain a fine powder which was stored at room temperature until further use.

d) <u>Pea flour</u>

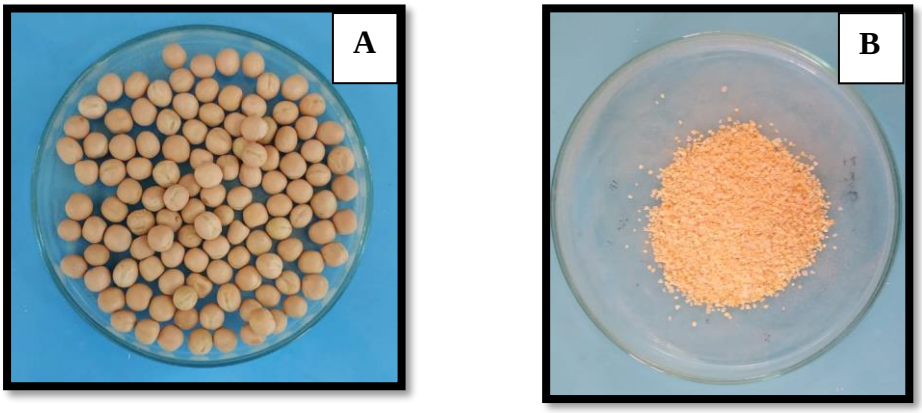


Fig 04 : Pea flour A) Yellow peas dried B) Peas ground to powder

Yellow peas were purchased from a local supermarket and milled using a mixer. Further, the pea flour was sieved to obtain a fine powder which was used as a binder in the feed preparation.

e) <u>Fish meal</u>

Fig 05 : Fish meal A) fish meal bought from a local vendor B) fish meal weighed and added to the feed

Fish meal was bought from a local vendor and used as a protein source feed additive.

3.2 Preparation of probiotic consortium

The previous studies by Dr. Samantha Fernandes (Fernandes et al., 2019) stated that using 4 bacterial strains isolated from a salt pan can be used as a water additive to enhance the immunity of *L. vannamei*. This study focuses on assessing the probiotic application of these strains as a feed additive.

The bacterial strains SK07, SK27, ABSK55, and TSK71 (*Bacillus subtilis, Bacillus amyloliquefaciens, Pseudomonas* sp. and *Bacillus licheniformis*) were mass cultured in 1000 mL of Nutrient broth and incubated for 48 hours in a shaker incubator. After incubation, the bacteria were harvested by centrifuging at 5000 rpm for 5 - 6 mins at 4 ºC. The pellet obtained was washed with 1XPBS twice followed by the addition of 10 % sucrose to the tubes as a cryoprotectant before freezing. The cells were frozen at -20 ºC for 2 hours and later transferred to a -80 ºC freezer. The frozen bacterial pellet was lyophilized for 2 days at -110ºC.

Fig 06 : Formulated feed A) Feed pellet machine B) lyophilized probiotic consortium C) Dough D) Formulated feed

3.3 Feed quality analysis

3.3.1 Estimation of physical parameters of the formulated feed

Physical parameters such as colour, size, sinking rate, and water stability were determined.

Shrimps are benthic organisms which mostly like settle at the bottom in the ocean and feed with high sinking rate is preferred (Obaldo et al., 2002). For sinking rate 10 pellets of each formulated feed (with probiotics, without probiotics, and control feed) were added to a beaker containing seawater (10 ppt salinity). The number of pellets was counted at different time intervals and the sinking rate was estimated at the time intervals of 0, 3, and 5 minutes

Fig 07 : Determination of sinking rate of the feed

3.3.2 Biochemical estimation of the feed

a) Estimation of Carbohydrates

DNSA method was used for the determination of reducing sugars in the probiotic feed (Miller, 1959). Standard solution and their dilutions of varying concentrations were prepared using Glucose (1 mg/mL). For sample, 1 mg of all three feeds (F+P, F-P and control) were added to different test tubes and diluted with 1 ml distilled water. 0.5 ml of DNSA reagent were added to the tubes and mixed thoroughly. Then the tubes were kept in boiling water bath for 10 minutes and absorbance was measured at 540 nm spectrophotometrically.

b) Estimation of Protein

Protein estimation was done by Folin-Lowry method (Lowry et al., 1951). Feeds (F+P, F-P and control) weighed (1 mg) precisely were dissolved in 1 ml distilled water for protein estimation. 1 mg/ml standard solutions were prepared using BSA followed by other dilutions. To the test tubes 5 ml of Reagent C (Copper alkali solution) was added and mixed. Tubes were kept for 10 minutes incubation at room temperature. Further, 0.5 ml of Folin-ciocalteue's reagent was added in all the test tubes and left for 30 minutes incubation in the dark at room temperature. Optical density of the samples were measured at 660 nm using spectrophotometer.

c) Screening of feed for presence of antioxidant activity

DPPH method (Blois, 1958) was used to estimate the antioxidant activity of all three feed (F+P, F-P and control). 0.1 mg/ml Ascorbic acid was used for the preparation of the standard solution. The dilutions were prepared of varying concentrations using ethanol as diluent. 0.3 mM of 1 mL DPPH was added to all the tubes. Samples (1 mg) were weighed and added to different test tubes. The control test tubes were made by adding 1 ml of DPPH in ethanol. The tubes were kept for incubation in the dark for half an hour and the optical density was measured at 517 nm using a spectrophotometer.

3.4 Laboratory trials for in-vivo assessment of the formulated feed on *Litopenaeus vannamei*

3.4.1 Experimental setup for maintenance of *Litopenaeus vannamei*

Litopenaeus vannamei post-larvae (pL-10) was procured from a hatchery in Chennai, Tamil Nadu, India. Tanks were already set up holding approximately 30 liters of water (salinity-10 ppt, pH 7, and temperature – 26 – 28 ºC) with aeration supply a day before the arrival of the shrimps. The shrimps were acclimatized for three weeks before the experiment was started.

Three experimental glass tanks were set up: (i) Tank treated with formulated feed along with probiotics (dosage 10^9 CFU/mL), (ii) Tank treated with formulated feed without any probiotics, and (iii) Control tanks which were fed with commercial shrimp feed. In each tank, the shrimps were stocked at a stocking density of 40 shrimps /tank.

Feeding was carried out twice a day i.e. 9:30 a.m. and 5:30 p.m. by placing the shrimp feed on a check tray to mimic the aquaculture farm condition. The tanks were maintained with aerator filters and cleaned once a week by changing 50 % of the water in the tank.

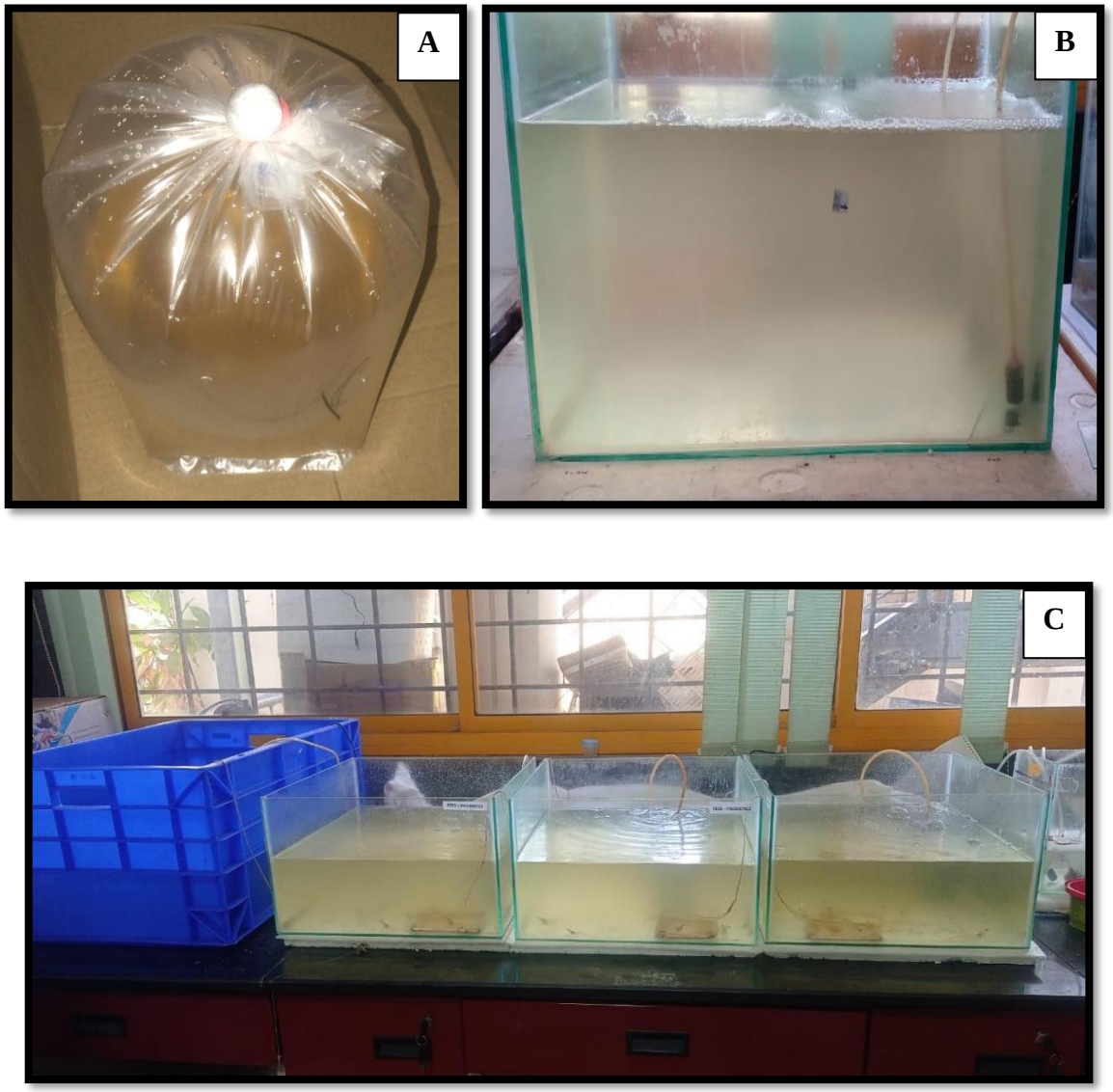


Fig 08 : Larval collection and experimental setup (A) specific pathogen free brood stock larvae of *L. vannamei* (B) post larvae Day-10 acclimatized in stock tank (C) Experimental setup

3.4.2 In-vivo analysis of the formulated feed on the growth and immunity of *Litopenaeus vannamei*

3.4.2.1 Growth Analysis of *Litopenaeus vannamei*

The effect of feed (control, formulated feed without probiotics SKSF4, and formulated feed with probiotics SKSF4) on shrimps was recorded within the time intervals of 10 days for 80 days. The weight gain, specific growth rate, and feed efficiency were calculated as follows (Fernandes et al., 2019):-

$$\textit{Weight gain (g)} = \textit{Final weight (g)} - \textit{Initial weight (g)}$$

$$\textit{Specific growth rate (SGR\%)} = \frac{[\ln(\text{final weight in grams}) - \ln(\text{initial weight in grams})]}{\text{Time}} \times 100$$

$$\textit{Feed efficiency} = \frac{\textit{Final weight (g)} - \textit{Initial weight (g)}}{\textit{Total feed intake}}$$

3.4.2.2 Evaluation of immunological parameters

The effect of the formulated feed on the immune system of *L. vannamei* was assessed after 80 days of the experiment.

