i

Formulation of a holiday feed with natural additives to enhance the immunity of *Xiphophorus helleri*

A Dissertation for

Course code and course title: MBT 651- Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc. in Marine Biotechnology

By

MALAVIKA VINO

22PO500009

ABC ID: 369483666095

202207108

Under the supervision of

Dr. Samantha Fernandes D'Mello

School of Biological Sciences and Biotechnology

Biotechnology Discipline

GOA UNIVERSITY

Date: 8th April 2024

Examined by:

Seal of the school

ii

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Formulation of a holiday feed with natural additives to enhance the immunity of *Xiphophorus helleri*" is based on the results of investigations carried out by me in 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 in 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.

Ms. Malavika Vino

22P0500009

Marine Biotechnology

School of Biological Sciences and Biotechnology

Date: 8th April'2024

Place: Goa University

iii

COMPLETION CERTIFICATE

This is to certify that the work entitled, "**Formulation of a holiday feed with natural additives to enhance the immunity of *Xiphophorus helleri.***" is a bonafide work carried out by Ms. Malavika Vino under my supervision at the School of Biological Sciences and Biotechnology, Goa University in partial fulfilment of the requirements for the award of the degree of **Master of Science in Marine Biotechnology** at the School of Biological Sciences and Biotechnology, Goa University.

Dr. Samantha Fernandes D'Mello

Marine Biotechnology

Prof. Bernard F. Rodrigues

Sr. Professor and Dean of

School of Biological Sciences and Biotechnology

Date: 8/4/24

Place: Goa University

Dean of School of Biological Sciences
& Biotechnology
Goa University, Goa-403206

गोंय विद्यापीठ
ताळगांव पठार
गोंय - ४०३ २०६
फोन : +९१-८६६९६०९०४८

(Accredited by NAAC)

Goa University
Taleigao Plateau, Goa - 403 206
Tel : +91-8669609048
E.mail : registrar@unigoa.ac.in
Website : www.unigoa.ac.in

Subject: IAEC Approval Certificate_forSmall Animal Experimentation

Given below are the decisions of Institutional Animal Ethics Committee (IAEC) with respect to the research proposal submitted for IAEC approval. Please quote the IAEC approval reference number in all your future correspondence for animal issue/other compliances in relation to this project.

IAEC Approval Reference No	GUZ/IAEC/23-24/N13
Date of IAEC meeting	12/09/2023
Project code number	IAEC/23-24/N13
Title of the research project	Study of immunostimulants for Xiphophorus helleri
Name of the Principal Investigator and/or other associated staff & Position	Dr. Samantha Fernandes D'Mello
Duration of the approved project	06 months.
IAEC approval status of the protocol	Approved
Animal sp. /strain/age/sex etc. and numbers approved by the IAEC Yearwise	40 Xiphophorus helleri
Expiry period of IAEC approval	One Year
Any other remarks	

Instructions to the investigators:

1. Investigators are responsible for keeping proper records of animals issued and used as per format 'C' & 'D'.
2. All legal and ethical provisions including IAEC /CPCSEA guidelines and animal welfare regulations should be strictly followed during entire course of animal housing and experimentation.
3. Animal experiment should be started only after approval of funding and the IAEC clearance.
4. Any deviation in the animal protocol/ procedure must be brought in the notice of IAEC for its advice.
5. Submission of periodical/annual progress report and final report on completion of the project would be mandatory in all cases.

To:

Dr. Samantha Fernandes D'Mello

Assistant Professor
School of Biological Sciences and Biotechnology

Savita Kerkar 13/9/23
Prof. Savita Kerkar
Member Secretary,IAEC
Goa University

CONTENTS

PREFACE

In the recent decades, the aquariculture industry has experienced unprecedented growth, emerging as a significant sector within global pet trade. The allure of ornamental fish lies in their unique patterns, mesmerising behaviours, and aesthetic colours. Ornamental fishes like gold fish, sword tails, gouramies, guppies show their best colour when in their natural environment and hence to give it a natural habitat like essence in captivity.

In captive conditions these fishes suffer from various diseases and also lose their natural colour and glow. As fishes cannot de-novo synthesize the pigments. They depend on two factors for the pigmentation, one, their environmental habitat and two, their dietary intake.

This project emphasis on formulating a feed that has natural feed additives like seaweeds, crustacean shells, and pigment producing bacteria all which are natural to their habitat and including them in the diet to see a positive effect on their growth, colour and immunity.

The work was started to find an alternative solution to antibiotics that would act as an immunostimulant without any side effects and environmental hazard and also using waste materials (crustacean shells) and seaweeds.

As one reads through the work, all the details from selection of feed additives to screening of them for nutrient requirements and finally their effect on sword tails in-vivo has been entailed in great detail.

ACKNOWLEDGEMENT

It gives me great pleasure in expressing my humble gratitude to DBT, for funding my M.Sc. dissertation.

I wholeheartedly acknowledge the Dean of the School of Biological Sciences and Biotechnology, Goa University, Prof. B.F. Rodrigues; the Vice-Dean (Research) of SBSB, Prof. Krishnan; and the Vice-Dean (Academics) and Programme Director of M.Sc. Marine Biotechnology, Prof. Sanjeev Ghadi; for providing the necessary facilities to carry out this research work.

I pay my sincere thanks to my guide Dr. Samantha Fernandes D'Mello for her constant help, support, and guidance in the course of my dissertation work.

I would like to express my gratitude towards the other faculty members of the Discipline of Biotechnology Prof. Savita Kerkar, Dr. Sanika Samant, Dr. Meghanath Prabhu, Ms. Dviti Mapari, Ms. Snesha Bhomkar and Ms. Snigdha Mayenkar for all the knowledge I have acquired through their teachings during my M.Sc. programme. A sincere gratitude to Dr. Avelyno D'Costa from Zoology and Dr. Nikita Lotlikar from Marine Science for helping me at various stages of my project work.

I am also thankful to the research scholars, especially Ms. Diksha, Ms. Deepti, Ms. Priti, Ms. Veda and Ms. Devika who were of great help throughout the study. I am very grateful to our non-teaching staff Mr. Serrao, Mrs. Sanjana, Mr. Sameer, Mr. Ashish, Mr. Parijat, Ms. Sandhya, Ms. Jaya for helping me with all the basic needs required for my project work.

Words fail to express the profound sense of gratitude I owe towards my mother, Mrs. Suvarna Vino, my father Mr. Vino P.R and my brother Mr. Saurav Vino for always supporting and motivating me, and The Almighty for the constant inspiration, moral support and the invaluable assistance rendered.

Lastly, I would like to thank my friends Ms. Manasi, Ms. Mariya, Ms. Varisha, Mr. Sushant, Mr. Sairaj for their constant support, and motivation.

I apologize if I have overlooked anyone. Omission of any acknowledgement doesn't show lack of gratitude.

TABLES

FIGURES

ABBREVIATIONS

Abbreviation	Entity
°C	Degree Celsius
Min	Minutes
%	Percentage
µg	Microgram
µL	Microlitre
g	Gram
C+S	Crustacean shell
RT	Room temperature
Control	Commercial feed

ABSTRACT

Ornamental fish industry or Aquariculture suffers from huge economic losses due to disease outbreaks. Increased use of antibiotics has led to antibiotic resistance and also causes other environmental hazards. This study explores the use of feed additives as immunostimulants as an alternative solution to antibiotics. Various natural feed additives like seaweeds (*Ulva*), crustacean shells, Probiotics (yellow and orange pigment producing bacteria) has been screened for nutrient characterisation. Crustacean shells and *Ulva* have shown high nutritional qualities compared to other samples.

The growth performance of fish fed with experimental feed additives showed significant increase in crustacean shell and consortium tanks.

In- vivo assessments like phagocytic activity, total plasma protein, phagocytosis index has been assessed to see high in fish fed with crustacean shells. While, consortium and yellow bacteria fed tanks also show significantly high results.

The feed cytotoxicity analysis reveals no adverse effect of feed additives as compared to the control feed except for orange pigment producing bacteria which showed high cytotoxicity value compared to control feed and also observed to have highest mortality rate.

The findings suggest that crustacean shells, *Ulva* and RKO3 (yellow pigment producing bacteria) are eco-friendly, immunostimulant, that enhance growth and maintains natural colour of swordtail fish, which is an effective alternative to antibiotics.

CHAPTER 1: INTRODUCTION

1.1 Background

Fish farming or pisciculture is a centuries old practice of breeding and rearing fish species, where ornamental fish farming is done specifically for their aesthetic values. Ornamental fisheries contribute to a large proportion of our global and domestic economy. India exports fish worth ₹20,000 crore a year. Of that, the share of ornamental fish is around ₹300 crore. (S. Raja et al., 2014). The global market size of ornamental fish was valued at USD 5.88 billion in 2022 and is expected to reach USD 11.30 billion by 2030 (Ornamental Fish Market Size, Share |Global Industry Report 2019-2025, 2019)

Ornamental fisheries have major roles in commercial world especially in terms of foreign exchange. This industry has come forward in the past few years as recognised as an avenue of employment to aqua culturists and younger generations. It provides great opportunities for entrepreneurs because of its low investment, less water and time requirements. (Many parts of India are engaged in this business and is supported by. the government through many initiatives like Pradhan Mantri Matsya Sampada Yojana which aims to meet the national, social and economic goals, livelihood sustainability and socio-economic upliftment of the fisher community.

Almost all of the ornamental fishes marketed in India are exotic. The ornamental freshwater fishes are broadly classified as live bearers and egg laying. Mollies, guppies and platies are live bearers while, gold fish, tiger barbs, koi, carp gouramies and fighters are egg layers.

Xiphophorus helleri commonly known as sword tail are popular fresh water ornamental fish belong to family Poecilidae. Native to central America they are translocated to 31 other countries including India. It is considered as commercially important ornamental fish after Gold fish due to its aesthetic value, food habit, and reproductive traits (Ghosh et al., 2008).

Studies have also reported evidence of biocontrol of mosquitoes by *Xiphophorus helleri* (Mckay,1928). Their appearance, invasive nature to adapt to any harsh habitat conditions and ability to give birth to live offsprings, makes them a good model organism for research purposes. *Xiphophorus* species are regularly used in genetic studies, and have been reported in the study of melanoma research since 1920s.

Sword tails are omnivorous in diet and was introduced in Queensland and Western Australia which fed on silt/ biofilm, aquatic insects (dipteran and ephemeropteran larvae), plant material (algae), and small crustaceans (Arthington, 1989; Maddern, 2011).

The ornamental fish industry faces significant challenges primarily due to various pathogenic infections. Bacterial infections are major causes of diseases in ornamental fishes and most of them are antibiotic resistant. Vaccines were initially used however; single vaccines are effective only against one type of pathogen.

All ornamental fishes like sword tails are affected by various bacterial, fungal diseases which leads to a huge lose to the breeders and to the aquaculture industries. Sword tails in particular have been affected by shimmies which is caused by poor water qualities (Aquarium Science – based on science and logic) skinny disease or chronic wasting syndrome in fish, which causes lethargy, anorexia, skin inflammation, ulceration, edema. This is mainly due to intestinal parasites like *Spironucleus* and internal parasites like tapeworms or mycobacteriosis cotton mouth or *columnaris* is caused by *Flavobacterium columnare* (Declercq et al., 2013) fin and tail rot disease caused by *Pseudomonas fluroscens* or fungal infection (Fisheries, 2020) ich or white spot disease is caused by ciliated protozoan *Ichthyophthirius multifiliis* (Tørud & Håstein, 2008) Other major problems include swim bladder issues, velvet disease and bulging eyes.

Antibiotics have been in demand in the aquaculture industries for urgent needs, usually delivered orally or through formulated feed (Schar et al., 2020) However, many fishes were unable to actively metabolize these antibiotics and most of it was passed out to the environment, unused. It was reported that 75% of the antibiotics was excreted to water (Burridge et al. 2010).

The overuse of antibiotics in aquaculture industries lead to antibiotic resistance and accumulation of toxin build up in the environment that is harmful to both aquatic and human lives. For this reason, many countries have banned the use of antibiotics in aquaculture industries. (Pepi & Focardi, 2021).

An alternate solution to this major concern was formulating feed additives that increased the overall growth performance and increased immune resistance to certain pathogens. The widely used alternatives include probiotics, prebiotics, synbiotics, acidifiers, plant extracts, nucleotides and immunostimulants such as beta-glucan and lactoferrin (LF) as mentioned before (Misra et al. 2006; Yokoyama et al. 2006; Dawood et al. 2015, 2016, 2017; Dawood & Koshio 2016a; Hossain et al. 2016).

Feed additives are non-nutritive products added to the basic feed mixture to enhance growth or other productive function. They include phytogenic compounds, organic acids, immune – stimulants, yeast products, probiotics, prebiotics, enzymes, synbiotics etc.

Feed blocks are new feeding technology developed to slowly dissolve and release food periodically for fish. This is used as a food supply to fish when the owner is away or to enhance the health and growth of fish. There are two types of feed blocks. Calcium block in which feed is embedded in calcium and gelatin-based feed in a gel. (Taiyo holiday vacation blocks food-taiyo group)

1.2 Aim and Objectives

Aim: To formulate a holiday feed and evaluate the potentials of various natural feed additives for enhancing immunity and skin pigmentation of *Xiphophorus helleri*.

Objectives:

1. To screen various nutrient sources for feed additive potential.
2. Formulation of a 'Holiday feed' for *Xiphophorus helleri*.
3. Assessing the nutritional profile of the formulated feed.
4. In-*vivo* assessment of the test feed on *Xiphophorus helleri.*

1.3 Hypothesis

The use of probiotics (orange and yellow pigment producing bacteria) and *Ulva* (seaweed), crustacean shells as feed additives enhance immunity, colour and overall growth performance in various ornamental fish like swordtails.

1.4 Scope

Over the past decade, the ornamental fish industry has been experiencing a significant growth, with hobbyist investing around 10 to 12,000 INR for a medium sized aquarium setup. Farmers and hobbyists suffer from huge losses due to bacterial, viral, fungal infections which causes discarding of the whole stock, sterilization of the whole units etc.

Another challenge in Ornamental fish industry is to replicate accurate natural colour of fish in captivity. Fish cannot synthesize pigments *de- novo* and depend on diet for pigment production. In captive conditions, ornamental fishes lose their natural glow and colour. Their colour fades due to absence of natural pigment enhancing components in the commercial feed.

