TO INVESTIGATE THE NUTRACEUTICAL PROPERTIES OF GINGER IN AQUACULTURE FEED FORMULATION

Dissertation

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

AQUACULTURE

(Minor Subject: Aquatic Environment Management)

By
Priya Rawat
(L-2017-F-01-D)

Department of Aquaculture
College of Fisheries

Ludhiana – 141004

2021

CERTIFICATE I

This is to certify that the dissertation entitled, **"TO INVESTIGATE THE NUTRACEUTICAL PROPERTIES OF GINGER IN AQUACULTURE FEED FORMULATION"** submitted for the degree of **Ph.D.,** in the subject of **AQUACULTURE** (Minor Subject: **AQUATIC ENVIRONMENT MANAGEMENT**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Ms. PRIYA RAWAT** Registration No. **L-2017-F-01-D** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

_____

(Dr. Vaneet Inder Kaur)
Major Advisor
Principal Scientist (Fisheries)
Department of Aquaculture
College of Fisheries
Guru Angad Dev Veterinary and
Animal Sciences University,
Ludhiana – 141004, Punjab,
India

CERTIFICATE I (A)

This is to certify that the dissertation entitled, **"TO INVESTIGATE THE NUTRACEUTICAL PROPERTIES OF GINGER IN AQUACULTURE FEED FORMULATION"** submitted for the degree of **Ph.D.,** in the subject of **AQUACULTURE** (Minor Subject: **AQUATIC ENVIRONMENT MANAGEMENT**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by Ms. **PRIYA RAWAT** Registration No. **L-2017-F-01-D.** The work under **objective-II (To develop ginger extract enriched *Artemia* with nutraceutical properties for aquaculture feed formulation),** funded by **Science & Engineering Research Board -Department of Science & Technology (SERB-DST)**, Government of India under ***Overseas Visiting Doctoral Fellowship*** **(Award No. ODF/2018/001006)** was carried under my supervision and that no part of this work has been submitted for any other degree.

The assistance and help received during the course of the investigation have been fully acknowledged.

26/04/2021

(Dr. Kartik Baruah)
Overseas Guide
Associate Senior Lecturer
Department of Animal Nutrition & Management
Faculty of Veterinary Medicine & Animal Science
Swedish University of Agricultural Sciences (SLU), Uppsala Sweden

CERTIFICATE II

This is to certify that the dissertation entitled, **"TO INVESTIGATE THE NUTRACEUTICAL PROPERTIES OF GINGER IN AQUACULTURE FEED FORMULATION"** submitted by **Ms. Priya Rawat** Registration No. **L-2017-F-01-D** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of **Ph.D.,** in the subject of **AQUACULTURE** (Minor Subject: **AQUATIC ENVIRONMENT MANAGEMENT**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

(Dr. Vaneet Inder Kaur)
Major Advisor

(Dr. Asha Dhawan)
External Examiner
Former Dean
College of Fisheries
GADVASU, Ludhiana
House No. 31 Green Park
Civil Lines, Ludhiana

(Dr. Meera D. Ansal)
Head of the Department

(Dr. Sanjeev Kumar Uppal)
Dean, Postgraduate Studies

ACKNOWLEDGEMENT

First and foremost, I bow my head to Him, the merciful ***"God",*** *for his blessing hand and bestowing a creative and healthy environment throughout my academic and research endeavor.*

Words in my lexicon fail to elucidate my profound sense of veneration and indebtedness to my Major Advisor ***Dr. Vaneet Inder Kaur,*** *Principal Scientist (Fisheries), Department of Aquaculture, College of Fisheries, GADVASU, Ludhiana. I sincerely thank her, for invaluable guidance, parental concern, outstanding cooperation, love, support, patience, encouragement, motivation, enthusiasm, constructive criticism and critical evaluation during the entire duration of my Ph.D. study and research. I could not have imagined having a better advisor and mentor for my Ph.D. study.*