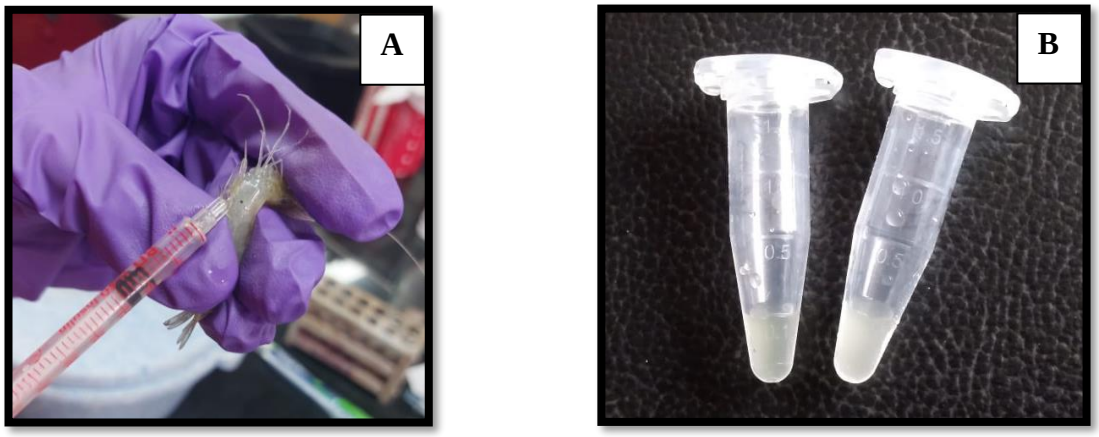


Fig 09 : Collection of haemolymph *from L. vannamei* (A)Extraction of haemolymph from ventral sinus cavity (B) Haemolymph sample collected

a) Total Haemocyte Count

A 26 gauge needle along with 1 ml syringe prefilled with 200 µL anticoagulant solution was used to extract haemolymph (100 µL) from the ventral sinus cavity of shrimp. Shrimps were randomly selected from experimental tanks receiving formulated feed containing probiotic SFSK4, tanks without probiotic SFSK4, and control feed, respectively.

10 µL aliquot of the haemolymph-anticoagulant combination was placed onto a Neubauer hemacytometer, and the haemocytes were counted at 400X magnification under a compound microscope. The total haemocyte count was determined in terms of number of cells/mL (Fernandes et al., 2021).

$$\textit{Total haemocyte count} = \frac{\textit{No of cells counted x Dilution factor x } 10^4}{\textit{No of squares counted on a haemocytometer (cells/ml)}}$$

b) Total plasma protein

Haemolymph was extracted from the ventral sinus cavity of shrimps using a 26 gauge needle coupled with a 1 ml syringe containing 200 µL of anticoagulant solution. In 3 tubes 100 µL of haemolymph was collected from shrimp of each experimental tank and 3 ml of Bradford reagent was added. The tubes were incubated for 10 minutes at room temperature and the protein concentration was determined spectrophotometrically at 600 nm. 1 mg/mL of Bovine Serum Albumin (BSA) was used as the standard. The total plasma protein was expressed in terms of mg/mL (Fernandes et al., 2021).

c) Phagocytotic activity assay

The haemocytes were isolated from the haemolymph by centrifugation at 2500 rpm (i.e. 1169 ×g) for 5 minutes at 4 °C. The mixture of haemocytes along with 100 µL of latex beads (polystyrene particles with a diameter of 1.1 µm, Sigma) in an anticoagulant solution was prepared on a glass slide. The mixture was then placed in a moist chamber and incubated for 30 minutes at 28 °C, followed by fixation using 2.5% glutaraldehyde. After fixation, the slides were rinsed with phosphate-buffered saline to eliminate non-adherent haemocytes, dried, and stained with Giemsa stain (Himedia, S011) (Fernandes et al., 2021b). The count of phagocytizing cells was conducted by observing 50 randomly selected cells under a magnification of 1000 X. Phagocytosis was calculated as follows

$$\textbf{\textit{Percentage phagocytosis}}\ (\%) = \frac{\textbf{\textit{Number of cells ingesting beads}}}{\textbf{\textit{Number of cells observed}}} \times \mathbf{100}$$

d) Respiratory Burst (RB)

The Respiratory burst activity of the haemocytes was determined by the reduction of nitro blue tetrazolium (NBT). Briefly, 100 µL haemolymph was incubated for 30 minutes at room temperature in a 24-well microtiter plate. The supernatant was discarded and 50 µL of 0.2 % NBT was added to each well and further incubated overnight at 28 ºC. The supernatant was again discarded and the haemocytes were fixed using 200 µL of absolute ethanol. The haemocytes were washed using 200 µL of 70 % ethanol and air dried. 2 mol/KOH (120 µL) and dimethyl sulfoxide (140 µL) were used to dissolve the formazan. The contents were transferred to a 96well microtiter plate and the absorbance was read at 620 nm (Fernandes et al., 2021).

e) Phenoloxidase Concentration (PO)

Preparation of haemocyte lysate supernatant (HLS)

Haemolymph was collected from the ventral sinus cavity of the shrimps using a 26-gauge needle coupled to a 1 ml syringe which was prefilled with 200 µL of the anticoagulant. The haemocyte pellet was obtained by centrifuging the haemolymph at 2,500 rpm for 10 min at 4 ºC. The Haemocyte pellet was washed with ice-cold cacodylate (CAC) buffer (0.01 M sodium cacodylate, 10Mm $CaCl_2$, 0.45 M NaCl, 26mM $MgCl_2$, pH 7.0). The haemocyte and CAC buffer were sonicated to obtain a homogenized solution followed by centrifugation at 10,000 rpm for 20 min at 4 ºC. The haemocyte lysate supernatant obtained was used as an enzyme source (Fernandes et al., 2021).

Determination of Phenoloxidase concentration

The haemocyte lysate supernatant was used as an enzyme source to determine the activity of Phenoloxidase. Phenoloxidase activity was assessed spectrophotometrically by observing dopachrome formation using D, L-3,4-dihydroxyphenylalanine (D,L-DOPA) as the substrate and trypsin as the stimulant. A volume of 200 µL of HLS was incubated with 0.1% trypsin (200 µL) in CAC buffer for 30 minutes at room temperature, followed by the addition of 200 µL D,L-DOPA (0.3% in CAC buffer). Each reaction mixture was diluted with CAC buffer (600 µL), thoroughly mixed, and measured at 490 nm. Absorbance was measured against a blank containing CAC buffer, D,L-DOPA, and trypsin to monitor substrate oxidation. The enzyme activity was calculated as the increase in absorbance of 0.001/min, representing one unit of enzyme activity (Fernandes et al., 2021).

The enzyme activity was calculated using formula given by (Gulzar & Amin, 2012):

$$\boldsymbol{Enzyme\ activity} = \frac{\boldsymbol{absorbance} \times \boldsymbol{volume\ of\ assay} \times \boldsymbol{dilution\ factor}}{\boldsymbol{extinction\ coefficient\ of\ dopachrome} \times \boldsymbol{volume\ of\ enzyme} \times \boldsymbol{time}}$$

3.5 Cytotoxicity evaluation

3.5.1 LC_{50} Analysis of probiotic feed SFSK4

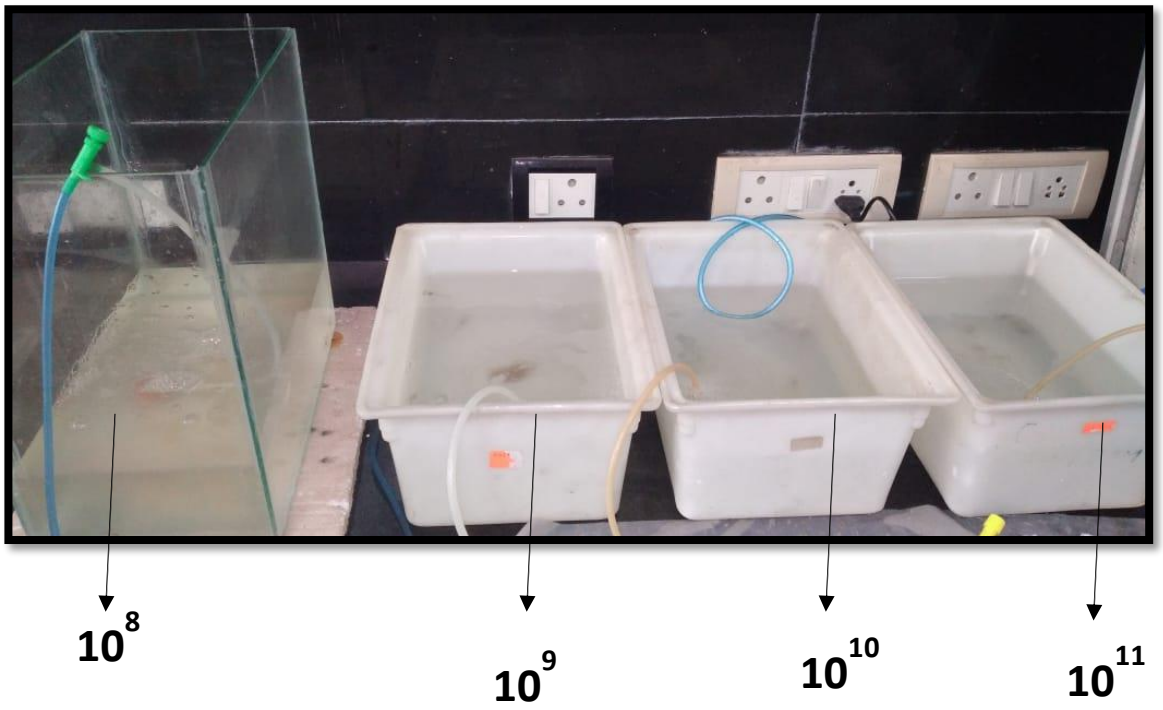


Fig. 10 : LC50 and cytotoxicity analysis of the feed with probiotics on *L. vannamei* post larvae

This experiment was carried out to find out if the formulated feed along with probiotic SKSF4's (*Bacillus subtilis, Bacillus amyloliquefaciens, Pseudomonas* sp. and *Bacillus licheniformis*) maximal dosage could be toxic to the post larvae of *L. vannamei*.

Formulated feed with 4 different concentrations of probiotics i.e.,10^8,10^9,$10^{10}$and 10^{11} CFU/mL was prepared accordingly.

8 post-larvae shrimps of *L. vannamei* were administered to 4 tanks which were fed with the formulated feed with probiotics containing bacterial consortium SKSF4 in increasing

concentrations of 10^8 ,10^9,10^{10} and 10^{11} CFU/mL and the survival rate was determined every day for 7 days (Fernandes et al., 2021).