Two factors are the main drivers of pigmentation in fishes (A) feed additives, pigments and (B) rearing environment setup i.e., tank colour and light. Natural feed additives like seaweeds and crustacean shells and probiotics when incorporated as a feed additive enhance immunity, resistance to pathogens and also maintains the natural colour of fish.

While fish keeping demands less maintenance compared to other pets, it still necessitates regular tasks such as feeding and weekly tank cleaning. Overfeeding can lead to turbidity in the water, which poses a risk to fish health. Even a brief lapse in regular feeding, as short as two days, can result in the death of many fish.

This problem can be tackled by adding feed blocks or commonly known as “Holiday feeds”. As the name suggests, these blocks of feed slowly diffuse in the tanks and lasts for few days to weeks depending on the block size, stocking density and tank size. The block does not disintegrate or make the water turbid, provided there is proper aeration maintained in the tank.

Overfeeding problems that cause both diseases in fish and turbidity of water is also considered in feed blocks.

The present study focuses on assessing the nutritional and immunostimulant properties of various natural feed additives including economical crustacean waste products as a source of immunity booster and growth enhancer. The study aims to formulate an economical feed with natural sources that are made into feed blocks to increase the shelf life of feed and also reduce the aquarium tank maintenance. This is done by designing a calcium sulfate or gelatin-based feed blocks that is added to uniformly aerated tanks and the concept lies in the slow diffusion of feed into the tanks for over a period of 7 days, 14 days and 21 days. The impact of the formulated feed with natural feed additives and feed blocks have been analysed on *X. helleri.*

CHAPTER 2: REVIEW OF LITERATURE

2.1 Ornamental fish culture

2.1.1 Global trend

Ornamental fish market was valued USD 6.27 Billion globally in 2022, and is anticipated to grow at CAGR of 10.25% from 2023 to 2032. The household use of ornamental fish rose to 71.2% in 2022 after the global pandemic (FAO,2022).

Many countries are focusing on developing marine ornamental fish setups and is anticipated to reach CAGR of 9.5% from 2023 to 2030. But due to the difficulties of maintenance, water requirements and equipment, the growth is expected to be slow in comparison to the freshwater ornamental fisheries (Madhu et al., 2023).

Statistical data of the past few years show an increase in the overall domestic interest in aquaculture as a hobby. According to the University of Florida, Institute of Food and Agricultural Sciences, in European countries ornamental fish cultivation has become a profitable income due to low transportation costs, low maintenance and increased foreign exchange (Agricultural Statistics at a Glance 2021).

Key companies and market shares are emerging in the field by introducing new fish, and related products which increase the market contribution. Qian Hu corporation limited in December 2021 entered an agreement with AquaEasy Pvt Ltd. to assist the shrimp farmers in implementing sustainable practices which increases the output and profits while reducing risks and expenses (Ornamental Fish Market Size, Share | Global Industry Report 2019-2025, 2019).

In 2021 November, a Japanese fish food brand Hikari partnered with Imperial Tropicals for feed formulation that is suitable for all types of fish, whether bred or caught wild.

2.1.2 Freshwater Ornamental aquaculture

Ornamental fish farming also known as aquariculture is the second most preferred hobby in which aesthetic fishes are kept in confined glass tanks as pets (Gopakumar, 2009). Aquarium keeping requires less space and low maintenance.

In India among freshwater ornamental fish culture 98% are cultured and 2% are captured wild while in case of marine ornamental fish due to high maintenance and high cost the trade is dominated by freshwater ornamental culture (Maheswari et al., 2017). Marine ornamental culture offer a beautiful and diverse array of fish and coral. Marine aquarium and marine fishes are expensive to maintain the high salinity and water chemistry and the cost and maintenance and equipment like protein skimmers, high powdered lighting systems, expensive filtration system, frequent water changes precise temperature control.

The indigenous and exotic freshwater ornamental fishes have high market demands which is bred and reared for commercial purposes. Inclusion of systems like RAS (recirculating aquaculture system), Biofloc technology, Aquaponics, In-pond raceway systems are used culture freshwater fish in a controlled environment that maximizes production, improve water use efficiency, reduce man power. These systems help deliver sustainable production at low cost (Das et al.,2022).

2.2 *Xiphophorus helleri*

Xiphophorus helleri commonly known as swordtail are popular fresh water ornamental fish belonging to family Poecilidae. Native to central America, they are translocated to 31 other countries including India (Froese and Pauly, 2007). It is considered as commercially important ornamental fish after gold fish, due to its aesthetic value, food habit, and reproductive traits (Ghosh et al., 2008).

X. helleri are sexually dimorphic, males develop a 'sword' from the lower rays of the caudal fin; hence the common name "swordtail". Males reach 140 mm, females reach up to 160 mm (Page and Burr, 1991).

The genus contains almost 30 species, and the members of this species are known to commonly hybridise. The commercially produced ones contain many colours (Tamaru et al., 2001) than the wild type (Dawes et al.,1995; Balon et al., 2004).

Wild populations are light greenish in colour with a red or brown mid lateral stripe and male's sword is black edged. They are live bearers with large brood size, short gestation period, multiple broods per year (Milton and Arthington et al., 1983; Dawes et al., 1995; Maddern et al., 2011). The optimal temperature for breeding is described as 22-26 ^{0}C (Milton and Arthington,1983).

Their appearance, invasive nature to adapt to any harsh habitat conditions and the ability to give birth to live offsprings makes them a good model organism for research purposes. *Xiphophorus* species have been used in genetic studies, and have developed many interspecific hybrids, especially in melanoma research.

Swordtails are omnivorous in nature and feed on both live and artificial feed (James and Sampath.,2003). Fish feed and its constituents plays a vital role as an exogenous factor in fish rearing. It enhances both reproductive and overall growth.

2.3 Infections

A major loss to the industry is infections caused by various microorganisms, which is mentioned in table 2.3.

Table 1: Pathogens and diseases caused by them in *X. helleri*.

Causative organism	Diseases	Symptoms	Reference
1. *Flavobacterium columnare*	Columnaris or cotton mouth disease.	Small white bump or fungus like appearance over mouth.	(Plumb,1997)
2. *Pseudomonas fluroscens*	Fin and tail rot disease.	Erosions, discolorations, disintegration of fins and tails.	(Declercq et al., 2013)
3. S*pironucleus*, *Mycobacterium*	Skinny disease/ chronic wasting syndrome	Lethargy, anorexia, skin inflammation, ulceration, edema.	(Matthews et al. 2001)
4. *Ichthyophthirius multifiliis*	Ich or white spots syndrome	Ich, white spots on body.	(Tørud& Håstein, 2008) (Durborow et al., 1988).

2.4 Immunostimulants

Bricknell and Dalmo (2005) stated "An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the hosts resistance against diseases that in most circumstances are caused by pathogens".

Immunostimulants comprise a group of biological and synthetic compounds that improve both specific (antibody and agglutination titre) and non-specific immunity (lysozyme, phagocytic, bactericidal activity, respiratory burst activity) against the infectious diseases in different fish and shellfish species (Robertsen et al, 1994; Anderson and Siwycki et al.,1995; Amar et al., 2004; Ai et al., 2006; Andrzej et al., 2006; Behera et al., 2011; Kaleeswaran et al., 2011; Safarpour et al., 2011; Chakrabarti et al., 2012; Srivastava and Chakrabarti et al., 2012; Ambas et al., 2013).

Natural immunostimulants are economical, biodegradable and biocompatible. Many natural sources of immunostimulants have been discovered for its various potentials as an immunity enhancer, colour enhancer and for improved growth. Plant, animal and bacterial sources were recorded as natural immunity boosters in fish and shell fish immunology in the past few decades.

2.5 Natural feed additives

2.5.1 *Ulva* seaweed

Seaweeds are excellent dietary source of vitamins, minerals, proteins, carbohydrates and other bioactive compounds. Green macroalgae exhibit rapid growth, higher amino acids, vitamins, proteins (kumar et al.,2008). *Ulva* species are potential sources of aqua feeds (Valente et al., 2006). They have low protein content of 5-30 % of dry weight but have been reported to have other high nutritional values (Anh et al., 2013). Improved growth up to 5 % *Ulva* diet was reported in *Oreochromis niloticus* by (Guroy et al., 2007). Improved growth performance, feed utilization efficiency, digestibility with digestive enzyme activities, intestinal development, innate immunity and disease resistance by *Ulva* was reported in *Paralichthys olivaceus* (Tharaka et al., 2020)

The supplemented level of *Ulva* in diets depend on the fish species, age, sex, size. Seaweeds are a potential source of immunostimulants due to its various bioactive components. Many brown and green seaweed extracts are known to enhance growth and immune response in many fish species. Many researchers also focused on the delivery of seaweeds as an immunostimulant, some using whole seaweeds (Satoh et al.,1987) and others using various extracts e.g. methanol (Peixoto et al., 2019). *Ulva* is a polysaccharide derived from green seaweed species of family Ulvaceae. It is a potent anticancer, antidiabetic, anti-inflammatory properties through antioxidant modulations.

2.5.2 Crustacean shells

Chitin is the most abundant polysaccharide found on the exoskeletons of insects and crustaceans. They are reported to stimulate a defence mechanism by enhancing the macrophage activities, haemolytic complement activity, leukocyte respiratory burst activity and cytotoxicity. Chitin supplementation had stimulating effect on the growth and survival of *Macrobrachium rosenbergii.* In shrimps, chitin has been used as an immunostimulant and was known to improve the disease resistance against *V. alginolyticus*. Crustacean wastes like shrimp head and crab offal also yield considerable amounts of carotenoids and astaxanthin, and are thereby finding increasing use in fish feed for colour enhancement and immunostimulant activity.

Crab and shrimp exoskeletons are used to recover high value-added products like chitin, chitosan, glucosamine, and astaxanthin. Crustacean shells (blue crab, tiger prawns) stimulate macrophage activity, antioxidant and immunostimulant properties (Kawakami et al., 2008). Crustacean shells have been used as components in fish meal due to its high nutritive values.

2.5.3 Pigment producing bacteria

Ornamental fishes like gold fish, swordtails lose their natural glow and skin colour under captivity. Microorganisms produce different types of pigments including carotenoids, flavins, indigo etc based on the environmental factors (Duffose et al., 2006). These pigments act as antioxidants, antimicrobials and antibacterials (Scolnik and Bartley et al., 1995). Researchers have reported probiotics such as *Streptococcus faecium* which was reported to improve growth, protein and lipid content of Nile tilapia. (Flores et al., 2003). *Bacillus cereus* 0.5 g/kg improved growth performance of juvenile common dentex (Hidalgo et al., 2006).

2.5.4 Feed Block

Feed blocks or holiday feed blocks are long lasting, slow releasing nutritive particles in a well aerated tank. This block is prepared to slowly release the feed particle over a period of 7, 14 or 15 days depending on the size of tank and number of fishes in the tank (Herzog et al., 2021).

This is an alternative solution to feed the fish without a regular manual feeding and also maintains the water quality without making the water turbid.

MATERIALS

Chemicals:

1. DPPH (2,2- diphenyl-1-picrylhydrazyl)
2. DNSA (Dinitro salicylic acid)
3. FCR (Folin Ciocalteau reagent)
4. Ascorbic acid
5. Gallic acid
6. Glucose
7. Beta carotene
8. 70% ethanol
9. Methanol
10. Chloroform
11. Petroleum ether
12. Glutaraldehyde
13. DMSO (Dimethyl sulfoxide)
14. DMF (N, N-Dimethylformamide)

APPARATUS

1. Weighing balance
2. Laminar Air flow
3. Autoclave
4. Centrifuge
5. Lyohilizer (ScanVac, coolsafe)
6. Refractometer
7. pH meter (pH 700, Eutech instruments, Thermo Fischer Scientific, India)
8. Spectrophotometer (UV-Vis, Shimadzu, US)
9. Hot air oven
10. -80 °C freezer (Blue star ultra-low temperature freezer)
11. Refrigerator
12. Water bath

CHAPTER 3: METHODOLOGY

3.1 Preparation of natural feed additives.

3.1.1 *Ulva* species

It was collected from Anjuna Beach, Goa, latitude 15.584534 º and longitude 73.736489 º It was washed with seawater and dried in an oven at 55 ºC. The dried *Ulva* was ground into fine powder and sieved. To obtain a crude extract, the seaweed was washed with tap water to remove all mud and epiphytes and was dried at 60 ºC in an oven. The dried seaweeds were milled using a blender, sieved (<0.5 mm), and stored in plastic bags until extraction. The crude polysaccharides were extracted via the hot water extraction method (Klaew et al., 2021) Briefly, 10 g of a dry seaweed sample was boiled in 1000 mL of distilled water (1:100 w/v) for 90 minutes at 110 °C in an autoclave. The solid residue was filtered from the hot water extract. The supernatant was then freeze-dried until further use.

3.1.2 Crustacean shells

Blue crab (*Callinectes sapidus*) and Tiger prawn shells (*Penaeus monodon*) were procured from Magson supermarket, Goa. The shells were washed with tap water, cleaned from all dirt and dried in an oven at 55 ºC overnight. The dried shells were then ground into fine powder, sieved (<0.5 mm) and stored in glass containers (Klomklao et al., 2009).

3.1.3 Pigment producing bacteria

The pigment producing bacteria used in the study were obtained from Prof. Savita Kerkar's culture collection. The bacteria were sub-cultured on Nutrient agar and incubated at room temperature for 48 hours (Wei et al., 2005). For mass culturing, the orange bacteria RK03 and the yellow bacteria RK01 was individually inoculated in LB broth and kept in a rotary shaker for 2 days. The broth was centrifuged at 6,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed twice with 1X PBS buffer. The pellet was dissolved in 10

% sterile sucrose solution and kept at -80 ºC. The bacteria were lyophilized for further process (Fernandes et al., 2021).

3.1.4 Lyophilisation

The vial containing bacterial pellets with cryoprotectant and crude *Ulva* extract was removed from the -80 °C freezer and kept for freeze drying. (lyophilizer, Scanvac, America). After a period of 5 to 6 hours, the powdered bacterial biomass and crude *Ulva* was weighed and added to the basal diet for feed formulation.