With immense pleasure, I wish to express my sincere thanks and profound sense of gratitude to my overwhelming Overseas Guide ***Dr. Kartik Baruah,*** *Associate Senior Lecturer, Department of Animal Nutrition & Management, Faculty of Veterinary Medicine & Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden for his constant support, help & inspiring and guiding my way through this unpredictable Ph.D. journey. It has been a privilege to work with him.*

I profess my heartfelt gratefulness and sincere regard to ***Dr. Meera D. Ansal,*** *Dean College of Fisheries, GADVASU, Ludhiana for providing the facilities for conducting the present study at College of Fisheries.*

I would like to thank members of my advisory committee, ***Dr. Meera D. Ansal,*** *Head, Department of Aquaculture and Dean PG'S Nominee,* ***Dr. Shashi Nayyar,*** *Senior Biochemist,* ***Department of Veterinary Physiology and Biochemistry,*** ***Dr. Anuj Tyagi,*** *Assistant Professor, Department of Aquatic Environment and* ***Dr. Abhed Pandey,*** *Assistant Professor, Department of Aquaculture for their invaluable suggestions and everlasting guidance throughout the pursuance of this research study.*

I feel elated in expressing sincere thanks and gratefulness to ***Dr. Anuj Tyagi,*** *Assistant Professor (Fisheries). The guidance and help rendered by him is highly appreciated. I will definitely remember his helping gratitude.*

It is my proud privilege to express my gratitude to ***Dr. Kulbir Singh Sandhu,*** *Former Dean, College of Fisheries, and* ***Dr. Sanjeev Kumar Uppal,*** *Dean, Postgraduate Studies, GADVASU, for providing me the facilities for conducting the present study.*

I feel privileged to express my sincere thanks to ***Science & Engineering Research Board - Department of Science & Technology (SERB-DST), Govt. of India*** *for providing me such a prestigious* ***Overseas Visiting Doctoral Fellowship (OVDF)*** *and a great financial support for one year with the help of which my dream came true regarding doing research in abroad. I couldn't ask for more. Thanks a ton from the bottom of my heart.*

I sincerely thank ***Mr. Ravinder*** *(Lab Assistant),* ***Mr. Balbir Singh*** *(FSI),* ***Mr. Gurmeet Singh*** *(FSI) and the entire field workers for their support and help in conducting the work efficiently throughout my experiment.*

I feel privileged to express my sincere thanks to ***Dr Sara Ostermann, Dr. Parisa and Dr. Tytti*** *for their guidance and help in conducting the molecular work at SLU, Sweden.*

I am extending my thanks to ***Johan Karlson*** *(Administrative Chief) and* ***Anagreeta*** *(Laboratory Chief) for their support and help in conducting the work efficiently throughout my experiment.*

No words can adequately express my indebted feelings to the most affectionate and caring Family, my Father ***Mr. S.P.S Rawat*** *and my Mother* ***Mrs. Anita Rawat*** *for their selfless love, care, blessings, moral support, emotional support and valuable guidance throughout the period of my Ph.D.*

Luckily, I have the luxury of being surrounded by delightful people who care about my vision by providing incessant encouragement and altruistic help by my best friend ***Dr. Vinkit Dhiman****, my sister* ***Parul Rawat*** *and brother* ***Nikhil Rawat.*** *Without their inspiration & moral support, it would not have been possible for me to complete this unpredictable Ph.D. journey.*

Last and most importantly I am thankful to my grandparents Late ***Ram Singh Rawat*** *and Late* ***Shakumbari Rawat*** *for their unconditional love and blessings which led to materialize this dream. I wish, I could hug them and show them my dissertation.* ***I would like to dedicate my Ph.D. dissertation to my beloved grandparents.***

Ludhiana ***Priya Rawat***

Title of the Dissertation	:	TO INVESTIGATE THE NUTRACEUTICAL PROPERTIES OF GINGER IN AQUACULTURE FEED FORMULATION
Name of the Student and Admission No.	:	Priya Rawat L-2017-F-01-D
Major Subject	:	Aquaculture
Minor Subject	:	Aquatic Environment Management
Name and Designation of the Major Advisor	:	Dr. Vaneet Inder Kaur Principal Scientist (Fisheries)
Degree to be Awarded	:	Ph.D.
Year of Award of Degree	:	2021
Total Pages in Thesis	:	161 + VITA
Name of University	:	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141004