3.5.2 Cell viability assay

Cytotoxicity of the feed (F+P, F-P, control) to the haemocyte count was measured using trypan blue dye exclusion assay 50 µL of haemolymph and 50 µL of 0.4% trypan blue in 1 x PBS were mixed in a vial. 10 µL of this mixture was added to the haemocytometer and 100 cells were counted. Viable cells appeared colourless due to intact cell membrane whereas the non-viable cells took up the stain and appeared blue in colour (Fernandes et al., 2021). The total cell viability of haemocytes was determined using formula:

$$\textbf{\textit{Viable cell count}}\ (\%) = \frac{\textbf{\textit{No. of viable cells}}}{\textbf{\textit{Total cells counted}}} \times \mathbf{100}$$

3.6 Primary cell culture of *Litopenaeus vannamei*

3.6.1 Primary cell culture of Hepatopancreatic cells

L-15 Complete media was prepared consisting of 10 % FBS and 1 % antibiotic and antimycotic solution.

Shrimps from stock tanks were selected randomly and the hepatopancreas were dissected out in a laminar air flow chamber. The hepatopancreatic cells were mechanically dislodged in 1X PBS and transferred to a vial containing 1 mL of L-15 complete media. The cells were centrifuged at 2500 rpm for 10 minutes at 4 ºC. supernatant was discarded and the pellet was resuspended in 1 mL complete L-15 media. The cell suspension was transferred to the T-25 flask containing 4 mL of complete media and incubated at 37 ºC. The cells were observed for growth and attachment after 48 hours of incubation.

Cells were dissociated by trypsinization and passaged in a new T25 flask containing the same media and the cell viability and growth was observed (Han et al., 2013).

3.6.2 Transfection of hepatopancreas with SV40 vector

Transfection of hepatopancreatic cells by SV40 vector was done using cell bio HiPer transfection teaching kit CCK042.

Briefly, 5 x 10^4 cells of the growing hepatopancreatic cells were seeded in the 24-well microtiter plate containing 500 µL of transfection media and incubated at 37 °C, with 5% CO_2 for 24 hours.

Preparation of transfection mixture I: 50 µL of complete medium and 2 µL of CCK042F (Transfection reagent 1) were added to a vial and the mixture was vortexed for 3-5 seconds.

Preparation of transfection mixture II: To 45 µL complete medium, 5 µL of CCK042H (plasmid DNA) and 2 µL of CCK042G (Transfection reagent 2) were added.

Transfection mixture 1 and Transfection mixture 2 were mixed and the mixture was incubated at room temperature for 15 - 20 mins. After incubation, the transfection mixture was gently mixed by pipetting up and down. 50 µL of this transfection mixture was added to the experimental well drop by drop. The plate was gently rocked to mix the contents and incubated at 37 °C and 5 % CO_2 for 48 hours. The mixture was not added to the control wells.

After 48 hours of incubation, the growth medium in all the wells was replaced by 500 µL 1X DPBS and gently mixed. 500 µL of fixative was added to the wells and incubated for 10 mins at room temperature. Fixative was removed and the wells were washed twice with 1X DPBS. 500 µL of staining solution was added to the wells and incubated at 37° C for 24 hours. After 24 hours, the cells were observed for production of blue colour under the inverted microscope.

3.6.3 MTT assay

100 µL of cell suspension was seeded in a 24-well micro titre plate containing 400 µL of complete L-15 and incubated at 37 °C in a 5 % CO_2 atmosphere for 48 hours. After incubation, the plate was removed out and 50 µL of MTT reagent was added and the

plate was incubated in dark for 6 hours in the incubator. After 6 hours, the plate was removed out and 100 µL of solubilizing solution was added to dissolve the formazan crystals formed in each of the experimental well. Negative control well was maintained which contained complete media and MTT.

3.6.4 DAPI staining

Approximately 5×10^5 hepatopancreatic cells were seeded to the 24-well microtiter plate containing a sterile coverslip and 500 µL of complete L-15 media. This was then incubated at 37 ºC for 48 hours. After 48 hours the excess media was removed and the coverslip containing adhered cells was washed with 1X PBS to remove the traces of the media. The cells were then fixed with 2.5 % glutaraldehyde for 10 minutes and again washed with 1X PBS to remove non-adhered cells. 0.5 % triton X solution was added to the cells to allow cell permeability and incubated for 10 minutes in the dark. After washing with 1X PBS, the cells were flooded with 0.1 µg/mL of DAPI and incubated for 20 minutes in the dark. The cells were observed under a fluorescence microscope at 358 nm.

3.6.5 Statistical analysis of data

Statistical analysis of the data was carried out using SPSS software version 23.0 (IBM Corporation, USA, 2015). The results were expressed as mean with their corresponding standard deviation (S.D.). The results were subjected to one-way analysis of variance (ANOVA). The data was considered to be statistically significant at $p < 0.05$ for nutrients screening and in-vivo studies. Subsequently, *post-hoc* multiple comparisons with Duncan's test were used to assess the significant difference between the different treatment groups.

CHAPTER - IV

4. ANALYSIS AND CONCLUSION

4.1 Feed preparation

Feed was prepared according to prior studies (Shinde, 2023 – dissertation report) using 5 feed ingredients mentioned in the table. Each ingredient was calculated according to Pearson's square method and used to formulate the feed for *L. vannamei* (Table 4)

Table 01. Feed ingredients selected based on the nutrition they provide

Sr. No.	Name of ingredient	Source of nutrition
1.	Pea flour meal	Binder and Carbohydrate
2.	*Spirulina platensis*	Protein, vitamin, minerals
3.	Shrimp waste	Lipids, carbohydrate and Protein source
4.	*Artemia salina*	Protein and carbohydrate
5.	Fish meal	Protein

0.1 g of lyophilized probiotics contained 4×10^9 CFU/mL of bacteria.

Table 02. Quantity of ingredients used to prepare the shrimp feed

Sr. No.	Ingredients	For 20 g of dry feed
1.	Shrimp waste meal	3.18 g
2.	Spirulina	3.62 g
3.	Artemia	3.62 g
4.	Pea flour	6.37 g
5.	Fish meal	3.18 g
6.	Bacterial consortium	0.1 g

The dough was made using the above ingredients and probiotics were added post-steaming before obtaining small pellets using a pellet machine.

4.2 FEED QUALITY ANALYSIS

4.2.1 Estimation of physical parameters of the formulated feed

a) Sinking rate

Table 03. Results of sinking rate of feed

Feed	At 3 mins	At 5 mins
F+P	50 %	80 %
F-P	60 %	80 %
CONTROL	50 %	70 %

The sinking rate of the feed was estimated to be 80 % which is termed as ideal sinking rate for benthic feeders (Obaldo et al., 2002).

b) Feed stability

The feed (F+P, F-P, and Control) was stable in water (assessed upto 24 hours) and was easily accepted by the shrimps.

4.2.2 Biochemical estimation of the formulated feed

a) Carbohydrate estimation of the feed

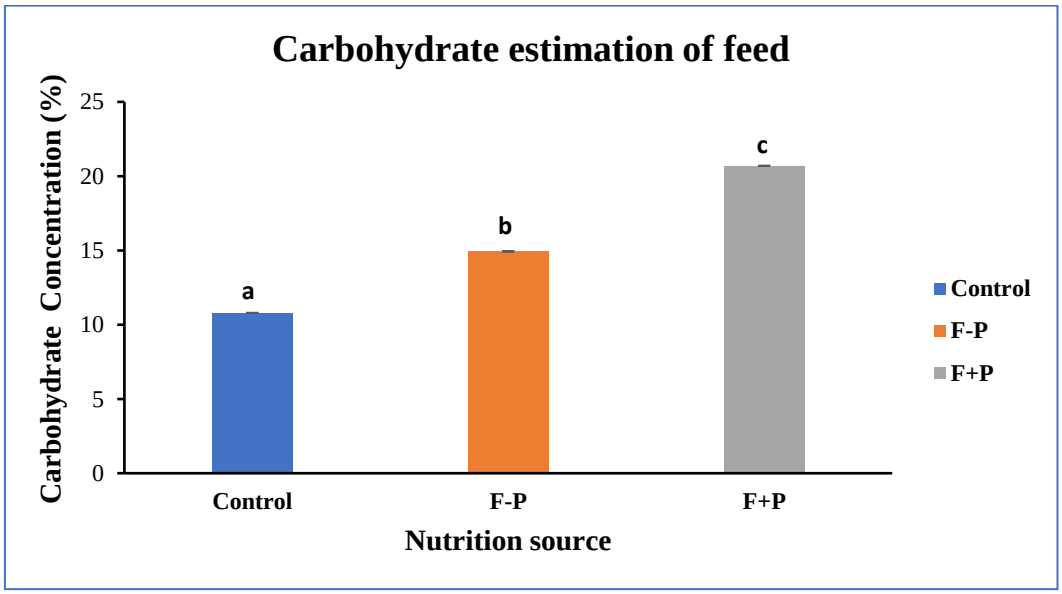


Fig 11 : Carbohydrate estimation of the feed

As seen in the Fig. 11, the concentration of carbohydrates was found to be significantly higher *($p < 0.001$)* in F+P (20.69 ± 0.3%) as compared to F-P (14.13 ± 0.5%) and control feed (10.79 ± 0.2%) respectively.

b) Protein estimation of the feed

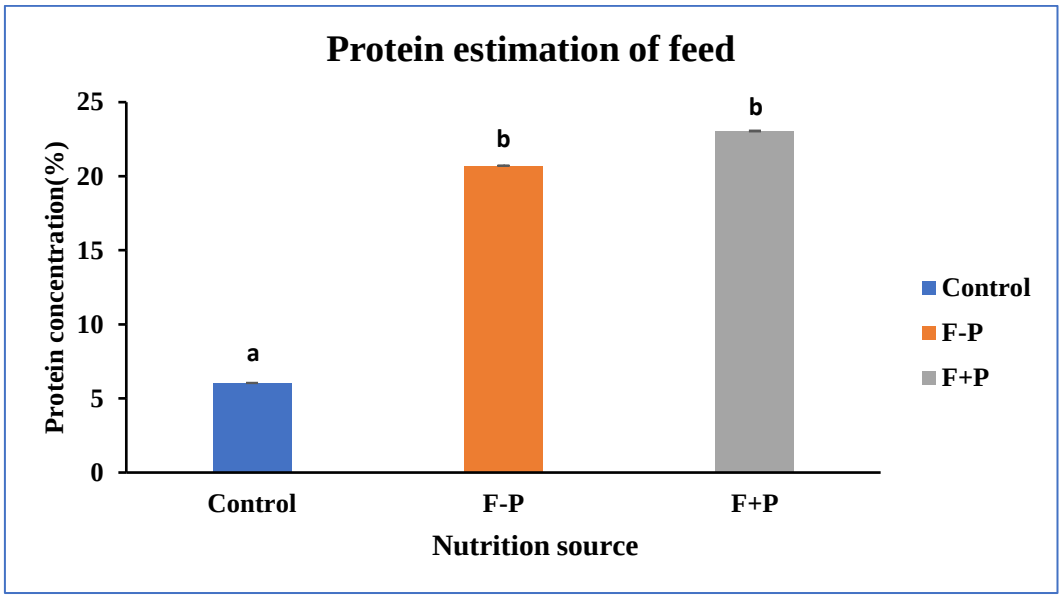


Fig 12: Protein estimation of the feed

According to Fig. 12, the concentration of proteins was found to be significantly higher *($p<0.001$)* in F+P (19.91 ± 0.5%) and F-P (19.13 ± 0.1 %) as compared to control feed (11.23 ± 0.1%).