Figure 1: (A) Oven dried crab shells (B) Oven dried shrimp shells (C) Dried powdered crab and shrimp shells.

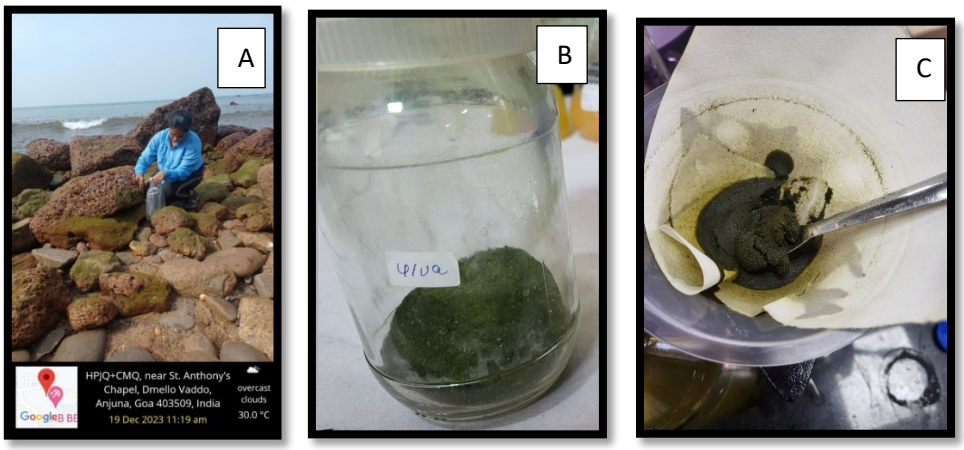

Figure 2: (A) Collection of *Ulva* sp. from Anjuna beach, Goa (B) Powdered *Ulva* sp. (C) Crude extract.

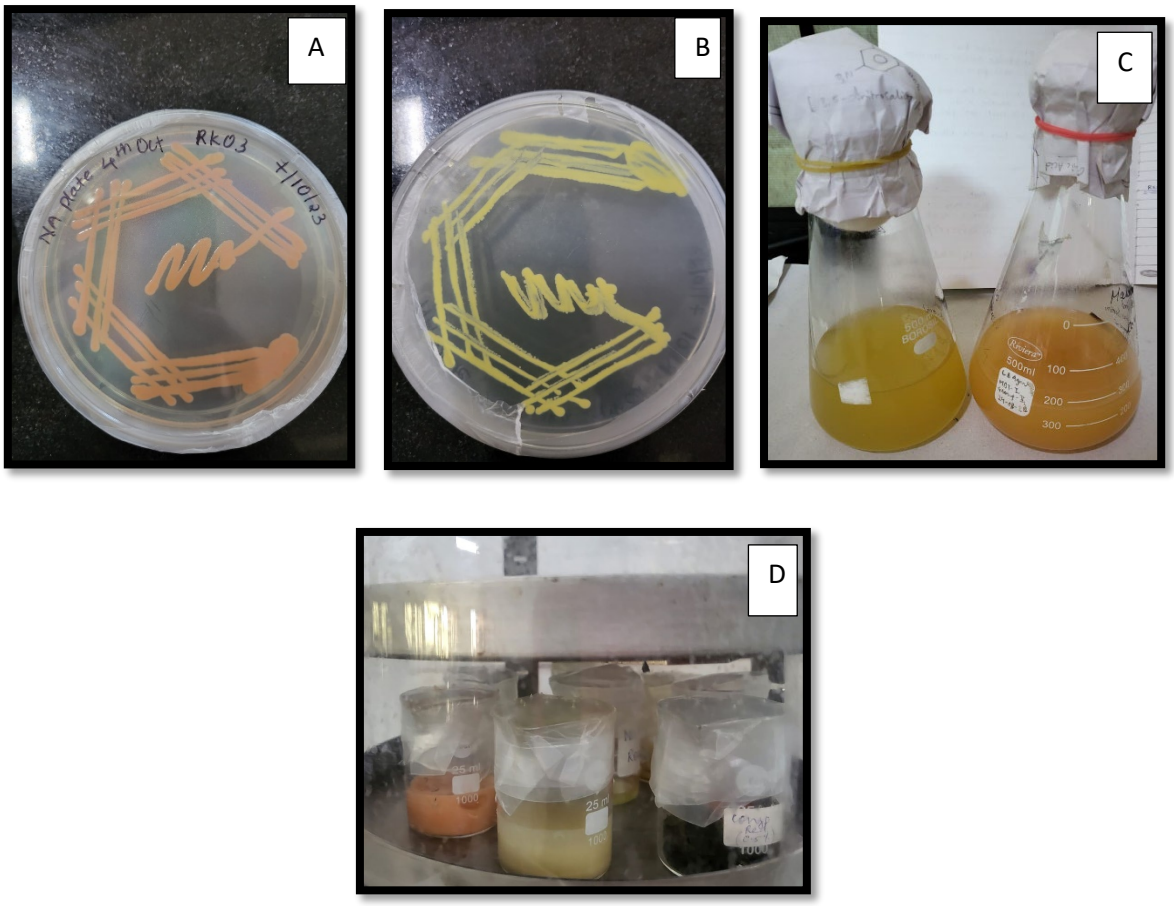

Figure 3: (A) Orange pigment producing bacteria subcultured on NA plates. (B) Yellow pigment producing bacteria subcultured on NA plates. (C) Broth inoculated with yellow and orange pigment producing bacteria. (D) Lyophilised samples.

3.2 Screening the potentials of natural feed additives

3.2.1 Estimation of proteins

Protein estimation was carried out by Bradford assay (Bradford et al., 1976). Bovine Serum Albumin (BSA) was used as the standard while 1 mg feed additives were dissolved in distilled water and used as the unknown/ test samples. 5 mL Bradford reagent was added to all the tubes and mixed thoroughly by vortexing. The absorbance was taken after 10 minutes at 595 nm. 1 mL distilled water and Bradford reagent was taken as the blank.

3.2.2 Estimation of carbohydrates

Carbohydrate estimation was carried out by DNSA method (Miller et al., 1959). Glucose was used as a standard solution (1 mg/mL). The feed additives (1 mg) were dissolved in 1 ml distilled water as diluent and used. DNSA reagent (0.5 ml) was added to all the tubes and mixed thoroughly. The set of tubes were then placed in a boiling water bath for 15-20 minutes. The optical density of all the sample tubes were measured at 540 nm spectrophotometrically.

3.2.3 Screening of antioxidant properties or scavenging activity

The scavenging activity was calculated by using DPPH method (Blois et al., 1958). This is used to measure the antioxidant properties that includes use of free radicals for assessing the potentials of substances that serves as a hydrogen or free radical scavengers (Ichikawa et al., 2019). Antioxidants are neutralizing chemicals that minimize oxidative damages by giving free radicals electrons. Ascorbic acid (1 mg/ml) was taken as the standard. The feed additives (1 mg) were taken into test tubes with ethanol as the diluent. 1 ml DPPH reagent were added to all the tubes and incubated in the dark for 30 minutes. The optical density of the samples was recorded spectrophotometrically at 517 nm.

3.2.4 Estimation of Phenol

The total phenolic compound was estimated using Folin Ciocalteau (FC) reagent as described by (Singleton et al., 1965). Gallic acid (1 mg/ml) was used as the standard for the test. The feed additives were added to individual test tubes with 3 mL of deionized water, and 250 µL of FC reagent, shaken, and allowed to stand protected from light for eight minutes. Then, 750 µL of a 7.5 % sodium carbonate solution was added and made up to a volume of 5 mL with distilled water. The solution was mixed manually until homogenized and kept in the dark at room temperature. The absorbance of the mixture was taken after 2 hours interval at 765 nm.

3.3 Preparation Of Feed

The screened feed additives (*Ulva* sp., crustacean shells, orange bacteria, yellow bacteria) were added to the Basal diets as 5 % immunostimulants (Menaga et al., 2021). The basal diet (20 g) was weighed according to 5 % body weight of fish initially. The Basal diet was into fine powder and 0.1 g of each feed additive was added. The feed was mixed with distilled water, kneaded into a dough, extruded using an extruding machine and kept for drying in the oven overnight at 50 ºC.

The dried feed was crushed using a mortar and pestle according to the fish mouth size. The stability, floatation and disintegration were standardised before the onset of the feeding trials.

For the consortium, initially 25 mg of each feed additives (*Ulva* sp., shells, orange and yellow bacteria) was measured and added to 20 g of basal diet. (James et al., 2006).

As the experimental trial continued, the percentage of feed additives added was substantially increased i.e. 1 g in 20 g of basal feed based on the body weight of the fish.

Figure 4: (A) Feed extruder machine (B) Extruded fish feed (C) Feed after crushing into floating micropellets.

3.3.1 Formulation of feed block

The formulated feed with feed additive for 20 days (4 g) was mixed with 2 g gelatin powder and made into a paste by addition of hot water. The paste was allowed to stand at 4 °C for 4 hours in a siliconized ice tray.

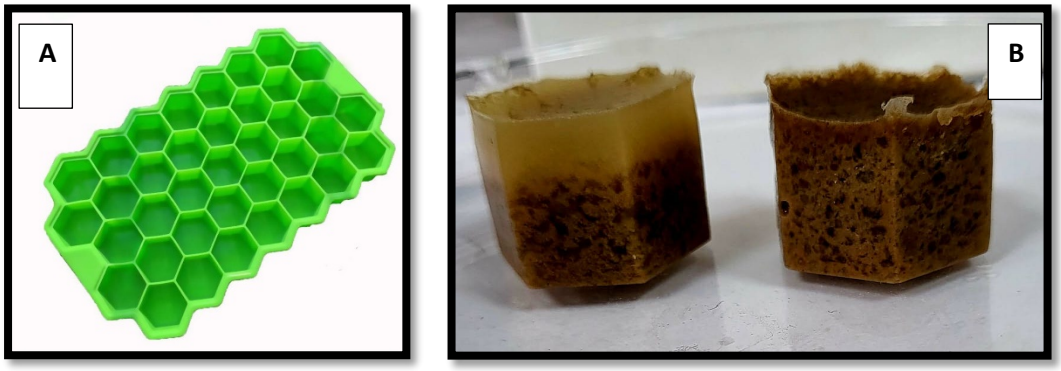

Figure 5: (A) Siliconized ice tray to solidify feed (B) Feed block after solidification.

3.3.2 Feed and feed block quality evaluation

The feed stability, colour, size and floatation rate of formulated feed and sinking rate of feed block was analysed for a period of 7 days. The prepared feed was evaluated for various parameters like protein estimation (Bradford et al., 1976), carbohydrates estimation (Miller et al., 1959), antioxidant properties by DPPH method (Blois et al., 1958), phenol estimation (Singleton et al.,1965) as described in section 3.2.

3.4 In-vivo assessment of the formulated feed additive and feed block on *X. helleri*

3.4.1 Maintenance and in-vivo assessment of formulated feed

X. helleri was bought from, Kannur, Kerala. 60 experimental fingerlings were randomly distributed into 6 tanks after acclimatizing them for 2 days at 26 ºC temperature, pH 7.4-8.9 and 0 psu salinity. The water quality was maintained throughout the experiment. The feeding was done twice a day at 9:30 am and 5:30 pm. The weight and length of the fish were recorded every month (Menega et al., 2023).

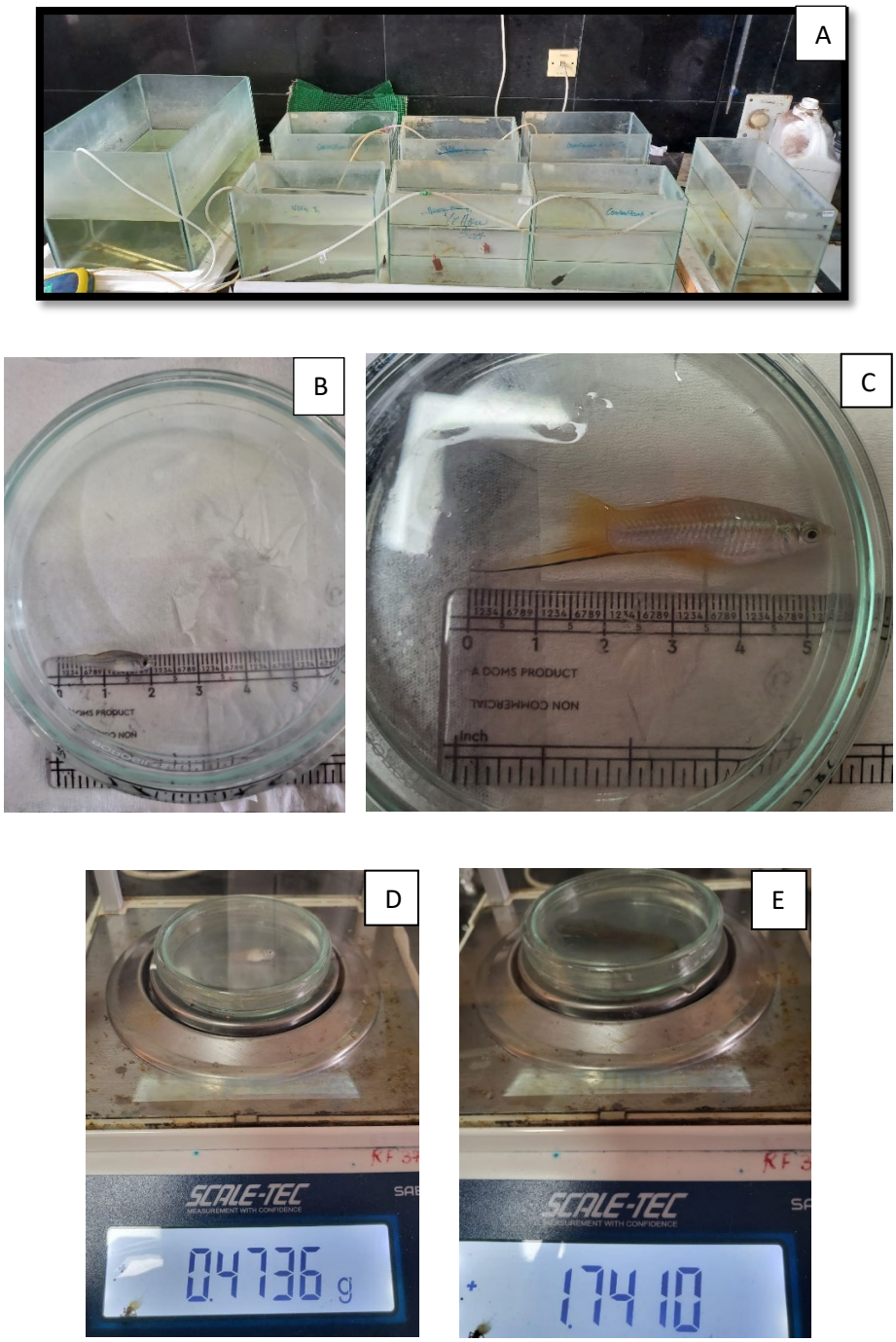

Figure 6: (A) Tank set up (B) Initial length of sword tail (C) Final length of sword tail at day 90 (D)initial weight of sword tail at day 0 (E) final weight at day 90.