ABSTRACT

The study was conducted by undertaking two experiments (Experiment I in India and Experiment II in Sweden). In Experiment I, feeding trial (120 day) was conducted to evaluate the efficacy of ginger powder (GP) as nutraceutical in rohu (*Labeo rohita*) in terms of growth, health status and resistance against *Aeromonas hydrophila* infection. Study was carried out in cemented outdoor tanks ($20m^2$) in triplicates with a stocking density of 30 fish $tank^{-1}$. Fish was fed with four experimental and one control diet [GP0 (control diet; no GP), GP5 (5g GP kg^{-1} GP0), GP10 (10g GP kg^{-1} GP0), GP15 (15g GP kg^{-1} GP0) and GP20 (20g GP kg^{-1} GP0)]. Results revealed significant ($P \leq 0.05$) improvement in fish growth (NWG, SGR, PER, FCR and condition factor), haematological parameters (RBC, WBC, Ht and Hb) and thyroid hormones (T3 and T4) in GP15. Other health related parameters like non-specific immune responses (RBA, total Ig and lysozyme activity), blood metabolic profile (HDL, cholesterol, triglycerides, AST, ALT total proteins, albumin, globulins, albumin/globulin ratio and glucose) and stress indices (SOD and LPO) were also improved in the same treatment (GP15) after prolonged feeding of 120 days. Results of 15 day challenge study of *L. rohita* with *A. hydrophilla* after 120 day feeding trial in terms of fish survival (%), level of protection (%) and post mortem symptoms too revealed ginger as an effective immune-modulatory agent. In Experiment II, prophylactic effect of ginger extract (GE) against *Vibrio parahaemolyticus* was determined in gnotobiotic *Artemia* as a model system, along with underlying mechanism of action behind the possible protective effects of GE was also unrevealed. The results showed that pre-treatment of *Artemia* larvae with GE @ 250 ug ml^{-1} led to a significant protection of the larvae upon challenge with *V. parahaemolyticus*. The results depicted that the increase in the survival of the GE pre-treated larvae was associated with a significant increase ($p \leq 0.05$) in the expression of a core set of genes related to the defense system of *Artemia* i.e. *hsp90*, *tgase1*, *hmgb*, *sod*, *pxn* and *lgbp*. In essence, due to easy availability, ginger can be recommended to be used in its crude form (ginger powder) as nutraceutical agent in carp culture for their sustainable production and in pure form (ginger extract) for the enrichment of *Artemia* to be used it as nutraceutical food for fish/shrimp larvae.

Key Words: Ginger powder, *L. rohita*, *Artemia*, ginger extract, nutraceutical

Signature of Major Advisor **Signature of the Student**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

@	-	At the rate
%	-	Percentage
ALT	-	Alanine Aminotransferase
ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Chemists
APHA	-	American Public Health Association
AST	-	Aspartate Aminotransferase
ATP	-	Adenosine Triphosphate
Av.	-	Average
BMR	-	Basal Metabolic Rate
BWG	-	Body Weight Gain
Ca^{2+}	-	Calcium
$CaCO_3$	-	Calcium Carbonate
CAT	-	Catalase
cDNA	-	Complementary Deoxyribonucleic acid
CE	-	Cholesterol Esterase
CF	-	Crude Fat
CFU	-	Colony Forming Unit
Cl^-	-	Chloride
cm	-	Centimeter
CO_3^{2-}	-	Carbonate
CP	-	Crude Protein
DHAP	-	Dihydroxy Acetone Phosphate