c) Estimation of antioxidant activity of the feed

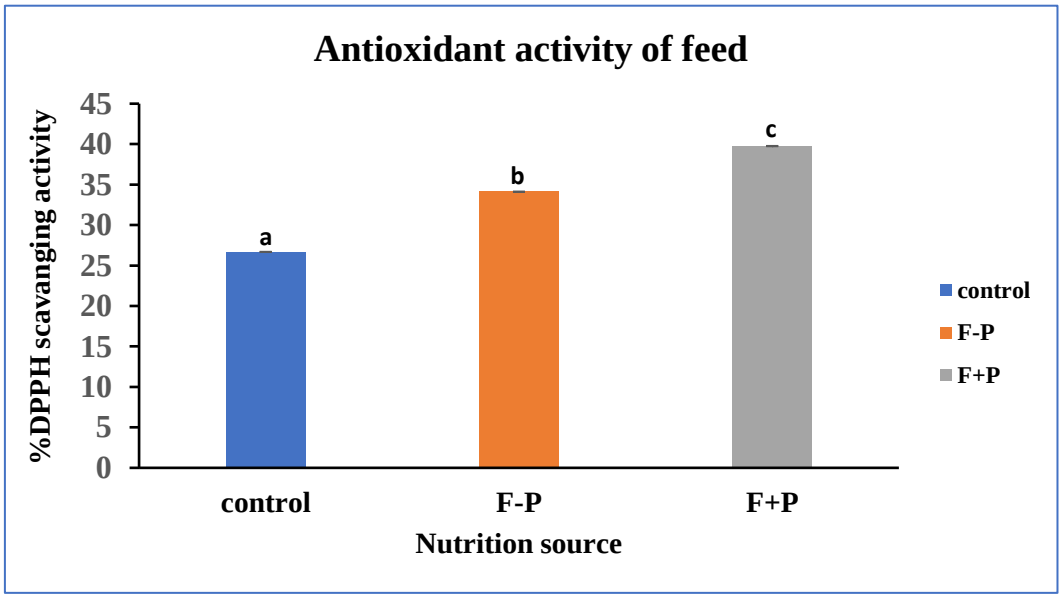


Fig 13 : Antioxidant activity of the feed

As seen in the Fig. 13, the antioxidant activity of F+P (39.75 ± 0.6%) was found to be significantly higher *(p<0.01)* as compared to F-P (34.11 ± 0.7%) and control feed (26.68 ± 0.9) respectively.

(Nesara and Paturi 2018) reported that the protein and carbohydrate content in the basal diet of *L. vannamei* was found to be 30 – 40 % and 25 – 35 % respectively. Further, (Li et al., 2017) reported that the basal feed for *L. vannamei* consists of a protein content of 30 % and a carbohydrate content of 20 %. Likewise reported by (Amaya et al., 2007), the commercial feed for *L. vannamei* consists 30 % crude protein and 15 - 20 % of carbohydrates.

4.3 Effect on Growth Performance

The effect of feed on the growth of *L. vannamei* was assessed in terms of weight gain of the shrimps at an interval of 10 days over 80 days.

As seen in Table. 04, after 80 days, the tank containing shrimps fed with F+P(2.81 ± 0.3 g) and F-P (1.83 ± 0.2 g) showed significantly higher ($p<0.05$) weight gain as compared to the shrimps fed with control feed (1.50 ± 0.3 g) respectively.

According to Table 05. Shrimps fed with F+P (7.67 ± 0.17) showed a significant ($p<0.001$) increase in specific growth rate as compared to shrimps fed with F-P (7.55 ± 0.22) and control feed (7.24 ± 0.29)

As observed in table 06. No significant difference was observed in the feed efficiency ratio among the treatment groups.

As in the above obtained results, the lower weight gain and specific growth rate in the shrimps is mainly due to high stocling density and inadequate resource allocation. As reported by (Araneda et al., 2008), higher stocking densities can often relate to lower growth rates. The inverse relation between stocking density and growth rate plays an important role in managing shrimps in aquaculture farming.

Table 04. Weight gain of *L. vannamei* for a period of 80 days

Treatments	20 days	40 days	60 days	80 days
Control	0.10	0.30 ± 0.07	0.75 ±0.07	1.50 ± 0.37[a]
F – P	0.11	0.39 ± 0.08	0.80 ± 0.11	1.83 ± 0.29[a,b]
F + P	0.12	0.45 ± 0.08	0.68 ± 0.33	**2.81 ± 0.30[b]**

Table 05. Specific growth rate of *L. vannamei* over a period of 80 days

Treatments	20 days (%)	40 days (%)	60 days (%)	80 days (%)
Control	16.11	10.51 ± 0.63	8.55 ± 0.17	7.24 ± 0.29[a]
F – P	16.64	11.29 ± 0.49	8.72 ± 0.22	7.55 ± 0.22[b]
F + P	16.27	11.31 ± 0.43	8.64 ± 0.28	7.67 ± 0.17[c]

Table 06. Feed efficiency ratio of *L. vannamei* over 80 days

Treatments	20 days	40 days	60 days	80 days
Control	16	16.42 ± 0.74	16.57 ± 0.79	16.62 ± 0.37[a]
F-P	16.07	16.49 ± 0.8	16.58 ± 0.11	16.63 ± 0.29[a]
F+P	16.02	16.49 ± 0.8	16.57 ± 0.15	16.63 ± 0.30[a]

4.4 Evaluation of immunological parameters

4.4.1 Total haemocyte count

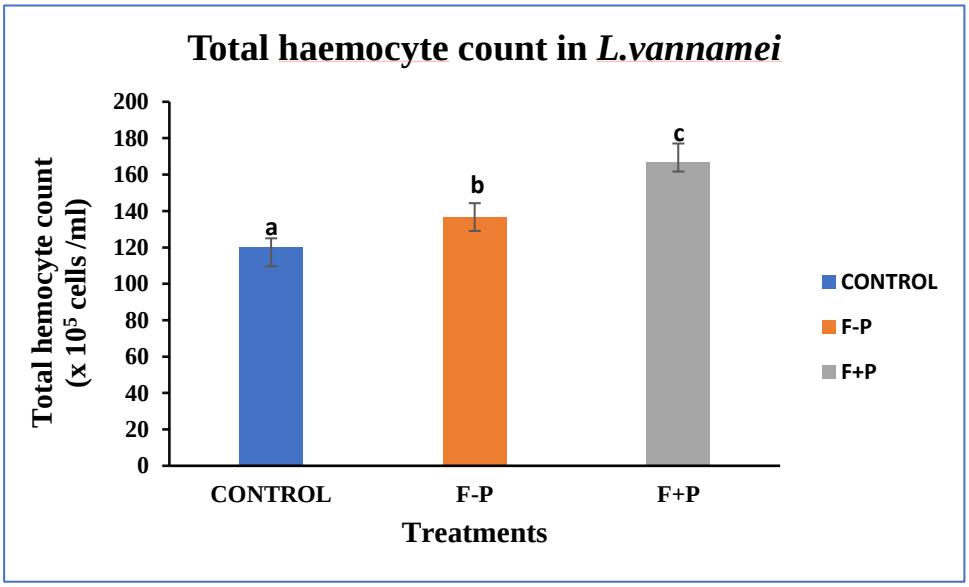


Fig. 14 : Total haemocyte count in shrimps treated with various experimental feed

As seen in Fig. 14, the Total haemocyte count in shrimps fed with F+P (167 ± 10.4 x 10^5 cells/ml) was found to be significantly higher *(p=0.001)* as compared to the shrimps fed with F-P (137 ± 7.63 x 10^5 cells/ml) and control feed (120 ± 5 x 10^5 cells/ml).

In *L. vannamei*, the administration of halotolerant bacteria such as *Bacillus licheniformis* TSK71, *Bacillus amyloliquefaciens* SK27, *Bacillus subtilis* SK07, and *Pseudomonas* sp. ABSK55 as probiotics at a concentration of 10^9 CFU/ml as a water additive for 120 days resulted in a notable increase in haemocyte count, reaching a peak of 143.67 x 10^5 cells/ml (Fernandes et al., 2021). Similarly, (Y.-C. Wang et al., 2019)demonstrated that the incorporation of *Lactobacillus fermentum, L. pentosus, Saccharomyces cerevisiae*, and *Bacillus subtilis* into feed at a dosage of 10^9 CFU per kg diet after a culture period of 56 days, led to an increase in haemocyte count, with a maximum value of 70× 10^5 cells/mL.

As compared to the literature, the formulated feed along with probiotics SFSK4 showed an increase in haemocyte count as reported by (Fernandes et al., 2021) and (Wang et al., 2019). Probiotics have the potential to produce compounds with immunomodulatory properties which directly stimulate the proliferation and production of haemocytes. Along with this, probiotics compete with pathogenic bacteria to reduce their colonization and growth in the gut. Due to this, a reduction in pathogen-induced immune suppression and integrity of immune system is maintained thus enhancing the total haemocyte count.

4.4.2 Total plasma protein

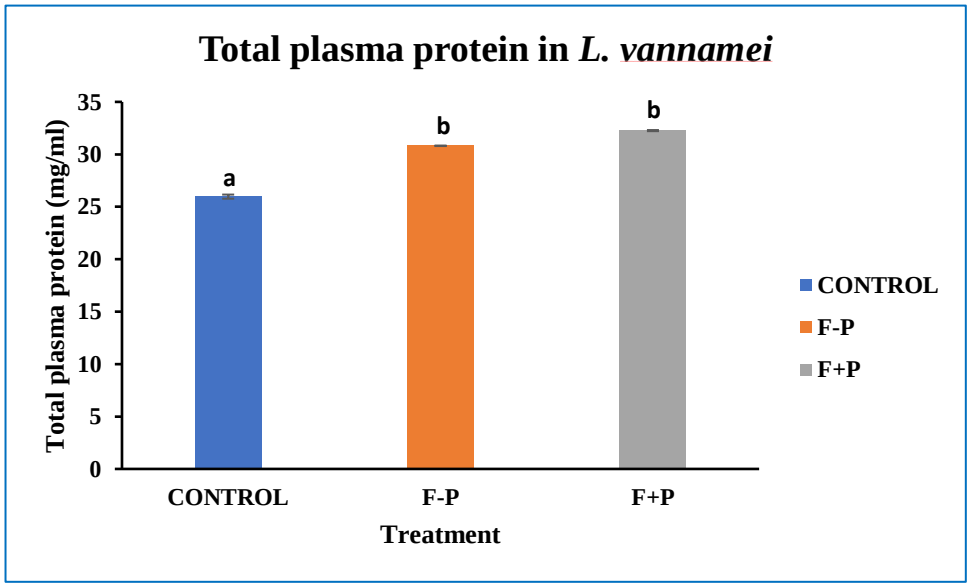


Fig 15 : Concentration of total plasma protein in shrimps treated with various experimental feed

Fig. 15 indicates that the concentration of total plasma protein was relatively higher *(p=0.004)* in shrimps fed with F+P (32.26 ± 0.05 mg/mL) and F-P (30 ± 0.02 mg/ml) as compared to the shrimps fed with control feed (25.96 ± 0.1 mg/ml).