3.4.2 Effect of feed additives on growth performance

The effect of feed additives (*Ulva*, crustacean shells, orange bacteria, yellow bacteria) on swordtail was recorded every month (Fernandes et al.,2021; Menega et al.,2023).

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

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

$$\textbf{Specific Growth Rate}(SGR\%) = \frac{[ln\ (\textbf{Final weight in grams}) - ln\ (\textbf{Initial weight})]}{\textbf{Time}} \times 100$$

3.5 Analysis of Water Quality

The physical and chemical parameters of the water quality were measured on monthly basis. The temperature of the fish tanks was recorded using a digital thermometer and the salinity was measured using a hand-held refractometer. The pH was recorded using a pH meter (pH 700, Eutech instruments, Thermo Fisher Scientific, India).

3.5.1 Nitrite test (APHA, 2012)

Nitrite concentration was determined spectrophotometrically according to APHA (2012). Briefly, 25 ml of water from different tanks was added to individual tubes, followed by the addition of 0.5 mL Sulphanilamide reagent. It was mixed and incubated at room temperature for 5 minutes, followed by addition of 0.5 mL N-(N-(1-napthyl) ethelene diamine hydrochloride solution. The samples were vortexed and kept for incubation at room temperature for 15-20 minutes. The absorbance was recorded at 543nm.

3.5.2 D.O. test (Winkler, 1888)

The Dissolved oxygen of the tanks was estimated by Winkler (1888), method. 1 mL of Winkler's B followed by Winkler's A was added to 250 mL test water sample from different

tanks, collected in amber coloured DO bottles to fix the samples The samples were mixed thoroughly and the precipitate was allowed to settle down for 30 minutes. 3 ml of 50 % dilute HCl was used to dissolve the precipitate completely. 50 ml of each sample was taken in a conical flask and titrated against $Na_2S_2O_3$ (Sodium thiosulphate) until pale yellow colour was developed, using 5 – 6 drops of starch as an indicator. The blue colour was titrated until colourless.

3.6 Immunological assays to assess cellular innate immune response

3.6.1 Microscopic characterisation of blood cells

The determination of blood parameters and characterisation will help diagnose fish disease and reflect alterations of the physiological status according to exogenous and endogenous factors. (Lehmann and Sturenberg 1981; Rowley et al., 1988; Goerlich and Hamers 1994; Hamers 1995).

Blood parameters and blood cell morphology was observed under panoptical staining, samples were stained using May-Griin- wald-Giemsa, slightly modified by Hamers (1995). 100 µL of blood was extracted using heparinized 26-gauge needle from the caudal vein of sword tail. A drop of blood was smeared on a glass slide, the sample was air dried, and fixed with methanol for 2 minutes. The non- adherent cells were washed using distilled water and the sample was kept in Giemsa stain for 30 minutes. The stain was washed with distilled water and air dried before observing under the microscope at 1000 magnification.

3.6.2 Total plasma protein

Plasma protein is the most clinically used sample to diagnose disease as it contains not only blood proteins but also proteins from all fish tissues (Li et al., 2016).

The total plasma protein was estimated by Bradford method (Bradford et al., 1976). BSA was used as a standard. 200µl of fish blood with anticoagulant was taken in a test tube with 3ml

Folin Ciocalteau reagent. The samples were allowed to stand for 10 minutes at room temperature and the optical density was recorded spectrophotometrically at 517nm.

3.6.3 Phagocytosis assay

Phagocytosis is a non-specific immune function involving the cellular uptake and intracellular processing of pathogens, foreign substance, cellular wastes and macromolecules (Koelner et al.,2004).

The phagocytic activity assay was performed using latex bead agglutination test (Anderson and Siwiski,1995; Fernandes et al., 2019) The phagocytic activity and the phagocytic index were determined using 1.1 μm size latex beads, (Sigma Aldrich, USA). Fish blood suspended in anticoagulant solution of 100 μL was mixed with 100 μL of latex beads on a clean grease free glass slide. The mixture was incubated for 30 minutes at RT, followed by fixation with 2.5 % glutaraldehyde. The slides were washed with Phosphate buffered saline (1X) to remove non-adherent blood cells, dried and stained with Giemsa stain (Himedia). The number of phagocytising cells were counted from 50 randomly observed cells at 100X magnification lens.

Phagocytosis was calculated as:

$$\textbf{Percentage phagocytosis} = \left(\frac{\textbf{number of cells ingesting beads}}{\textbf{numberof cells observed}}\right) \times \mathbf{100}$$

3.6.4 Respiratory burst

The interaction of neutrophils with microbes leads to activation of oxygen dependent respiratory burst following release of NADPH oxidase in plasma membrane of phagocytic vacuole. This is an important defence mechanism seen in fish. Thus, an increase in respiratory burst indicates an improved health status of fish (Ortuno et al. 2000).

The reduction of Nitroblue tetrazolium (NBT) by fish blood cells was recorded using Secombes and subsequently modified by Stasiack and Bauman method to determine the quantity of superoxide free radicals (Anderson et al., 1992). Anticoagulant mixed fish blood (100 µL) was placed in a 96 well microtiter plate and incubated at 27°C for 1 hour. After 1 hour, the supernatant was discarded and 0.2 % NBT (100 µL) was added and incubated at 27 °C for 1 hour. The supernatant was again discarded and fixed using absolute ethanol for 2 minutes followed by three washing steps using 70 % ethanol (200 µL) and dried. The formazan deposits were dissolved in 2 mol/L KOH (120 µL) and DMSO (140 µL). The absorbance was read at 620 nm using ELISA plate reader (Fernandes et al.,2021).

3.7 Carotenoid estimation

Certain fish belongs to astaxanthin converting group, they obtain and sustain their body colour through diet. To assess the impact of the diet on the body colour of *X. helleri*, the carotenoid content in fish supplemented with feed additives was estimated and compared to the control.

After 90 days of supplementing with carotenoid enriched feed, the skin of the fish was removed, pre weighed and capped in a 10 mL glass vial. 2.5 g of Anhydrous sodium sulphate was added. The sample was gently meshed with a glass rod against the side of a vial. 5 ml of chloroform was added and incubated overnight at 4ºC.

Chloroform forms a clear 1-2 cm layer above the caked residue. The optical density of sample was recorded at 452 nm with a blank of chloroform diluted to 3ml with absolute ethanol. (Rana et al.,2023).

$$Total\ \ carotenoid\ (\mu g/weight\ of\ tissue) = \frac{Absorbance}{0.25 \times sample\ weight\ (g) \times 10)}$$

3.8 Cytotoxicity evaluation

The feed safety assessment in fish blood cells was assessed by trypan blue dye exclusion test after feeding the fish for 2 months. 200 µL of fish blood was extracted from caudal vein in anticoagulant using 1 mL 26-gauge syringe. The blood was mixed with 0.4 % trypan blue for 2 minutes at RT. The viable cells have intact plasma membrane hence, they appear colourless under the microscope whereas the non-viable cells take up the stain due to disintegrated plasma membrane and appear blue under the microscope.

The cell viability (%) was calculated according to (Strober ,2015) by counting the number of stained (dead cells) and unstained (live cells) separately.

$$cell\ viability\ (\%) = \frac{number\ of viable\ cells}{Total\ number\ of\ cells\ counted} \times 100$$

3.8.1 LC_{50} Analysis

This is a toxicity test to determine the lethal concentration at which 50 % of the test organism experience lethal effects. The fingerlings were set up into 4 tanks fed with consortium of 0.1, 0.3, 0.5, and 0.7 g. The maximum dosage was 0.7 g and the survival rate was observed for 7 days (Fernandes et al., 2021).

3.9 Statistical analysis of data

Statistical analysis was performed by using IBM© SPSS version 23.0 statistical software (IBM Corporation, USA, 2021). The results were expressed as mean with their corresponding standard deviation (S.D.). The results were analysed statistically using one-way analysis of

variance (ANOVA). Subsequently, post-hoc multiple comparisons with Duncan's test were used to assess the significant difference between the different treatments/ feed additives

CHAPTER 4: ANALYSIS AND CONCLUSIONS

4.1 Screening of natural feed additives for nutrients

4.1.1 Estimation of carbohydrates of different feed additives and formulated feed

The carbohydrate content was significantly ($p< 0.001$) higher in *Ulva* sp. with 32.04 ± 1.83 %, followed by C + S with 27.46 ± 1.3 %. (Figure 7 (A)).

Among the formulated feeds, *Ulva*-based feed had the highest carbohydrate content of 33.6 ± 0.11 %, followed by orange bacteria with 22.7 ± 10.68 %. (Figure 7 (B)).

The result is in accordance to the study reported by Postama et al., that the carbohydrate concentration ranged from 20 – 51 %. The same study also reported that, the carbohydrate content range depends on the extraction methodology. Another study by Mohan et al., reported that, the dry weight of crustacean shells ranged from 17.50 % to 23.75 % (Mohan et al.,2015).

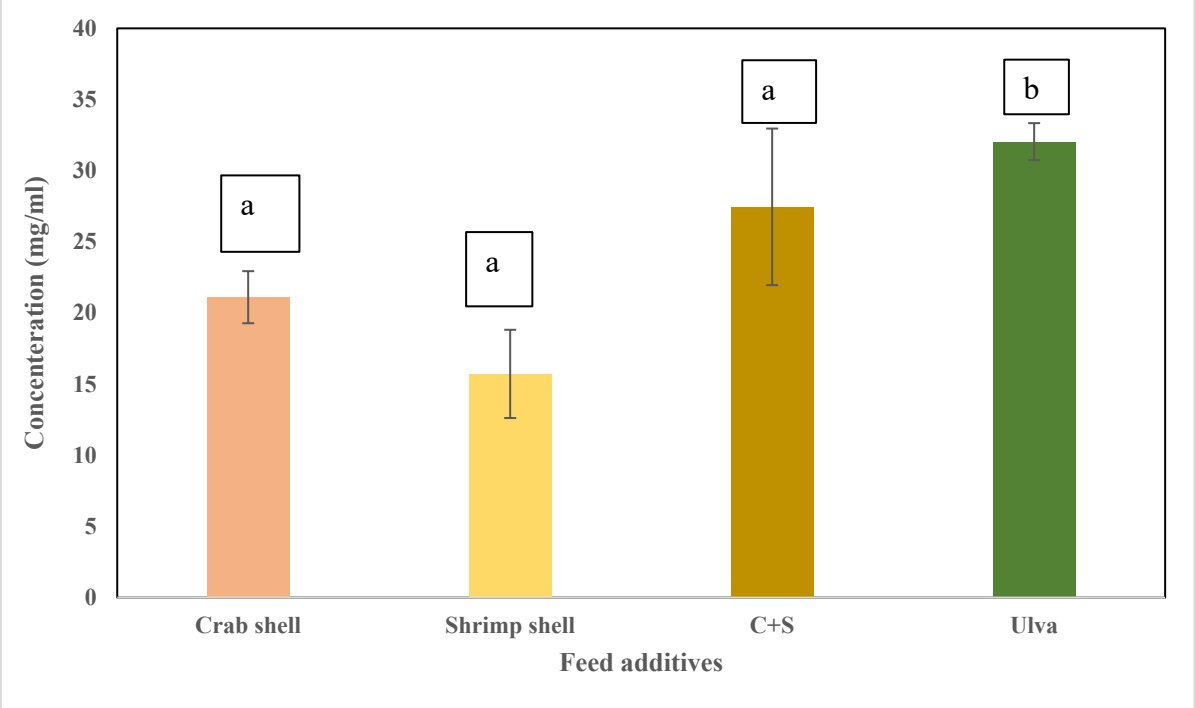

Figure 7(A): Estimation of carbohydrates from different feed additives.

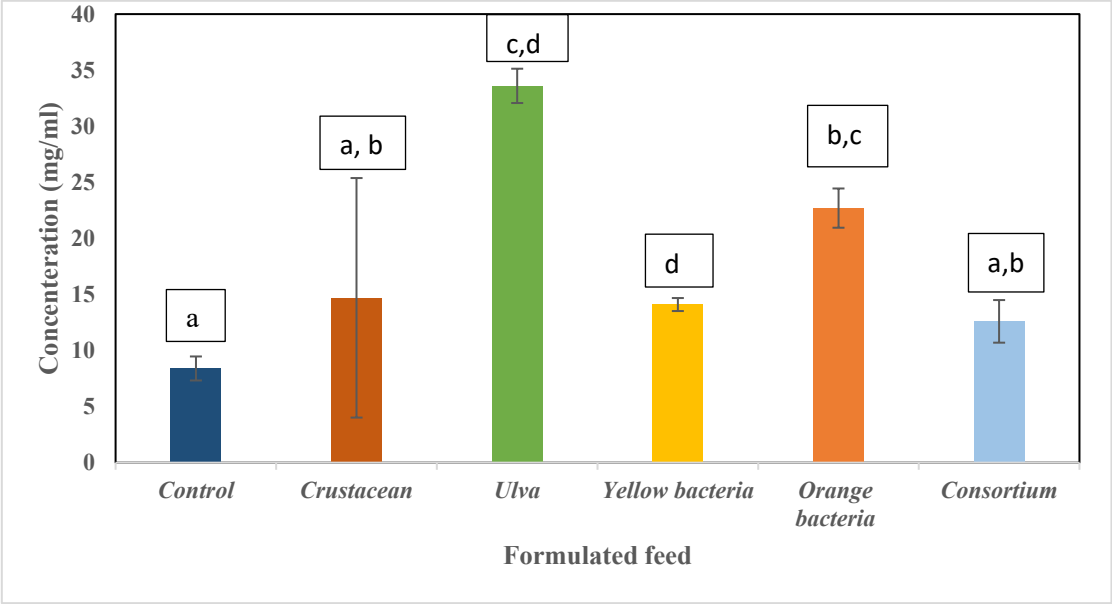

Figure 7 (B): Estimation of Carbohydrates in the formulated feed

Each value is the mean ± Standard Deviation of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; $p < 0.01$ and subsequent post hoc multiple comparison with Duncan's test).

4.1.2 Estimation of Protein concentration in feed additives and formulated feed

The Protein content in the feed additives were estimated to be, 13.80 ± 5.9 % (*Ulva* sp.), 13.50 ± 2.2 % (C + S), 12.40 ± 1.4 % (shrimp shell), 11.20 ± 2 % (crab shell). There was no significant ($p>0.05$) difference in protein content of different feed additives (Fig. 8 (A)). Among the formulated feed, the Protein concentration was significantly ($p<0.001$) higher in crustacean shells by 22.6 ± 3.09 mg/mL followed by the consortium 18.8 ± 5.92 mg/mL. (Fig. 8 (B)).

Natify et al., reported 14.2 % crude protein in *Ulva lactuca* feed additive fed to mottled rabbit fish (Natify et al.,2015).

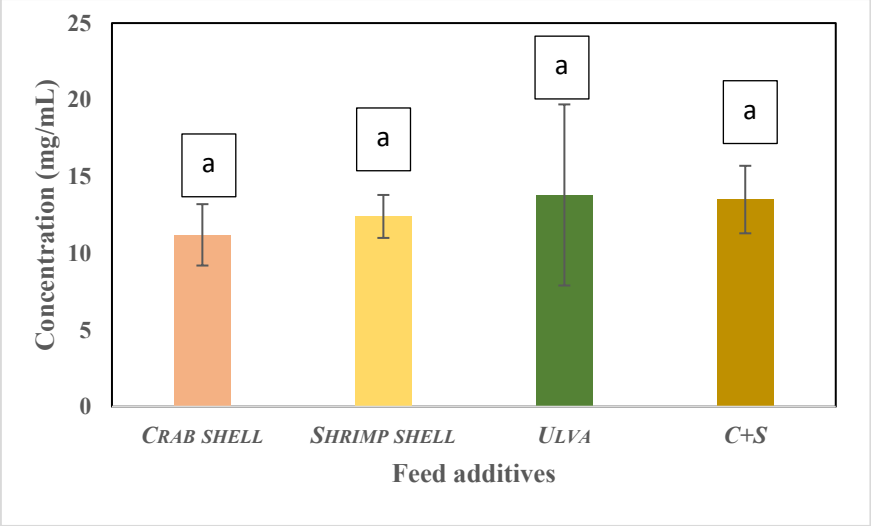

Figure 8 (A): Estimated protein concentration of feed additives.

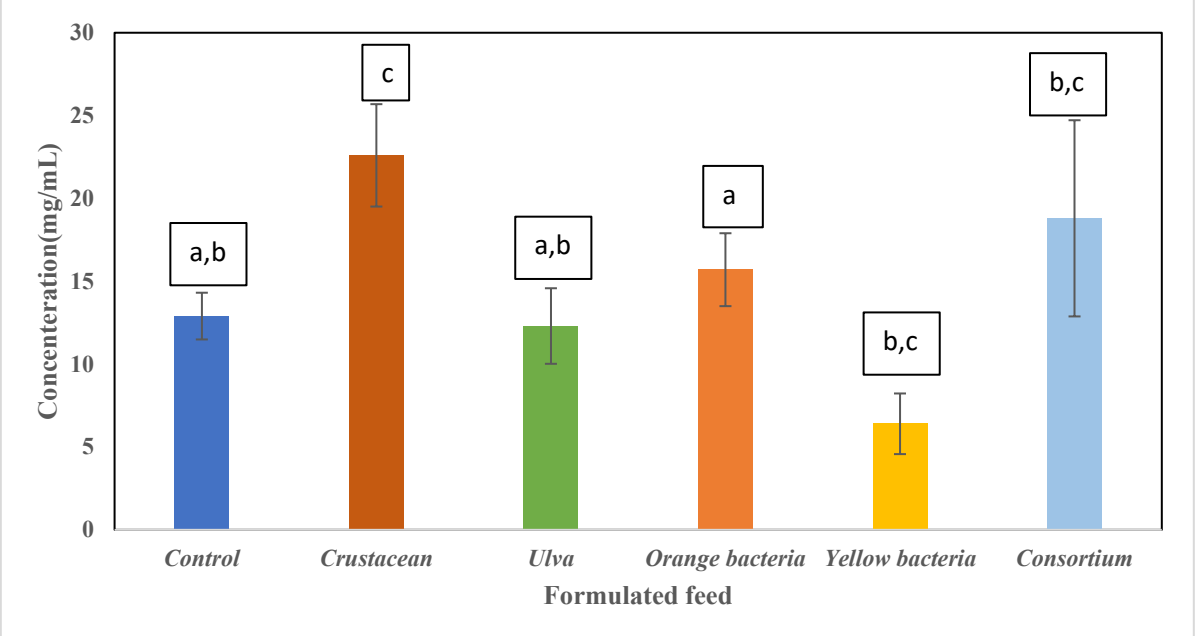

Figure 8 (B): Estimated protein concentration of feed formulated.

Each value is the mean ± Standard deviation of three replicates (n = 2). Within each bar, means with different superscript letters are statistically significant (ANOVA; $p < 0.01$ and subsequent post hoc multiple comparison with Duncan's test).

4.1.3 Estimation of Antioxidant activity of formulated feed

Yellow bacteria containing feed showed significantly ($p<0.01$) higher scavenging activity using DPPH method by 25 ± 1.3 mg/mL, followed by crustacean shells 18 ± 0.54 mg/mL and orange bacteria 15 ± 1.83 mg/mL.

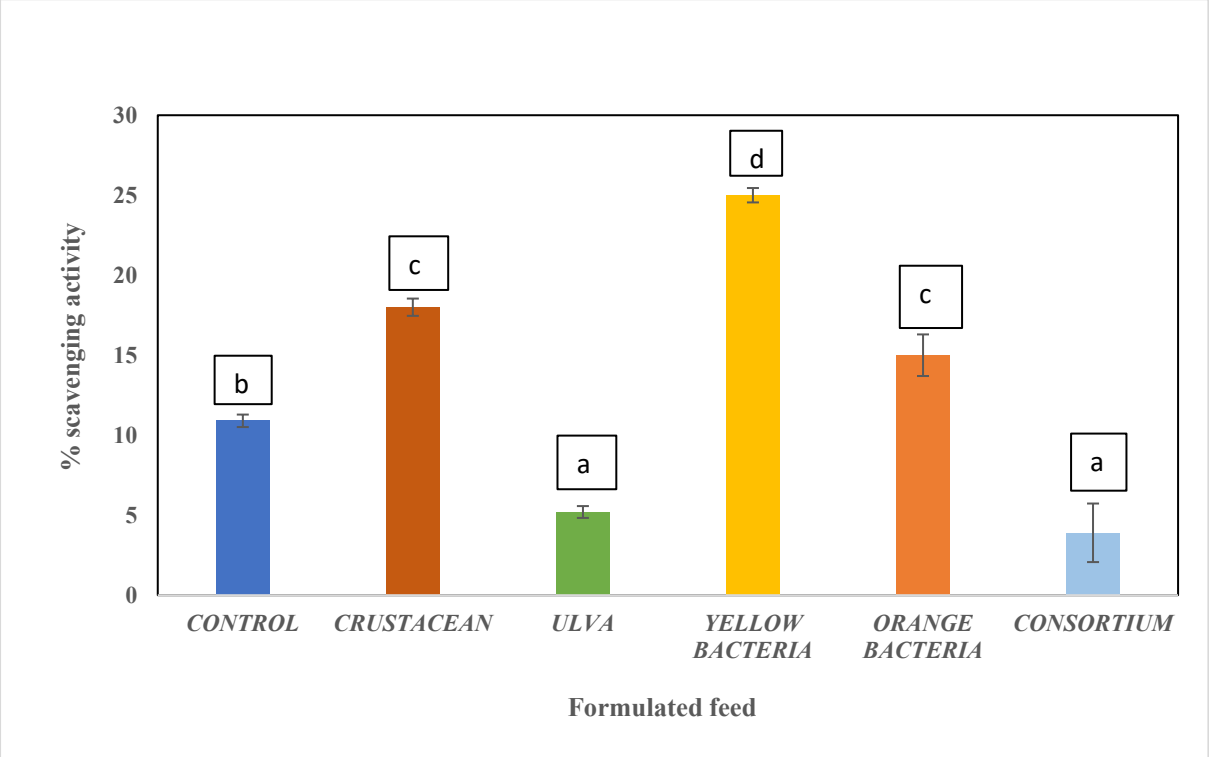

Figure 9: Estimated antioxidant activity shown by formulated feed.

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; p < 0.01 and subsequent post hoc multiple comparison with Duncan's test).

4.1.4 Estimation of phenolic compounds in feed additives and formulated feed

Phenol content of crustacean shells (C + S) was significantly *(p<0.001)* higher concentration (233.611 ± 3.917 mg/mL) than crab shells (177.84 ± 5.1 mg/mL), and shrimp shells (117.36 ± 4.34 mg/mL) individually.

Consortium showed significantly (*p<0.001*) higher phenolic compounds 349.85 ± 1.08 mg/mL compared to crustacean shells 183.99 ± 3.31 mg/mL and yellow bacteria containing feed 181.82 ± 3.13 mg/mL.

Maia et al., reported the total phenol content of shrimp shell waste from *Palaemon serratus* and *Palemon varians* ranged between 4.7 and 10.4 mg GAE/g (Maia et al.,2023).

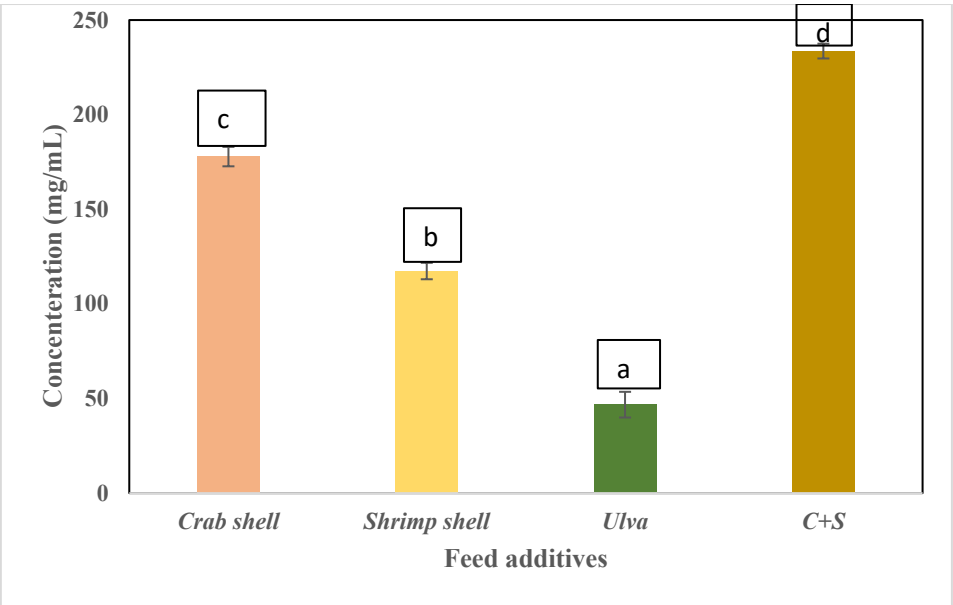

Figure 10 (A): Estimated phenol contents in feed additives.

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; p < 0.01 and subsequent post hoc multiple comparison with Duncan's test).

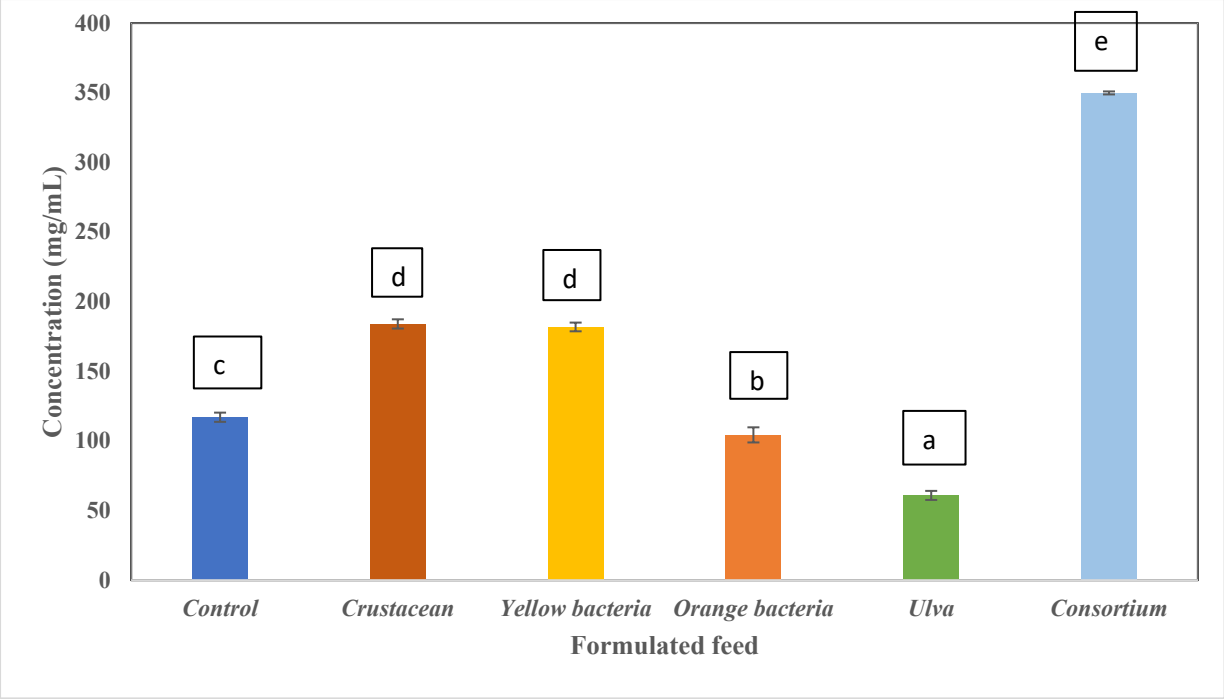

Figure 10 (B): Estimated phenol content in formulated feed.