This observation is confirmed with previous research findings, such as those by, where the use of probiotics led to elevated total plasma protein levels in shrimp. Specifically,

(Nedaei et al., 2023) reported that the incorporation of *Lactobacillus plantarum* in the diet resulted in a significant increase in total plasma protein level after a 97 day experimental period. Similarly (Fernandes et al., 2021) documented a rise in total plasma protein concentration upon administering probiotics SFSK4, comprising various bacterial strains, as a water additive during the culture of *L. vannamei.* These findings show the potential of probiotic to positively affect the immune parameters of shrimps, including total plasma protein levels, could contribute to enhanced immune function and overall health in aquaculture settings.

4.4.3 Phagocytic activity

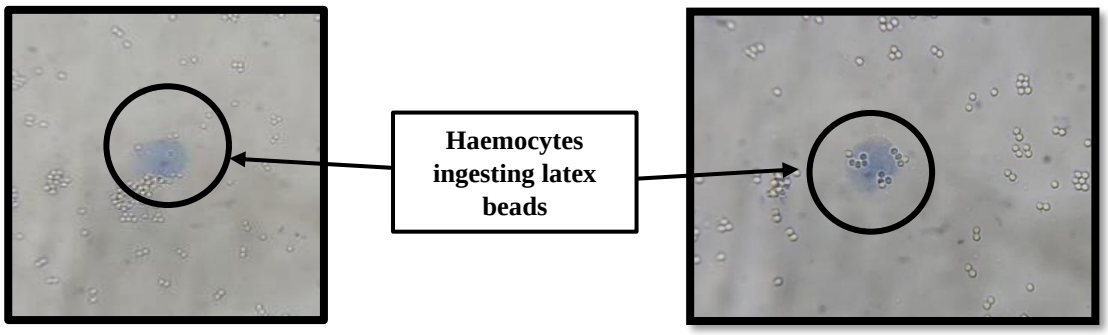


Fig. 16 : Phagocytic activity of haemocytes using latex beads

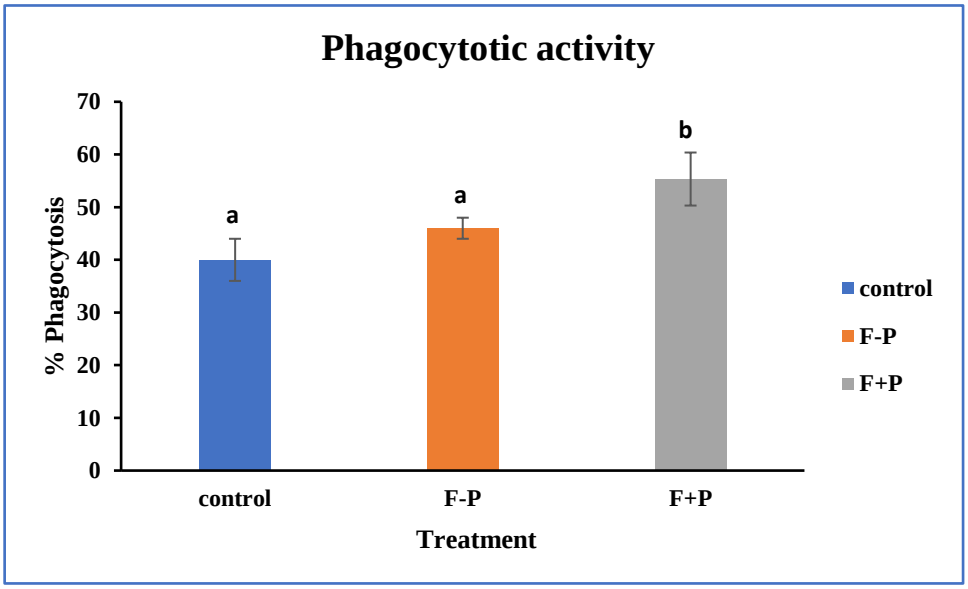


Fig 17 : Phagocytic activity of haemocytes in *L.vannamei*

As seen in Fig. 17, a significant increase *(p=0.008)* in phagocytotic activity was observed in the haemocytes of shrimps fed with F+P (55 ± 5%) as compared to (F-P 46 ± 2%) and control feed (40 ± 4%).

The observed increase in phagocytotic activity in shrimps fed with F+P shows the potential immunomodulatory effects of probiotics in *L. vannamei*. This finding is supported with previous research, such as the study conducted by (Lee et al., 2023), which reported a significant increase in phagocytotic activity of haemocytes in *L. vannamei* upon administration of probiotic FCMB by *Bacillus subtilis* at a dosage of 5 g kg^{-1} diet. Similarly, (Wang et al., 2019) documented an increase in phagocytotic activity by haemocytes of *L. vannamei* to 47 % following the administration of probiotics comprising *Lactobacillus fermentum, L. pentosus, Saccharomyces cerevisiae,* and *Bacillus subtilis* as a feed additive.

The observed increase in phagocytotic activity suggests that probiotics have the potential to increase the shrimp's innate immune response. Probiotics possess specific surface molecules such as lipopolysaccharides and also secrete molecules such as fatty acids, exopolysaccharides, and antimicrobial peptides. When ingested by the shrimps these molecules interact with phagocytic receptors present on immune cells mainly haemocytes enhancing the phagocytic activity. Also LYS and peroxinectin, a component of the immune system and part of the proPO pathway, is released following proPO system activation. It functions as an opsonin, facilitating haemocyte phagocytosis in shrimps thereby improving its ability to recognize and kill pathogens (Amparyup et al., 2013).

4.3.4 Phenoloxidase concentration

The enzyme activity of Phenoloxidase was determined spectrophotometrically and a six-fold increase in the concentration *($p<0.001$)* was observed in shrimps fed with F+P (13.39 ± 0.003 U/min) as compared to F-P (2.59 ± 0.002 U/min) and control feed (2.94 ± 0.003 U/min).

An increase in Phenoloxidase enzyme activity was noted in *L. vannamei* culture when treated with probiotics *Pediococcus pentosaceus* as a feed additive at a dosage of 1×10^8 CFU/g diet as reported by (Hong et al., 2022). Additionally, (Y. Wang & Gu, 2010) reported an increase in Phenoloxidase activity in *L.vannamei* culture following the administration of probiotics *Lactobacillus, acidophilus RS058, Rhodopseudomonas palustris GH642* and *Bacillus coagulans NJ105* at a dosage of $1 \text{ X } 10^7$ CFU/kg diet. The above literature confirms that use of probiotics in feed can increase the conversion of

prophenoloxidase to Phenoloxidase leading to increase in immune response against pathogens by melanization and encapsulation. Probiotics mainly trigger pattern recognition receptors (PRRs) or damage-associated molecular patterns (DAMP) leading to a cascade of reactions. These reactions can lead to the production of serine proteins which are mainly responsible for cleavage of prophenoloxidase to Phenoloxidase.

4.3.5 Respiratory burst

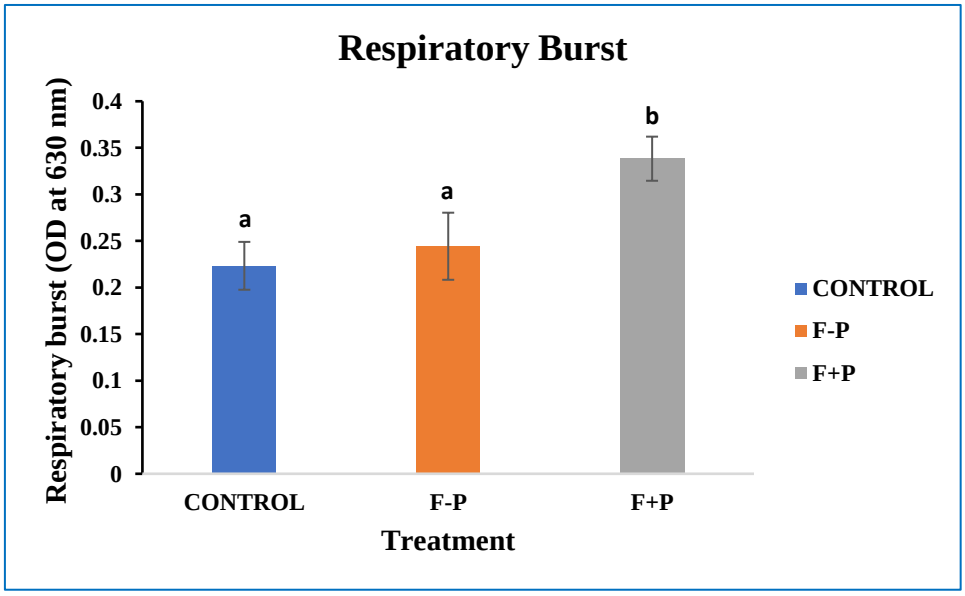


Fig. 18 : Respiratory burst activity of haemocytes in *L. vannamei*

As observed in Fig. 18, a rapid increase in the respiratory burst activity of haemocytes in shrimps fed with F+P (0.33 ± 0.02) at *($p<0.003$)* was observed as compared to the shrimps fed with F-P (0.24 ± 0.03) and control feed (0.22 ± 0.02).

The observed increase in respiratory burst activity in shrimps fed with F+P has been proven with previous research, such as the study by (S.-T. Chiu et al., 2021), which reported an increase in respiratory burst activity in shrimp following the feeding of a diet

containing *L. plantarum*. Additionally, (Y. Wang & Gu, 2010) documented a similar increase in respiratory burst activity in cultured *L. vannamei* upon treatment with specific probiotics as feed additives, indicating the immunomodulatory effects of probiotics in shrimp. Respiratory burst activity in *L. vannamei* refers to the rapid release of reactive oxygen species (ROS) by immune cells, primarily haemocytes, in response to pathogens or other stimuli, playing a crucial role in the shrimp's immune defense mechanisms.

4.4 Cytotoxicity evaluation

4.4.1 Determination of LC_{50} concentration

Table 07. Results of cytotoxicity evaluation of the feed

Experimental tanks	Dosage of probiotics (CFU/mL)	Initial no of shrimps	Survival rate		
			2 days	4 days	7 days
Probiotic dose 1	10^8	8	8	8	7
Probiotic dose 2	10^9	8	8	8	7
Probiotic dose 3	10^{10}	8	8	8	6
Probiotic dose 4	10^{11}	8	8	8	7

No significant difference ($p<0.05$) was observed between the mortality of shrimps in the tanks fed with the formulated feed incorporated with different doses of probiotics SFSK4 (10^8, 10^9, 10^{10} and 10^{11} CFU/mL), indicating that the feed does not show any toxicity when given at a high dosage up to 10^{11} CFU/mL.

4.4.2 Determination of Cell viability

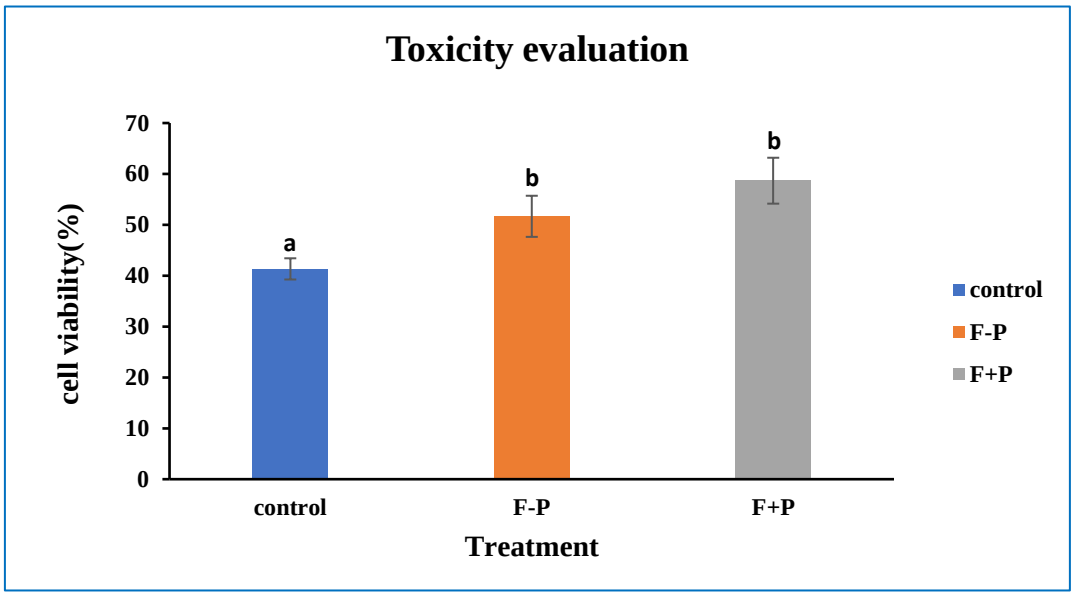


Fig 19 : Viable haemocyte count in *L. vannamei*

According to Fig. 19, the cell viability in shrimps fed with F+P (58.6 ± 4.5 %) and shrimps fed with F-P (51.6 ± 4 %) was found to be significantly higher (*p<0.004)* as compared to shrimps fed with control feed (41.3 ± 2%), indicating that the feed with probiotics does not impart any toxicity to the haemocytes of shrimps.

The above results are supported by (S.-T. Chiu et al., 2021) which revealed *L. pentosus* given at a dosage of 10^{10} CFU/kg diet was found to be nontoxic to *L. vannamei*. Further, (Fernandes et al., 2021) in her study determined that probiotic containing *Bacillus subtilis, Bacillus amyloliquefaciens, Pseudomonas* sp. *and Bacillus licheniformis* did not show any toxicity and was found to be avirulent to the shrimps when given at a maximum dosage of 10^{11} cells/mL as a water additive.

4.5 Primary cell culture of *Litopenaeus vannamei*

4.5.2 Primary cell culture of hepatopancreatic cells

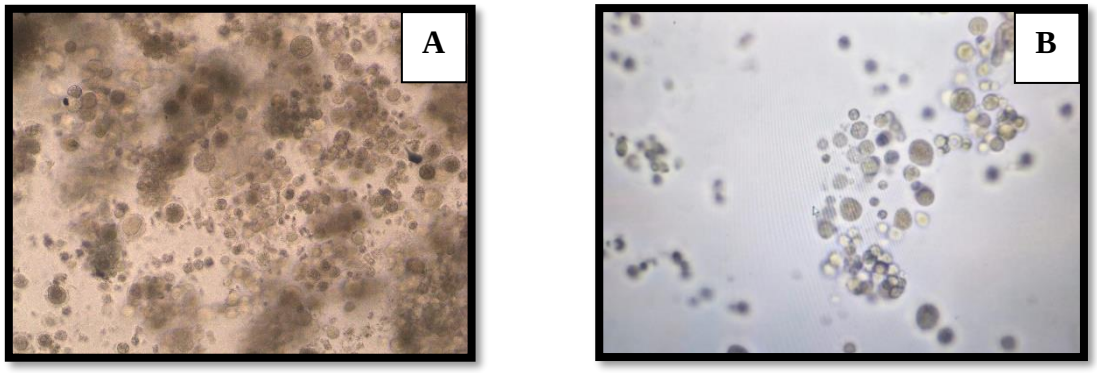


Fig. 20 : Primary cell culture of hepatopancreatic cells observed at 200 X magnification A) Hepatopancreatic cells cultured in L-15 media B) Hepatopancreatic cells after 3 passages

According to Fig.20, Hepatopancreatic cells were observed to attach when incubated for 72 hours in L-15 media with 10 % FBS and 1% antibiotic and antimycotic solution. A decrease in the number of cells was observed after 3 passages.

In a study conducted by (George & Dhar, 2010) revealed that hepatopancreatic cells were observed to decrease in number after exposure to trypsin. Conventional methods such as trypsin solution or mechanical detachment are ineffective, as shrimp cells struggle to reattach and proliferate in vitro after being dislodged (Sloane, 1975). According to (Jayesh et al., 2013) 1 % collagenase V can act as a better dissociation solution when compared to 0.25 % trypsin and 1% acutase which can enhnce the chances of cell survival on passaging.

4.5.2 Transfection of hepatopancreatic cells with SV40 Vector

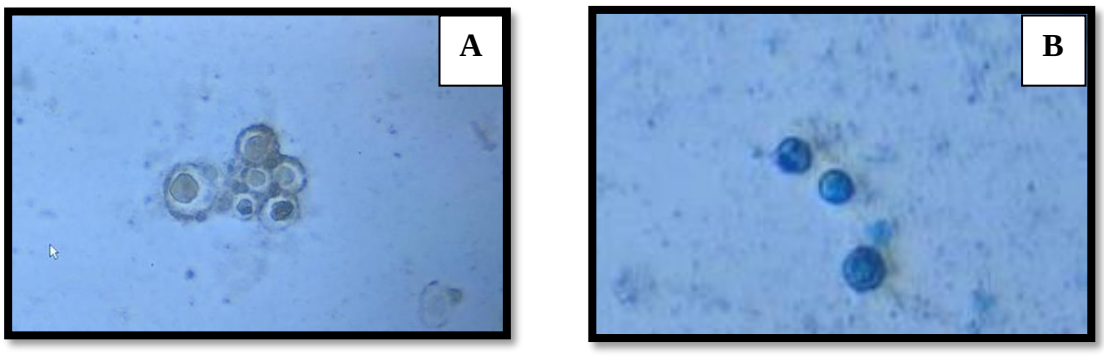


Fig 21 : Transfection of hepatopancreatic cells with SV40 Vector A) control hepatopancreatic cells B) Transformed hepatopancreatic cells

As seen in Fig. 21, Hepatopancreatic cells when stained with X-gal showed blue-green pigmentation in the experimental well indicating the hepatopancreatic cells were transfected with the SV 40 vector and could utilize x-gal as a substrate whereas the control well did not show any significant colour change.

As reported by (Hu et al., 2008), the presence of the transferred SV40T gene and its expression in transduced lymphoid cells of *Penaeus chinensis* increases its potential to attach and proliferate. Notably, the transfected cells exhibited accelerated growth rates and an extended replication lifespan in comparison to their cells which were not transfected. According to (Shi et al., 2016), incorporation of an SV40 vector in the genome of shrimp cells is mainly accomplished by Non homologous end joining of repair mechanism. Once the DNA is integrated in the host mechanism, the double stranded breaks leads to random integration of the viral vector in the genome. Once integrated into genome, SV40 vector replicates with the host DNA leading to expression of foreign gene in the invertebrate. These studies suggest that retrovirus-based immortalization-inducing

gene delivery system holds promise as a valuable tool for the establishment of stable shrimp cell line.

4.5.3 Cell viability assay

a) MTT assay

The MTT assay serves as a means to gauge cellular metabolic activity, providing insights into cell viability, proliferation, and cytotoxicity.

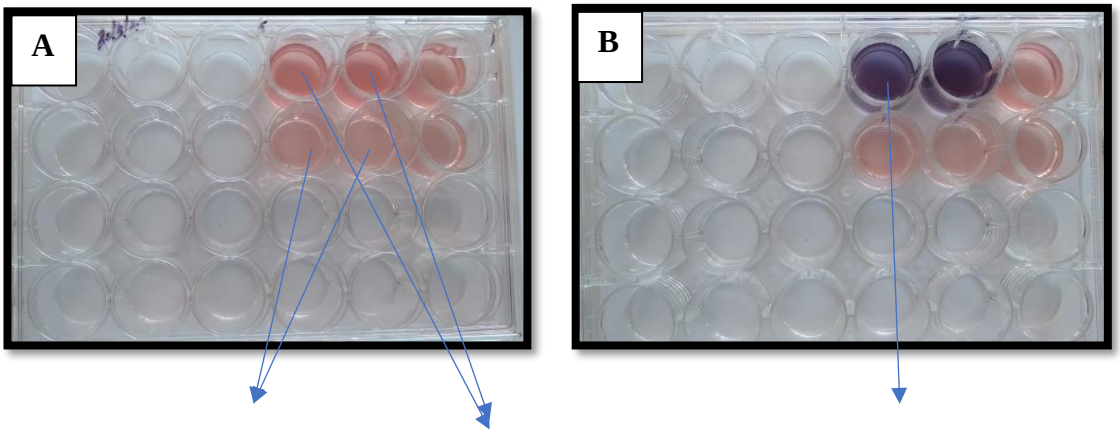


Fig. 22 : MTT assay of the hepatopancreatic cells (A) Cells before incubation with MTT (B) Cells after incubation with MTT

As observed in Fig. 22, Formazan crystals were produced when attached cells were incubated with MTT reagent for 6 hours. The formazan crystals were solubilized in a solubilizing solution appearing purple in colour whereas no colour change was observed in the negative control well which contained MTT reagent and media without cells.

According to the study conducted by (P. Kumar et al., 2018), the MTT assay enhances the conversion of the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] to an insoluble purple formazan by mitochondrial reductase activity, indicating cells are viable.

b) <u>DAPI staining</u>

DAPI is significant for its ability to selectively stain DNA, enabling precise visualization and analysis of cellular nuclei in cells.

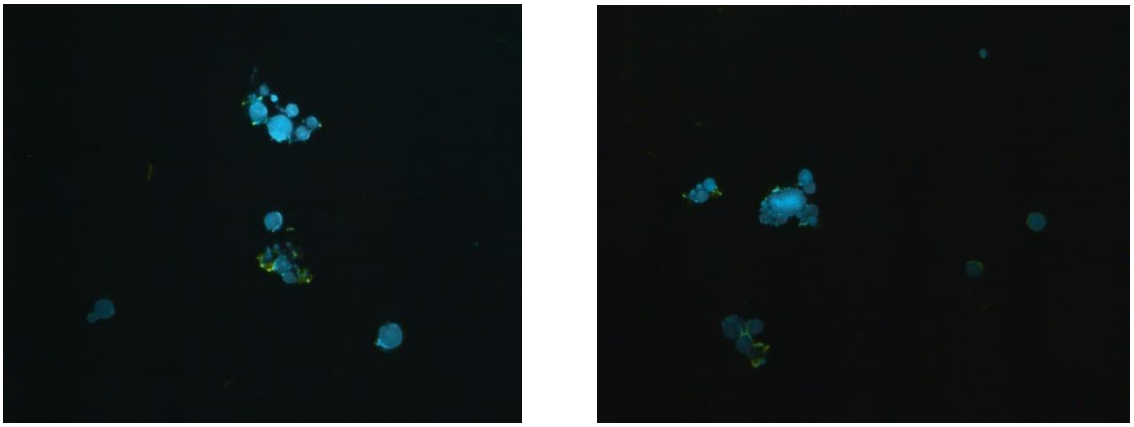


Fig. 23 : Hepatopancreatic cells stained with DAPI

Hepatopancreatic cells stained with DAPI were observed under 400 X magnification in the fluorescence microscope. In Fig. 23 Bright blue coloured cells were observed indicating the cells are viable.