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; $p < 0.01$ and subsequent post hoc multiple comparison with Duncan's test).

Table 2 (A): Summary of the biochemical analysis of natural feed additives.

Feed additives test	**Crab**	**Shrimp**	***Ulva***	**C+S**
Carbohydrate (mg/mL)	21.11 ± 1.83	15.72 ± 3.1	**32.04 ± 1.3**	27.46 ± 1.83
Protein (mg/mL)	11.20 ± 2	12.40 ± 1.4	**13.80 ± 5.9**	13.50 ± 2.2
Phenol (mg/mL)	177.84 ± 5.13	117.36 ± 4.34	46.75 ± 6.78	**233.61 ± 3.91**

Table 2(B): Summary of the Biochemical analysis of formulated feed using natural feed additives

Formulated feed constituents	Control	Crustacean	*Ulva*	Yellow bacteria	Orange bacteria	Consortium
Carbohydrate (mg/mL)	8.4 ± 1.07	14.7 ± 1.75	**26 ± 0.11**	14.1 ± 1.53	22.7 ± 10.68	12.6 ± 2.25
Protein (mg/mL)	12.9 ± 1.41	**22.6 ± 3.09**	12.3 ± 2.28	6.4 ± 1.83	15.7 ± 2.2	18.8 ± 5.92
Phenol (mg/mL)	117.04 ± 3.31	183.99 ± 3.31	60.87 ± 3.25	181.82 ± 3.13	104.3 ± 5.43	**349.8 ± 1.08**
Antioxidants (%)	11 ± 0.39	18 ± 0.54	5 ± 0.45	**25 ± 1.3**	15 ± 1.83	4 ± 0.37

Table 3: Biochemical analysis of gelatin-based feed block

Tests	Test Feed block (5 days)
Carbohydrate (mg/mL)	14.9 ± 1.9
Protein (mg/mL)	18.8 ± 5.9
Phenol (mg/mL)	526.78 ± 23.4
Antioxidants (%)	6 ± 0.15

Commercial feed block like Tetra holiday feed for (14 days) has reported a protein content of 40.5 %, fat 5.5 % and crude fibre 4.5 %. (Tetra holiday). This shows that test feed has required protein content for 5 days.

The Holiday Feed Block, patented under CA1325129C in Canada and invented by Klaus-Jurgen V. Poeppinghausen, was assigned later to Tetra Werke Dr Rer Nat. The patent

application was approved on December 14, 1993. However, the patent for the product has expired since December 12, 2010.

4.2 In-vivo assessment of formulated feed additive and feed block on *X. helleri.*

4.2.1 Feed stability and acceptability.

As per the observation, the formulated feed had a floatation rate of 13 minutes as compared to the control feed (16 mins) which is an ideal parameter for surface feeders. Since the feed blocks were made in an ice cube shape, they sink to the bottom of the tank within seconds. The initial dry weight of block was found to be 2.36 g and after adding to the fish tank the weight was observed as 1.773 g on day 3. The sword tails were observed pecking on the gelatin-based feed block and it lasted for 5 days without disintegrating.

The Percentage floatation rate was calculated by (Solomon et al.,2011). The % floating rate of consortium was observed to be 70% at 20th minute and stayed till 30th minute. While, control feed, % floating rate was 100% till 30th minute.

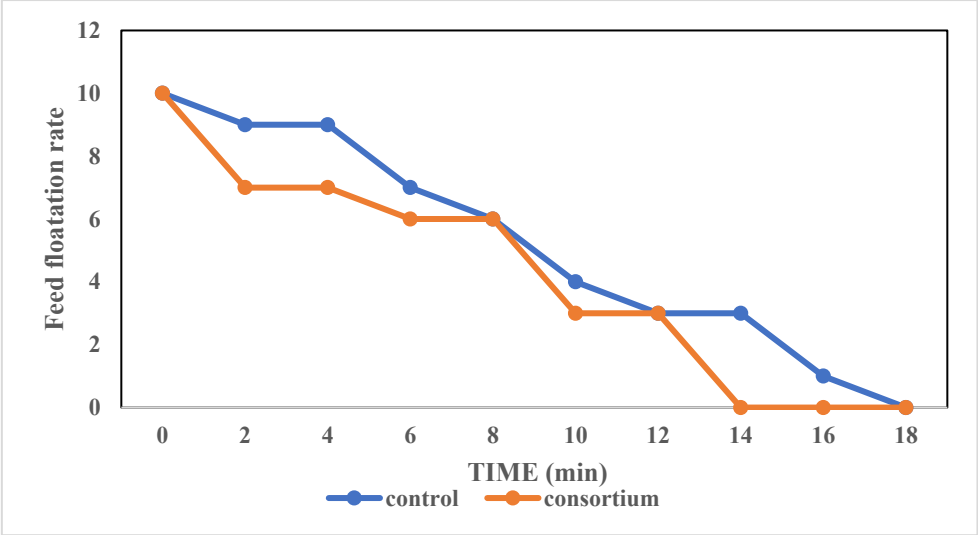

Figure 11: Formulated feed floatation rate compared to control feed.

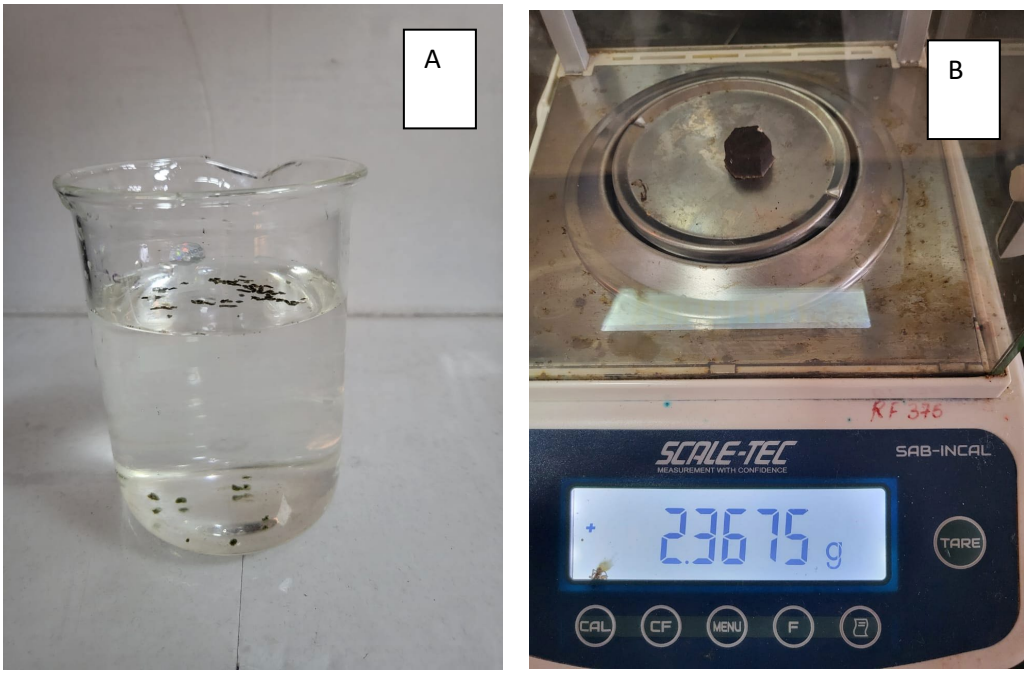

Figure 13: (A) Feed floatation rate assessment (B) Initial weight of Feed block

4.2.2 Effect of feed additives on growth performance

The effect of formulated feed on Sword tails was measured on a monthly basis by weighing their size and measuring the length.

The weight gain ranged from 0.154 ± 0.042 to 0.718 ± 0.07 g in control group and consortium shows a range of 0.142 ± 0.052 g to 0.741 ± 0.105 g over a period of 90 days. There is no significant *(p> 0.05*) weight gain difference between the treatment groups.

The specific growth rate (SGR) shows no significant (*p>0.05*) difference within each treatment groups. The SGR ranged from 3.22 ± 0.57 % to 2.18 ± 0.13 % in control and 4.53 ± 0.68 % to 2.55 ± 0.47 % in consortium over a period of 90 days. The feed efficiency ratio after 90 days of experimental trial was recorded as 7.54 ± 1.46 % in crustacean and 7.5 ± 1.05 % in consortium tank.

There is an overall growth of sword tails over a period of 90 days but no significant ($p>0.05$) difference within the treatment groups was observed.

A similar growth performance was observed by Menega et al., (2023) in swordtails supplemented with dietary pigment (50 mg/kg) isolated from the bacteria *Exiguobacterium profundum* (T1), *Chryseobacterium joostei* (T2), *Staphylococcus pasteuri* (T3*), Staphylococcus arlettae* (T4) served as treatments.

Table 4 (A): Graph showing weight gain of fish over a period of 90 days in gram

Treatments	30	60	90
Control (g)	0.277 ± 0.170	0.448 ± 0.116	0.718 ± 0.07
Crustacean shell (g)	0.301 ± 0.118	0.530 ± 0.175	0.754 ± 0.14
Orange Bacteria (g)	0.306 ± 0.168	**0.582 ± 0.202**	0.610 ± 0.18
Yellow Bacteria (g)	**0.360 ± 0.185**	**0.582 ± 0.224**	0.643 ± 0.23
***Ulva* (g)**	0.254 ± 0.114	0.564 ± 0.243	0.658 ± 0.19
Consortium (g)	0.282 ± 0.091	0.395 ± 0.213	**0.741 ± 0.10**

Table 4 (B): Graph representing the length of fish in cm over 90 days of experimental trial

Treatments	0	30	60	90
Control (cm)	1.8 ± 0.25	3.4 ± 0.45	4.7 ± 0.321	5.7 ± 0.152
Crustacean shell (cm)	1.5 ± 0.26	**3.6 ± 1.20**	**5.3 ± 0.472**	**6.4 ± 0.404**
Orange Bacteria (cm)	1.8 ± 0.15	3.2 ± 0.642	5.1 ± 0.321	6.1 ± 0.208
Yellow Bacteria (cm)	1.9 ± 0.10	3.5 ± 1.66	5.1 ± 0.264	6.2 ± 0.450
***Ulva* (cm)**	1.8 ± 0.058	2.9 ± 0.950	5.1 ± 0.153	6.1 ± 0.230
Consortium (cm)	**2.2 ± 0.75**	3.2 ± 0.585	5.0 ± 0.360	6.1 ± 0.305

Figure 4 (C): Feed efficiency ratio of fish fed with formulated feeds

Treatments	30	60	90
Control	2.8 ± 1.70	4.8 ± 1.16	5.9 ± 4.06
Crustacean shell	3.0 ± 1.18	4.9 ± 1.93	**7.5 ± 1.46**
Orange Bacteria	**3.1 ± 1.76**	5.2 ± 2.86	6.5 ± 1.81
Yellow Bacteria	2.9 ± 2.07	**5.4 ± 2.38**	6.4 ± 2.34
Ulva	2.0 ± 1.31	3.4 ± 3.34	6.6 ± 1.95
Consortium	2.8 ± 0.91	3.9 ± 2.13	**7.5 ± 1.05**

Table 4 (D): Specific growth rate of sword tail over 90 days of experimental trials.

Treatments	30	60	90
Control	5.7 ± 4.4	3.8 ± 2.3	2.5 ± 1.5
Crustacean shell	4.1 ± 0.8	2.6 ± 0.9	2.2 ± 0.3
Orange Bacteria	4.8 ± 3.4	2.5 ± 1.0	2.0 ± 0.3
Yellow Bacteria	3.6 ± 2.1	2.7 ± 0.8	2.0 ± 0.3
Ulva	3.0 ± 1.8	1.7 ± 1.8	2.1 ± 0.2
Consortium	3.7 ± 1.3	2.5 ± 0.6	2.3 ± 0.2

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; $p < 0.01$ and subsequent post hoc multiple comparison with Duncan's test).

4.3 Water quality assessment

Water quality parameters were assessed according to section 3.5 and maintained throughout the experimental trial.

The ideal water quality parameters for ornamental fish reported by (Menega et al.,2023) temperature (26 -30 ºC), pH (6.78 – 8.46), nitrite (0.1 – 0.2 mg/mL) and dissolved oxygen ranging from (4.2 – 5.3 mg/mL).

Table 5: Water quality tests of experimental tanks fed with various feed additives.

water quality tests	Control	Crustacean	*Ulva*	Yellow bacteria	Orange bacteria	Consortium
pH	8.29	8.11	8.14	8.05	7.99	8.34
Temperature	26 - 28^0C	26 - 28^0C	26 - 28^0C	26 - 28^0C	26 - 28^0C	26 - 28^0C
Salinity (psu)	0	0	0	0	0	0
D.O (mg/mL)	4.5 ± 0.15	4 ± 0.10	4 ± 0.06	3.9 ± 0.20	4.9 ± 0.15	3.8 ± 0.20
Nitrite (mg/mL)	0.26 ± 0.08	0.23 ± 0.07	0.28 ± 0.06	0.24 ± 0.04	0.30 ± 0.05	0.21 ± 0.07

4.4 Immunological assays to assess cellular innate immune response

4.4.1 Microscopic analysis of fish blood cells

The fish blood cells were stained using Giemsa stain and viewed under microscope at 1000 X magnification to observe fish blood cells. (Schütt et al., 1997).