According to (Kapuscinski, 1995), DAPI selectively binds to double-stranded DNA, however, it can also non-specifically bind to other cellular components such as RNA and certain proteins present in the cytoplasm. Usually nuclear membrane acts as a barrier to DAPI, allowing it to selectively stain DNA. This may result in the appearance of blue cells which is an indicator of membrane viability (Banerjee & Pal, 2008).

CONCLUSION

The research carried out provides a newly formulated economically feed with indigenous saltpan probiotic, SFSK4, which boosts the immunity of *Litopenaeus vannamei* by increasing the innate immunity.Notably, this formulated feed exhibits non-toxicity making it a safe feed additive.

Further, due to the non-availability of stable shrimp cell lines, the study also attempts to set the criteria for establishing a primary cell culture to promote the development of *Litopenaeus vannamei* cell lines.

FUTURE PROSPECT

1) Field-based study to test the effect of the feed in the local shrimp farms of Goa.
2) Establishment of stable and immortal shrimp cell lines of hepatopancreas and haemocyte cells.

REFERENCES

1. Amaya, E., Davis, D. A., & Rouse, D. B. (2007). Alternative diets for the Pacific white shrimp Litopenaeus vannamei. *Aquaculture*, *262*(2–4), 419–425. https://doi.org/10.1016/j.aquaculture.2006.11.001
2. Amparyup, P., Charoensapsri, W., & Tassanakajon, A. (2013). Prophenoloxidase system and its role in shrimp immune responses against major pathogens. *Fish & Shellfish Immunology*, *34*(4), 990–1001. https://doi.org/10.1016/j.fsi.2012.08.019
3. Anaya-Rosas, R. E., Rivas-Vega, M. E., Miranda-Baeza, A., Piña-Valdez, P., & Nieves-Soto, M. (2019). Effects of a co-culture of marine algae and shrimp (Litopenaeus vannamei) on the growth, survival and immune response of shrimp infected with Vibrio parahaemolyticus and white spot virus (WSSV). *Fish & Shellfish Immunology*, *87*, 136–143. https://doi.org/10.1016/j.fsi.2018.12.071
4. Araneda, M., Pérez, E. P., & Gasca-Leyva, E. (2008). White shrimp Penaeus vannamei culture in freshwater at three densities: Condition state based on length and weight. *Aquaculture*, *283*(1–4), 13–18. https://doi.org/10.1016/j.aquaculture.2008.06.030
5. Assavalapsakul, W., Smith, D., & Panyim, S. (2003). Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of Penaeus monodon. *Diseases of Aquatic Organisms*, *55*, 253–258. https://doi.org/10.3354/dao055253
6. Banerjee, D., & Pal, S. K. (2008). Dynamics in the DNA Recognition by DAPI: Exploration of the Various Binding Modes. *The Journal of Physical Chemistry B*, *112*(3), 1016–1021. https://doi.org/10.1021/jp077090f

7. Blois, M. S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. *Nature, 181*(4617), 1199–1200. https://doi.org/10.1038/1811199a0
8. Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., & Corner, R. (2010). Aquaculture: Global status and trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1554), 2897–2912. https://doi.org/10.1098/rstb.2010.0170
9. Cannon, J. G., O'Donnell, J. P., Lee, T., Hoppin, C. R., Long, J. P., Ilhan, M., Costall, B., & Naylor, R. J. (1975). N-Isopropyl derivatives of dopamine and 5,6-dihydroxy-2-aminotetralin. *Journal of Medicinal Chemistry, 18*(12), 1212–1216. https://doi.org/10.1021/jm00246a008
10. Chen, S. N., Chi, S. C., Kou, G. H., & Liao, I. C. (1986). Cell culture from tissues of grass prawn, Penaeus monodon. *Fish Pathology, 21*(3), 161–166. https://doi.org/10.3147/jsfp.21.161
11. Chiu, C.-H., Guu, Y.-K., Liu, C.-H., Pan, T.-M., & Cheng, W. (2007). Immune responses and gene expression in white shrimp, Litopenaeus vannamei, induced by Lactobacillus plantarum. *Fish & Shellfish Immunology*, *23*(2), 364–377. https://doi.org/10.1016/j.fsi.2006.11.010
12. Chiu, S.-T., Chu, T.-W., Simangunsong, T., Ballantyne, R., Chiu, C.-S., & Liu, C.-H. (2021). Probiotic, Lactobacillus pentosus BD6 boost the growth and health status of white shrimp, Litopenaeus vannamei via oral administration. *Fish & Shellfish Immunology*, *117*, 124–135. https://doi.org/10.1016/j.fsi.2021.07.024
13. Dantas-Lima, J. J., Corteel, M., Oanh, D. T. H., Bossier, P., Sorgeloos, P., & Nauwynck, H. J. (2012). Development of two haemocyte culture systems (in

attachment and in suspension) for shrimp immunity studies. *Aquaculture*, *366–367*, 17–26. https://doi.org/10.1016/j.aquaculture.2012.08.032

14. Fan, T.-J., & Wang, X.-F. (2002). In vitro culture of embryonic cells from the shrimp, Penaeus chinensis. *Journal of Experimental Marine Biology and Ecology*, *267*(2), 175–184. https://doi.org/10.1016/S0022-0981(01)00364-1
15. FAO (Ed.). (2020). *Sustainability in action*. https://doi.org/10.4060/ca9229en
16. FAO (Ed.). (2022). *Towards blue transformation*. FAO. https://doi.org/10.4060/cc0461en
17. Farzanfar, A. (2006). The use of probiotics in shrimp aquaculture: Table 1. *FEMS Immunology & Medical Microbiology*, *48*(2), 149–158. https://doi.org/10.1111/j.1574-695X.2006.00116.x
18. Felix, S., Samocha, T., & Menaga, M. (Eds.). (2021). *Vannamei shrimp farming*. CRC Press.
19. Fernandes, S., Kerkar, S., D'Costa, A., Costa, M., Mishra, A., Shyama, S. K., & Das, K. R. (2021a). Immuno-stimulatory effect and toxicology studies of salt pan bacteria as probiotics to combat shrimp diseases in aquaculture. *Fish & Shellfish Immunology*, *113*, 69–78. https://doi.org/10.1016/j.fsi.2021.03.017
20. Fernandes, S., Kerkar, S., Leitao, J., & Mishra, A. (2019). Probiotic Role of Salt Pan Bacteria in Enhancing the Growth of Whiteleg Shrimp, Litopenaeus vannamei. *Probiotics and Antimicrobial Proteins*, *11*(4), 1309–1323. https://doi.org/10.1007/s12602-018-9503-y
21. Garlock, T., Asche, F., Anderson, J., Bjørndal, T., Kumar, G., Lorenzen, K., Ropicki, A., Smith, M. D., & Tveterås, R. (2020). A Global Blue Revolution: Aquaculture Growth Across Regions, Species, and Countries. *Reviews in*

Fisheries Science & Aquaculture, *28*(1), 107–116. https://doi.org/10.1080/23308249.2019.1678111

22. George, S. K., & Dhar, A. K. (2010). An improved method of cell culture system from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of Penaeus vannamei. *In Vitro Cellular & Developmental Biology - Animal*, *46*(9), 801–810. https://doi.org/10.1007/s11626-010-9343-x
23. Gulzar, S., & Amin, S. (2012). Kinetic Studies on <i>β</i>-Galactosidase Isolated from Apricots (<i>Prunus armeniaca kaisa</i>). *American Journal of Plant Sciences*, *03*(05), 636–645. https://doi.org/10.4236/ajps.2012.35077
24. Han, Q., Li, P., Lu, X., Guo, Z., & Guo, H. (2013). Improved primary cell culture and subculture of lymphoid organs of the greasyback shrimp Metapenaeus ensis. *Aquaculture*, *410–411*, 101–113. https://doi.org/10.1016/j.aquaculture.2013.06.024
25. Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B., & Kautsky, N. (2003a). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science & Technology*, *38*(3), 255–266. https://doi.org/10.1046/j.1365-2621.2003.00671.x
26. Hong, N. T. X., Linh, N. T. H., Baruah, K., Thuy, D. T. B., & Phuoc, N. N. (2022). The Combined Use of Pediococcus pentosaceus and Fructooligosaccharide Improves Growth Performance, Immune Response, and Resistance of Whiteleg Shrimp Litopenaeus vannamei Against Vibrio parahaemolyticus. *Frontiers in Microbiology*, *13*, 826151. https://doi.org/10.3389/fmicb.2022.826151

27. Hoseinifar, S. H., Sun, Y.-Z., Wang, A., & Zhou, Z. (2018). Probiotics as Means of Diseases Control in Aquaculture, a Review of Current Knowledge and Future Perspectives. *Frontiers in Microbiology*, *9*, 2429. https://doi.org/10.3389/fmicb.2018.02429

28. Hsu, Y.-L., Yang, Y.-H., Chen, Y.-C., Tung, M.-C., Wu, J.-L., Engelking, M. H., & Leong, J. C. (1995). Development of an in vitro subculture system for the oka organ (Lymphoid tissue) of Penaeus monodon. *Aquaculture*, *136*(1–2), 43–55. https://doi.org/10.1016/0044-8486(95)01048-3

29. Hu, G., Wang, D., Wang, C., & Yang, K. (2008). A novel immortalization vector for the establishment of penaeid shrimp cell lines. *In Vitro Cellular & Developmental Biology - Animal*, *44*(3–4), 51–56. https://doi.org/10.1007/s11626-007-9076-7

30. Jamal, M. R. (2023). Can vannamei shrimp (Litopenaeus vannamei) revitalise Bangladesh's dying shrimp industry? *Aquaculture International*, *31*(3), 1637–1641. https://doi.org/10.1007/s10499-022-01045-9

31. Jayesh, P., Jose, S., Philip, R., & Bright Singh, I. S. (2013). A novel medium for the development of in vitro cell culture system from Penaeus monodon. *Cytotechnology*, *65*(3), 307–322. https://doi.org/10.1007/s10616-012-9491-9

32. Kapuscinski, J. (1995). DAPI: A DNA-Specific Fluorescent Probe. *Biotechnic & Histochemistry*, *70*(5), 220–233. https://doi.org/10.3109/10520299509108199

33. Kawai, T., & Akira, S. (2006). Innate immune recognition of viral infection. *Nature Immunology*, *7*(2), 131–137. https://doi.org/10.1038/ni1303

34. Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of Cell Viability by the MTT Assay. *Cold Spring Harbor Protocols*, *2018*(6), pdb.prot095505. https://doi.org/10.1101/pdb.prot095505

35. Kumar, V., Roy, S., Meena, D. K., & Sarkar, U. K. (2016). Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. *Reviews in Fisheries Science & Aquaculture*, *24*(4), 342–368. https://doi.org/10.1080/23308249.2016.1193841