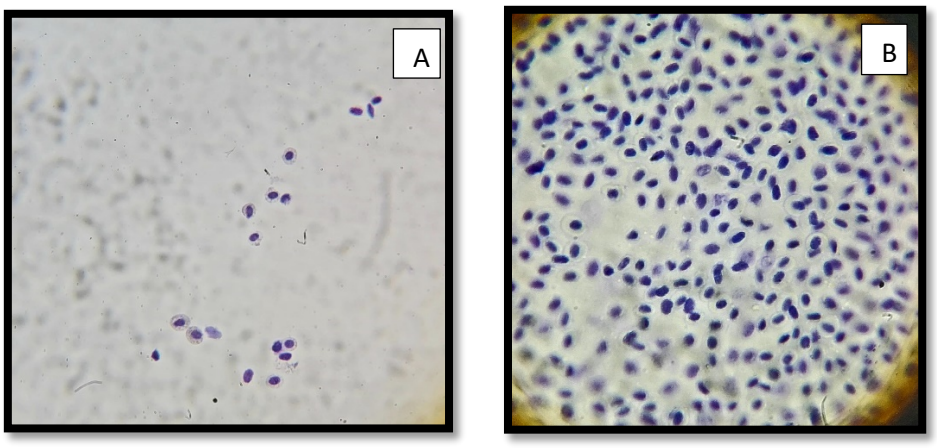

Figure 14 (A) (B): Blood cells observed under microscope at 100 X magnification lens.

4.4.2 Total plasma protein analysis.

Total plasma protein was estimated to be higher in crustacean shells fed fish by 18.6 ± 0.9 % and in yellow bacteria 16.8 ± 0.25 % compared to other treatments. There is no significant ($p>0.05$) difference in the plasma protein content within treatment groups.

In a study by Haque et al., the total plasma protein content in discus fish (*Symphysodon aequifasciatus)* treated with shrimp shell waste derived natural astaxanthin was 3.89 ± 0.05 mg/mL (Haque et al.,2020). This shows that crustacean shells (Tiger prawns shell and blue crab shell) contribute highly to the plasma protein content.

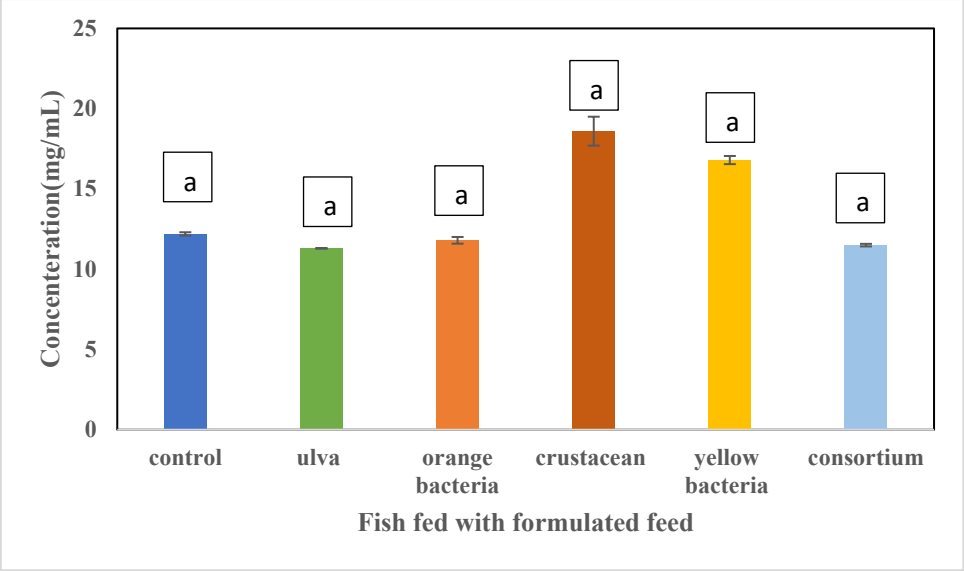

Figure 15: Graph representing total plasma protein in fish fed with different feed additives.

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; p < 0.01 and subsequent post hoc multiple comparison with Duncan's test).

4.5.3 Phagocytosis

The percentage phagocytosis activity was observed to be high in crustacean, *Ulva* and orange bacteria by 30 ± 1.1 %, 30 ± 2.8 %, 30 ± 1.6 %, respectively. There was no significant *(p>0.05*) difference between the treatments.

Fernandes et al., reported an increase in phagocytic activity treated with the probiotic consortium comprising (salt pan bacteria *Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus subtilis, Pseudomonas sp*.) by 46.15 ± 5.33 % as compared to control (36.74 ± 6.21 %) highest at day 120. (Fernandes et al., 2021). This is in accordance to percentage phagocytosis activity of sword tail fed with orange bacteria at day 90.

Another study by (Rodríguez & Ávila, 2016) reported an increase in phagocytosis content of tilapia *(Oreochromis niloticus*) fed with *Ulva* dietary feed at day 150 was reported to be 23.202 (*p < 0.001*).

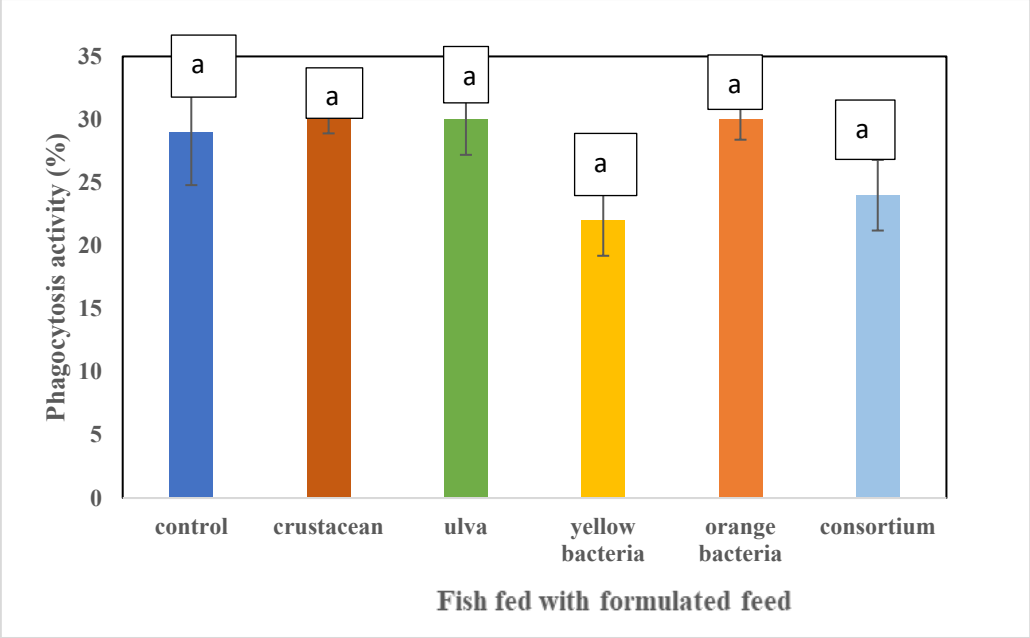

Figure 16: Graph depicting phagocytic activity in swordtail fish

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; p < 0.01 and subsequent post hoc multiple comparison with Duncan's test).

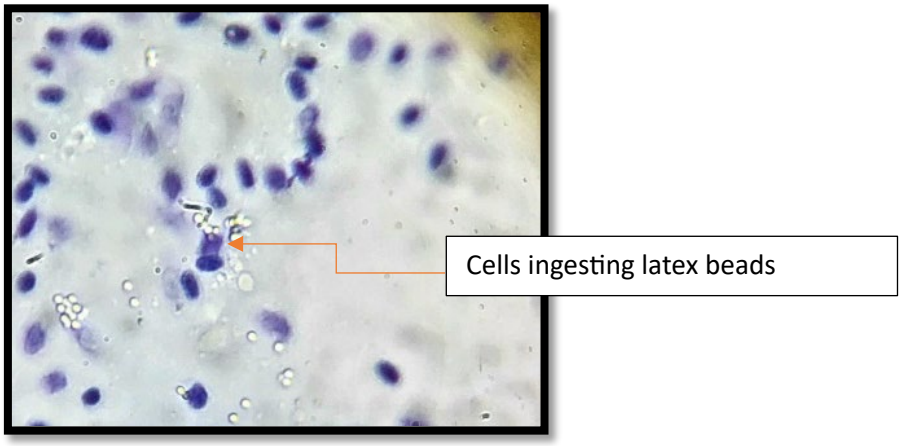

Figure 17: Cells ingesting latex beads observed at 100 X magnification lens.

4.4.4 Respiratory burst

Consortium (0.274 ± 0.018), orange bacteria (0.266 ± 0.077) and crustacean shells (0.247 ± 0.012) fed group showed significantly high ($p<0.001$) respiratory burst activity of optical density, respectively at 630 nm compared to 0.134 ± 0.024 (control), 0.126 ± 0.019 (yellow bacteria) and 0.112 ± 0.009 (*Ulva* species) group.

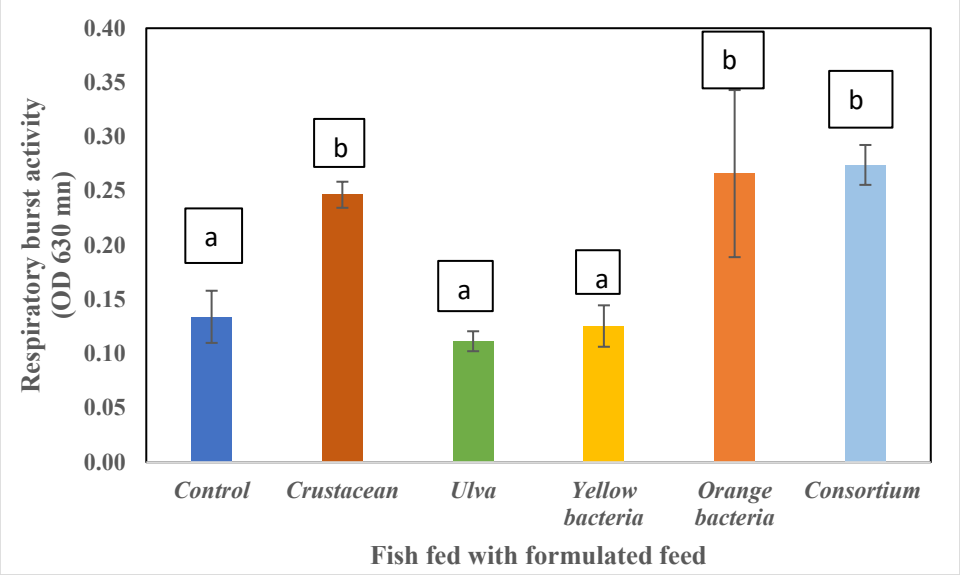

Figure 18: Respiratory burst (OD at 630 nm)

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means with different superscript letters are statistically significant (ANOVA; p < 0.01 and subsequent post hoc multiple comparison with Duncan's test).

4.5 Estimation of carotene of fish skin and fish tail.

Swordtails belong to group I based on their carotenoid biosynthetic capability i.e., they can convert lutein, zeaxanthin, or intermediates to astaxanthin but beta carotene is not the major precursor of astaxanthin. Hence, store astaxanthin directly from the diet into their body. Carotene estimation was done as a part of the colour maintenance in captivity. There is no significant difference in the carotenoid content in the fish between different treatments.

However, the consortium exhibited the capacity to increase the carotene content by 0.111 ± 0.011 mg/g compared to the control 0.091 ± 0.003 mg/g.

The mean carotenoid content of *X. helleri* treated with various plant derived bio- resources as dietary feed reported by Rana et al., shows 0.1742 ± 0.0851 mg/10 kg in commercial fed fish. While, China rose, marigold and carrot fed groups showed the following carotene content as 0.2053 ± 0.0955 mg/g, 0.3524 ± 0.2054 mg/g and 0.2341 ± 0.1128 mg/g respectively (Rana et al.,2023). This shows that the consortium comprising (Crustacean shells, *Ulva*, orange and yellow pigment producing bacteria) has lower carotenoid content compared to the study.

Our study reports a minor increase in the carotene content in the fish fed with consortium, however, the results were not significant as compared to the control.

Table 6: Estimated carotenoid content of fish fed with different feed additives

Beta carotene test	**Control**	**Crustacean Shell**	***Ulva***	**Yellow bacteria**	**Orange bacteria**	**Consortium**
Fish skin (mg/g)	0.091 ±0.003	0.107 ± 0.003	0.103 ± 0.011	0.101 ± 0.006	0.096 ±0.007	**0.111 ±0.011**

4.6 Cytotoxicity evaluation in sword tails fed with feed additives.

The cytotoxicity test by trypan blue dye exclusion was performed to analyse the percentage cell viability after feeding trials to determine the toxicity of the incorporated feed additives in the fish diet.

The test revealed higher cell viability in fish fed with crustacean shell 87 ± 2.828 %, similar to 85.5 ± 2.121(*Ulva)* and (85.5 ± 3.536) consortium compared to the control basal feed (commercially available) that has 72.2 ± 3.11 % cell viability. All experimental feed additives

showed significantly higher cell viability compared to the control feed except, orange bacteria fed fish that shows 49 ± 2.31 % which is much less compared to the control.

Orange bacteria tanks also showed high mortality compared to other experimental tanks indicating toxicity. While, all other feed additives showed comparatively higher viable count than control feed indicating no cytotoxicity**.**

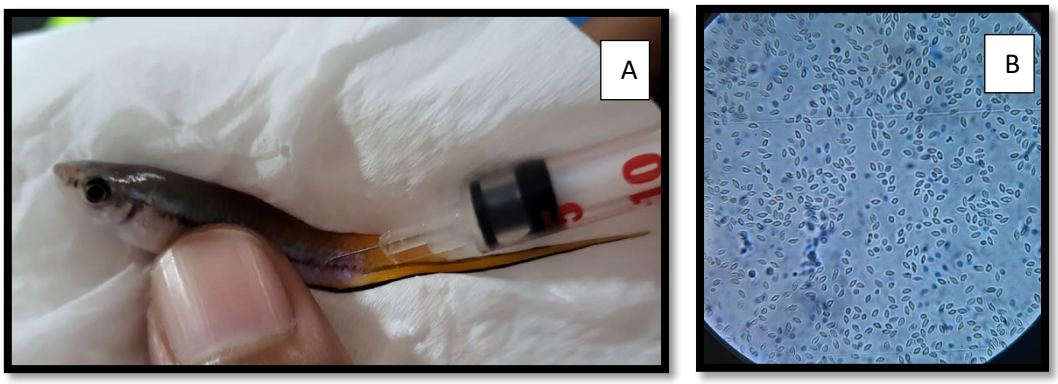

Figure 19(A): Blood extracted from caudal vein Fig.19 (B): Viable cell count using trypan blue dye exclusion test observed under 100 X magnification lens

Table 7: Cell viability counts of fish fed with different feed additives

Feed Formulated	Cell Viability In %
Control	72.2 ± 3.111
Crustacean Shell	**87 ± 2.828**
Ulva	85.5 ± 2.121
Yellow Bacteria	76 ± 1.414
Orange Bacteria	63 ± 2.828
Consortium	85.5 ± 3.536

4.7 LC_{50} evaluation

The LC_{50} test was conducted to determine the concentration of the consortium that would lead to a lethal effect on 50% of the fingerlings. In this experiment, six young fish aged between six and seven days were fed varying concentrations of the consortium (0.1 g, 0.3 g, 0.5 g, 0.7 g). Results showed that the group fed 0.5 g experienced an 80% mortality rate, while the group fed 0.7 g experienced 100% mortality within one day. The concentrations of 0.1 g and 0.3 g did not exhibit any harmful effects on the fish. However, at a dosage of 0.5 g, >50% mortality was observed, indicating LC_{50} to be 0.5 g.