36. Lakshmi, B., Viswanath, B., & Sai Gopal, D. V. R. (2013). Probiotics as antiviral agents in shrimp aquaculture. *Journal of Pathogens*, *2013*, 424123. https://doi.org/10.1155/2013/424123

37. Lee, M.-C., Lo, C.-T., Ho, T. H., Chen, L.-L., Nan, F.-H., Lai, H.-C., Wangkahart, E., & Lee, P.-T. (2023). Assessment of Bacillus subtilis fermented Caulerpa microphysa byproduct as feed additive on the growth performance, immune regulation and disease resistance of white shrimp (Litopenaeus vannamei). *Fish & Shellfish Immunology*, *142*, 109134. https://doi.org/10.1016/j.fsi.2023.109134

38. Li, E., Wang, X., Chen, K., Xu, C., Qin, J. G., & Chen, L. (2017). Physiological change and nutritional requirement of Pacific white shrimp *Litopenaeus vannamei* at low salinity. *Reviews in Aquaculture*, *9*(1), 57–75. https://doi.org/10.1111/raq.12104

39. Liu, G., Zhu, S., Liu, D., Guo, X., & Ye, Z. (2017). Effects of stocking density of the white shrimp Litopenaeus vannamei (Boone) on immunities, antioxidant status, and resistance against Vibrio harveyi in a biofloc system. *Fish & Shellfish Immunology*, *67*, 19–26. https://doi.org/10.1016/j.fsi.2017.05.038

40. Liu, H., Li, Z., Tan, B., Lao, Y., Duan, Z., Sun, W., & Dong, X. (2014). Isolation of a putative probiotic strain S12 and its effect on growth performance, non-specific immunity and disease-resistance of white shrimp, Litopenaeus

vannamei. *Fish & Shellfish Immunology*, *41*(2), Article 2. https://doi.org/10.1016/j.fsi.2014.08.028

41. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.
42. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426–428. https://doi.org/10.1021/ac60147a030
43. Nedaei, S., Noori, A., Valipour, A., Khanipour, A. A., & Hoseinifar, S. H. (2023). Dietary Effects of Lactobacillus plantarum Combined with Galactooligosaccharide on Immunological and Biochemical Parameters, Gut Microbiota, Digestive Enzyme Activity, Body Composition, and Stress Resistance in Narrow-Clawed Crayfish, Pontastacus leptodactylus (Eschscholtz, 1823). *Aquaculture Nutrition*, *2023*, 1–13. https://doi.org/10.1155/2023/3345916
44. Nisar, U., Zhang, H., Navghan, M., Zhu, Y., & Mu, Y. (2021). Comparative analysis of profitability and resource use efficiency between Penaeus monodon and Litopenaeus vannamei in India. *PLOS ONE*, *16*(5), e0250727. https://doi.org/10.1371/journal.pone.0250727
45. Obaldo, L. G., Divakaran, S., & Tacon, A. G. (2002). Method for determining the physical stability of shrimp feeds in water: Determining physical stability of shrimp feed. *Aquaculture Research*, *33*(5), 369–377. https://doi.org/10.1046/j.1365-2109.2002.00681.x
46. Ringø, E., & Song, S. K. (2016). Application of dietary supplements (synbiotics and probiotics in combination with plant products and β-glucans) in aquaculture. *Aquaculture Nutrition*, *22*(1), 4–24. https://doi.org/10.1111/anu.12349

47. Shi, Y., Xiang, J., Zhou, G., Ron, T. B., Tong, H.-I., Kang, W., Sun, S., & Lu, Y. (2016). The Pacific White Shrimp β-actin Promoter: Functional Properties and the Potential Application for Transduction System Using Recombinant Baculovirus. *Marine Biotechnology*, *18*(3), 349–358. https://doi.org/10.1007/s10126-016-9700-1
48. Sloane, N. H. (1975). Alpha-Naphthoflavone activation of 6-hydroxymethylbenzo(alpha)pyrene synthetase. *Cancer Research*, *35*(12), 3731–3734.
49. Song, S. K., Beck, B. R., Kim, D., Park, J., Kim, J., Kim, H. D., & Ringø, E. (2014). Prebiotics as immunostimulants in aquaculture: A review. *Fish & Shellfish Immunology*, *40*(1), 40–48. https://doi.org/10.1016/j.fsi.2014.06.016
50. Soto, D. & Food and Agriculture Organization of the United Nations (Eds.). (2009). *Integrated mariculture: A global review*. Food and Agriculture Organization of the United Nations.
51. Subasinghe, R., Soto, D., & Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in Aquaculture*, *1*(1), 2–9. https://doi.org/10.1111/j.1753-5131.2008.01002.x
52. Suva, M., Sureja, V., & Kheni, D. (2017). Novel insight on probiotic Bacillus subtilis: Mechanism of action and clinical applications. *Journal of Current Research in Scientific Medicine*, *3*(2), 136. https://doi.org/10.4103/jcrsm.jcrsm_47_17
53. Tacon, A. G. J. (2017). Biosecure Shrimp Feeds and Feeding Practices: Guidelines for Future Development. *Journal of the World Aquaculture Society*, *48*(3), 381–392. https://doi.org/10.1111/jwas.12406

54. Tacon, A. G. J. (2020). Trends in Global Aquaculture and Aquafeed Production: 2000–2017. *Reviews in Fisheries Science & Aquaculture*, *28*(1), 43–56. https://doi.org/10.1080/23308249.2019.1649634

55. *The State of World Fisheries and Aquaculture 2020*. (2020). FAO. https://doi.org/10.4060/ca9229en

56. Van Hai, N., & Fotedar, R. (2010). A Review of Probiotics in Shrimp Aquaculture. *Journal of Applied Aquaculture*, *22*(3), 251–266. https://doi.org/10.1080/10454438.2010.500597

57. Wang, Y., & Gu, Q. (2010). Effect of probiotics on white shrimp (*Penaeus vannamei*) growth performance and immune response. *Marine Biology Research*, *6*(3), 327–332. https://doi.org/10.1080/17451000903300893

58. Wang, Y.-C., Hu, S.-Y., Chiu, C.-S., & Liu, C.-H. (2019). Multiple-strain probiotics appear to be more effective in improving the growth performance and health status of white shrimp, Litopenaeus vannamei, than single probiotic strains. *Fish & Shellfish Immunology*, *84*, 1050–1058. https://doi.org/10.1016/j.fsi.2018.11.017

APPENDIX

1. Reagents for carbohydrate estimation by DNSA

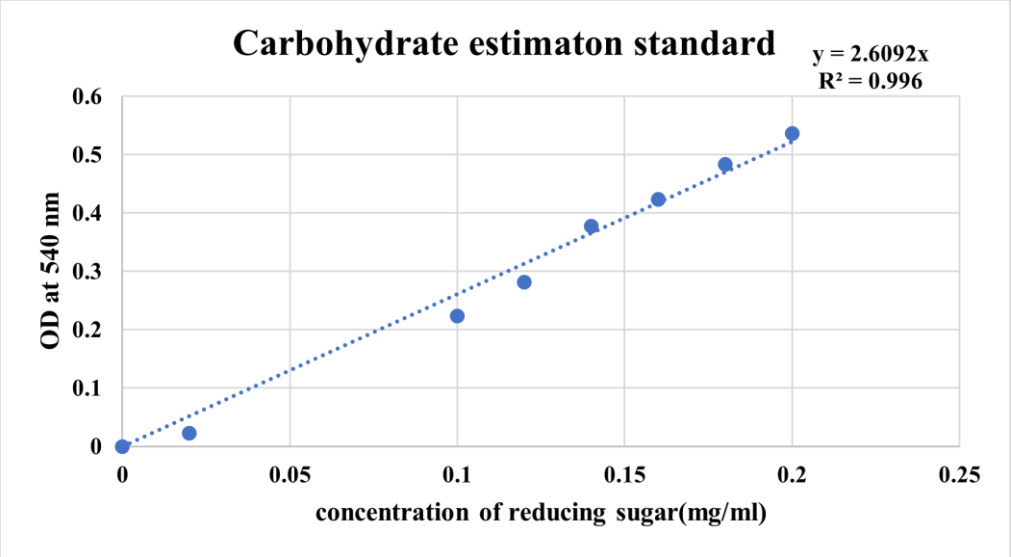
Sodium potassium tartrate: Dissolve 30 gms of sodium potassium tartrate in 50 mL of distilled water.

3,5-DNS solution: Dissolve 1 gm of DNS reagent in 20 mL of 2 M NaOH with help of magnetic stirrer.

2 molar NaOH: 1.6 g of NaOH in 20 ml of distilled water

DNSA reagent: Prepare fresh by mixing the reagents (1) and (2) make up the volume to mL with water.

Standard: 1 mg/mL glucose.



2. Reagents for protein estimation by Folin's Lowry

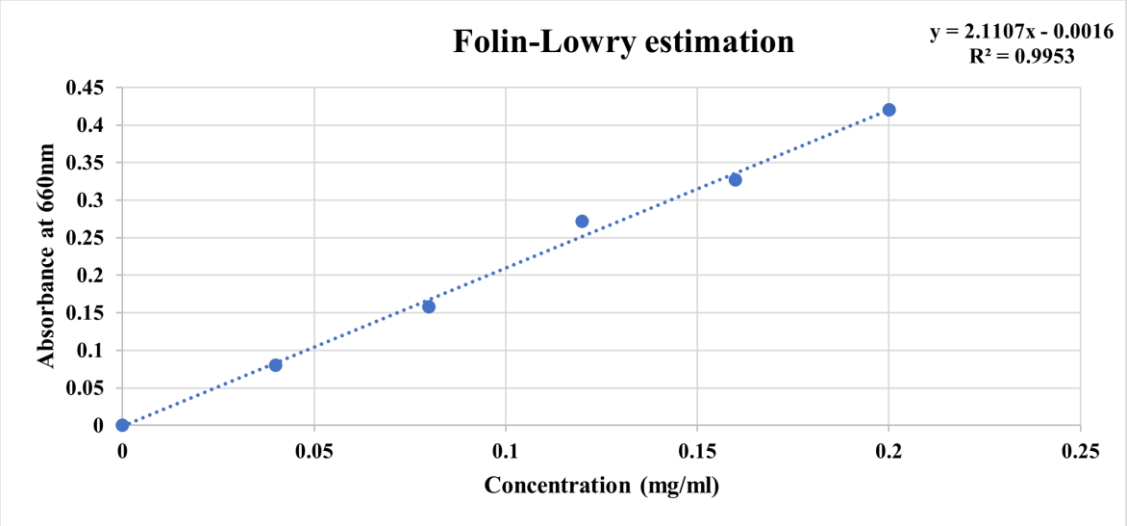
Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide Reagent

Reagent B: 0.5% copper sulphate in 1% potassium sodium tartarate. Prepare freshly by mixing stock solutions.

Reagent C (Alkaline copper solution): Add 50 ml of Reagent A and 1 ml of Reagent B prior to use.

Folin's reagent (Reagent D): Dilute Folin-Ciocalteau with an equal volume of 0.1 N NaOH

Standard: 1 mg/ml of BSA



3. Reagents for antioxidants assay by DPPH method

DPPH reagent : 11 mg DPPH (2,2-diphenyl-1-picrylhydrazyl) dissolved in 100 ml absolute ethanol to prepare 0.3 mM DPPH reagent

Standard: 0.1 mg/mL ascorbic acid