CONCLUSION

The study resulted in the formulation of an ecofriendly economical feed additive comprising of crustacean shells, *Ulva* and bacteria with immunostimulatory activity in *X. helleri*. The feed additives used in the study exhibited no cytotoxicity when tested in-vivo.

Furthermore, the analysis also emphasised on formulating a gelatin- based feed block which showed higher nutrient content than the commercial blocks available in the market.

FUTURE PROSPECTS

- Analysis of carotenoid content after prolonged feeding trials with consortium excluding orange bacteria, under dark and light conditions.
- Proteomic analysis to study the differential expression of fish proteins expressed after treatments.
- Development of holiday feed block of 14 days.

REFERENCES

1. Ako, H., C.S. Tamaru, L. Asano, B. Yuen and M. Yamamoto, 2000. Achieving natural colouration in fish under culture. UJNR Technical Report, pp: 28.
2. Anderson, D. P., Tadaaki Moritomo, & R de Grooth. (1992). *Neutrophil, glass-adherent, nitroblue tetrazolium assay gives early indication of immunization effectiveness in rainbow trout*. *30*(4), 419–429. https://doi.org/10.1016/0165-2427(92)90110-c
3. Anderson, D.P., Roberson, B.S. and Dixon, O.W., 1979. Cellular immune response in rainbow trout Salmo gairdneri Richardson to Yersinia ruckeri O-antigen monitored by passive hae- molytic plaque assay test. J. Fish Dis., 2:169-178.
4. Andrews, C., Exell, A. & Carrington, N. (1988). The Interpet Guide to Fish Health. London: Salamander Books.
5. Barakat, K.M., Ismail, M.M., Abou El Hassayeb, H.E. *et al.* Chemical characterization and biological activities of *Ulva*n extracted from *Ulva fasciata* (Chlorophyta). *Rend. Fis. Acc. Lincei* **33**, 829–841 (2022).
6. Barman D, Nen P, Mandal SC, Kumar V (2013) Immunostimulants for Aquaculture Health Management. J Marine Sci Res Dev 3: 134. doi: 10.4172/2155-9910.1000134
7. Baron M, Davies S, Alexander L, Snellgrove D, Sloman KA (2008) The efect of dietary pigments on the coloration and behaviour of fame-red dwarf gourami, Colisa Lalia. Anim Behav 75(3):1041–1051.
8. Bricknell I, Dalmo RA (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology* **19**: 457–472.
9. Das A, Biswas SJJoA, Biology M (2016) Carotenoids and pigmentation in ornamental fish. J Aquac Mar Biol 4(4):00093. https://doi.org/10.15406/jamb.2016.04. 00093
10. de Carvalho, C. C. C. R., & Caramujo, M. J. (2017). Carotenoids in Aquatic Ecosystems and Aquaculture: A Colorful Business with Implications for Human Health. *Frontiers in Marine Science*, *4*. https://doi.org/10.3389/fmars.2017.00093
11. Debnath, Sudip & Sarker, Dipta & Kundu, Pankaj & Parvez, Md & Arafat, Shaikh & Mathew, Roshmon & Alkhamis, Yousef & Rahman, Md Moshiur & Rahman, Sheikh. (2022). Growth, Survival and Body Protein Content of Swordtail (Xiphophorus helleri) Fed Live and Formulated Feeds. Advances in Animal and Veterinary Sciences. 10. 10.17582/journal.aavs/2022/10.2.335.341.

12. Declercq, A. M., Haesebrouck, F., Van den Broeck, W., Bossier, P., & Decostere, A. (2013). Columnaris disease in fish: a review with emphasis on bacterium-host interactions. *Veterinary research*, *44*(1), 27.

13. Del Rocío Quezada-Rodríguez, P., Fajer-Ávila, E.J. The dietary effect of *Ulva*n from *Ulva clathrata* on hematological-immunological parameters and growth of tilapia *(Oreochromis niloticus*). *J Appl Phycol* **29**, 423–431 (2017). https://doi.org/10.1007/s10811-016-0903-7

14. Umamaheswari, T., Rajakumar, M., & Chidambaram, P. (2017). Economics of ornamental fish farming industry in Madurai District, Tamil Nadu. *Indian Journal of Fisheries*, *64*. https://doi.org/10.21077/ijf.2017.64.special-issue.76302-44

15. El-Araby, D. A., Amer, S. A., & Khalil, A. A. (2020). Effect of different feeding regimes on the growth performance, antioxidant activity, and health of Nile tilapia, Oreochromis niloticus. Aquaculture, 528, 735572.

16. C.

17. GOPAKUMAR, G. (2009). Controlled Breeding and Larval Rearing Techniques of Marine Ornamental Fishes. *Asian Fisheries Science*, *22*(2). https://doi.org/10.33997/j.afs.2009.22.2.038

18. Gouveia, L., Rema, P., Pereira, O., & Empis, J. (2003). Colouring ornamental fish (Cyprinus carpio and Carassius auratus) with microalgal biomass. *Aquaculture Nutrition*, *9*(2), 123–129. https://doi.org/10.1046/j.1365-2095.2003.00233.x

19. Grassi, T.L.M., do Espírito Santo, E.F., de Siqueira Marcos, M.T. *et al.* Bacterial pigment for Nile tilapia feeding. *Aquacult Int* **24**, 647–660 (2016).

20. Growth, Survival and Body Protein Content of Swordtail (Xiphophorus helleri) Fed Live and Formulated Feeds. Advances in Animal and Veterinary Sciences. 10. 10.17582/journal.aavs/2022/10.2.335.341. Vallejos-Vidal, E., Reyes-López, F., Teles, M., & MacKenzie, S. (2016).

21. HIDALGO, M. C., SKALLI, A., ABELLAN, E., ARIZCUN, M., & CARDENETE, G. (2006). Dietary intake of probiotics and maslinic acid in juvenile dentex (Dentex dentex L.): effects on growth performance, survival and liver proteolytic activities. *Aquaculture Nutrition*, *12*(4), 256–266.

22. J. Ezhil, & Narayanan, M. (2012). Pseudomonas aeruginosa as a Potential Probiont and Pigment Enhancer in the Ornamental Cichlid, Pseudotropheus lombardoi. *Proceedings of the Zoological Society*, *66*(2), 154–158.

23. Jeney, G., & Anderson, D. P. (1993). An in vitro technique for surveying immunostimulants in fish. *Aquaculture*, *112*(2-3), 283–287.
24. 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.
25. Luo M, Lu G, Yin H, Wang L, Atuganile M, Dong Z (2021) Fish pigmentation and coloration: molecular mecha nisms and aquaculture perspectives. Rev Aquac 13(4):2395–2412
26. Luo, M., Lu, G., Yin, H., Wang, L., Atuganile, M., & Dong, Z. (2021). Fish pigmentation and coloration: Molecular mechanisms and aquaculture perspectives. *Reviews in Aquaculture*, *13*(4), 2395–2412.
27. M. Menaga, S. Felix, C. Mohanasundari and M. Charulatha (2023). Characterisation of Pigment Producing Bacteria Isolated from Bio Floc Ponds and its Colour Enhancement Effect in Xiphophorus helleri. Indian Journal of Animal Research. 57(10): 1415-1421. doi: 10.18805/IJAR.B-4384.
28. Madhu, K., Madhu, R., & Harikrishna, N. (2023). *International Workshop-cum-Training on Fisheries Management and Aquaculture ICAR-Central Marine Fisheries Research Institute MARINE ORNAMENTAL FISH CULTURE: MEANS FOR ECONOMIC GROWTH*.
29. Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & Shellfish Immunology*, *20*(2), 137–151. https://doi.org/10.1016/j.fsi.2004.09.006
30. Margareta A, Nikhlani A (2021) The efectiveness of supplementation of spirulina sp and astaxanthin within feed to color quality of comet fsh (Carassius auratus). Jurnal Ilmu Kelautan 7(1):1–6
31. Mehana, El-Sayed & Rahmani, Arshad & Aly, Salah. (2015). Immunostimulants and Fish Culture: An Overview. Annual Research & Review in Biology. 5. 477-489. 10.9734/ARRB/2015/9558.
32. Menaga, M., Felix, S., Charulatha, M., Mohanasundari, C., Gopalakannan, A. (2020). Comparison of fertilization prototype on biofloc development and its characteristics in GIFT Tilapia culture. Indian Journal of Animal Research. 3: 310-316.
33. Menaga, M., Felix, S., Mohanasundari, C., & Charulatha, M. (2021). Characterisation of Pigment Producing Bacteria Isolated from Bio Floc Ponds and Its Colour Enhancement

Effect in Xiphophorus helleri. *Indian Journal of Animal Research*, *Of*. https://doi.org/10.18805/ijar.b-4384

34. Mente E, Karalazos V, Karapanagiotidis IT, Pita C (2011) Nutrition in organic aquaculture: an inquiry and a dis course. Aquac Nutr 17(4):e798–e817
35. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3), 426-428.
36. Ng TT, Lau CC, Tan MP, Wong LL, Sung YY, Tengku Muhammad TS, Van de Peer Y, Sui LY, Danish-Daniel M (2023) Cutaneous transcriptomic profiling and can didate pigment genes in the wild discus (Symphysodon spp.). NZ J Zool.
37. Obtaining of Astaxanthin from Crab Exosqueletons and Shrimp Head Shells. (2021). *Biointerface Research in Applied Chemistry*, *11*(5), 13516–13523.
38. *Ornamental Fish Market Size, Share | Global Industry Report 2019-2025*. (2019). Grandviewresearch.com.
39. Pepi, M., & Focardi, S. (2021). Antibiotic-Resistant Bacteria in Aquaculture and Climate Change: A Challenge for Health in the Mediterranean Area. *International journal of environmental research and public health*, *18*(11), 5723.
40. Peterson, G. L. (1979). Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Analytical biochemistry, 100(2), 201-220.
41. *Pradhan Mantri Matsya Sampada Yojana | Department of Fisheries, MoCIT, GoI*. (n.d.). Pmmsy.dof.gov.in.
42. Rana, S., Bari, A. A., Shimul, S. A., Mazed, M. A., & Nahid, S. A. A. (2023). Enhancement of body coloration of sword-tail fish (Xiphophorus helleri): Plant-derived bio-resources could be converted into a potential dietary carotenoid supplement. Heliyon, 9(4), e15208.
43. Rashidian, Ghasem & Simona, Rainis & Prokic, Marko & Faggio, Caterina. (2020). Effects of different levels of carotenoids and light sources on swordtail fish (Xiphophorus helleri) growth, survival rate and reproductive parameters. Natural Product Research. 10.1080/14786419.2020.1723091.
44. Sathyaruban S, Uluwaduge DI, Yohi S, Kuganathan S (2021) Potential natural carotenoid sources for the colouration of ornamental fish: a review. Aquac Int 29(4):1507 1528.
45. Sathyaruban, S., Uluwaduge, D.I., Yohi, S. *et al.* Potential natural carotenoid sources for the colouration of ornamental fish: a review. *Aquacult Int* **29**, 1507–1528 (2021).

46. Schar, D., Klein, E.Y., Laxminarayan, R. *et al.* Global trends in antimicrobial use in aquaculture. *Sci Rep* **10**, 21878 (2020).
47. Selvakumar Dharmaraj, & Kandasamy Dhevendaran. (2011). *Application of Microbial Carotenoids as a Source of Colouration and Growth of Ornamental Fish Xiphophorus helleri*. *3*(2), 137–144.
48. Supplementation of Feed Additives on Aquaculture Feeds: A Review. (2020). *International Journal of Pharmaceutical Research*, *13*(01).
49. The response of fish to immunostimulant diets. *Fish & shellfish immunology*, *56*, 34–69.
50. Thépot, V., Campbell, A. H., Paul, N. A., & Rimmer, M. A. (2021). Seaweed dietary supplements enhance the innate immune response of the mottled rabbitfish, Siganus fuscescens. *Fish & Shellfish Immunology*, *113*, 176–184.
51. Thépot, V., Campbell, A. H., Rimmer, M. A., & Paul, N. A. (2020). Meta-analysis of the use of seaweeds and their extracts as immunostimulants for fish: a systematic review. *Reviews in Aquaculture*, *13*(2), 907–933. https://doi.org/10.1111/raq.12504
52. Tørud, B., & Håstein, T. (2008). Skin lesions in fish: causes and solutions. *Acta Veterinaria Scandinavica*, *50*(Suppl 1), S7. https://doi.org/10.1186/1751-0147-50-S1-S7
53. Wieczorek, R., & Horley, P. (2015). The replicas of rongorongo objects in the musée du quai Branly (Paris). *Journal de La Société Des Océanistes*, *140*, 123–142.

APPENDIX I

CHEMICAL PREPARATION

1. Bradford reagent

Coomassie brilliant blue G-250 (100 mg) dissolved in 50 ml 95% ethanol. 100ml 85% phosphoric acid added to this solution, the solution diluted to final 1 L volume.

2. DPPH reagent

Dissolve 1 mg of DPPH powder in 13 ml ethanol.

3. DNSA reagent

Mix 3 g of sodium potassium tartrate in 50 mL distilled water and mix 1 g of DNSA powder in 20 mL of 2 M NaOH. Mix the solutions together.