DO	-	Dissolve Oxygen
dl	-	Deciliter
DM	-	Dry Matter
DNA	-	Deoxyribonucleic Acid
dscam	-	Down Syndrome Cell Adhesion Molecule
EAA	-	Essential amino acid
EDTA	-	Ethylenediamine Tetraacetic acid
EE	-	Ether Extract
ef-1	-	Elongation Factor
FASW	-	Filtered Artificial Sea Water
FCE	-	Feed Conversion Efficiency
FCR	-	Feed Conversion Ratio
g	-	Gram
g %	-	Gram Percent
gapdh	-	Glyceraldehyde 3-Phosphate Dehydrogenase
GE	-	Ginger Extract
GP	-	Ginger Powder
GPO	-	Glycerol Phosphate Oxidase
GPX	-	Glutathione Peroxidase
h	-	Hour
H_2O_2	-	Hydrogen Peroxide
H_2SO_4	-	Sulphuric Acid
Hb	-	Haemoglobin
HDL	-	High Density Lipids

hmgb	-	High Mobility Group Box
hsp70	-	Heat Shock Protein 70
hsp90	-	Heat Shock Protein 90
Ht	-	Haematocrit
IFCC	-	International Federation of Clinical Chemistry
IU	-	International Unit
K-value	-	Condition Factor
K^+	-	Potassium
kcal	-	Kilo Calories
kg	-	Kilogram
l	-	Litre
LDH	-	Lactate Dehydrogenase
lgbp	-	Lipopolysaccharide and b-1,3-Glucan-Binding Protein
LPO	-	Lipid Peroxidisation
m	-	Meter
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCHC	-	Mean Cell Haemoglobin Concentration
MCV	-	Mean Corpuscular Volume
MDA	-	Malondialdehyde
MDH	-	Malate Dehydrogenase
mg	-	Milligram
mg l^{-1}	-	Milligram per litre
Mg^{2+}	-	Magnesium

min.	-	Minute
ml	-	Milliliter
mm	-	Millimeter
MOA	-	Methyl Orange Alkalinity
mmol	-	Millimole
mRNA	-	Messenger Ribonucleic acid
Na^+	-	Sodium
NADH	-	Nicotinamide Adenine Dinucleotide
NBT	-	Nitroblue Tetrazolium Chloride
NFE	-	Nitrogen-Free Extract
NH_3-N	-	Ammonical Nitrogen
NH_4^+	-	Ammonium Ion
nm	-	Nano Mmeter
NWG	-	Net Weight Gain
OD	-	Optical Density
PA	-	Phenolphthalein Alkalinity
PBS	-	Phosphate Buffer Saline
PCV	-	Packed Cell Volume
PER	-	Protein Efficiency Ratio
pH	-	Power of Hydrogen
PMS	-	Phenazine Methosulphate
PO_4^-	-	Phosphate
ppm	-	Parts per million
proPO	-	Prophenoloxidase

pxn	-	Peroxinectin
RBA	-	Respiratory Burst Activity
RBC	-	Red Blood Cell
RNA	-	Ribonucleic Acid
ROS	-	Reactive Oxygen Species
RPM	-	Revolutions per minute
RT-qPCR	-	Quantitative Real-Time PCR
SGR	-	Specific Growth Rate
SO_4^{2-}	-	Sulphate
SOD	-	Super Oxide Dismutase
spp	-	Species
SPSS	-	Statistical Package for the Social Sciences
TA	-	Total Alkalinity
TBA	-	Thiobarbituric Acid
TBLG	-	Total Body Length Gain
TG	-	Trigycerides
tgase1	-	Transglutaminase 1
tgase2	-	Transglutaminase 2
TH	-	Total Hardness
TP	-	Total Proteins
WBC	-	White Blood Cell
µg	-	Micro Gram
µM	-	Micro Molar

CHAPTER I

INTRODUCTION

Aquaculture is the world's fastest growing food production sector, which has made encouraging progress in the past two decades by playing an imperative role in feeding the world population in the form of fish and shellfish in addition to its contribution towards livelihood/employment generation. Further, global population is expected to shoot up to around 9.8 billion by 2050 and indeed it is world's greatest challenge to feed such a huge population by meeting nutritional security demands (FAO, 2020). As a result, to meet this continuous increasing demand, there is major shift of aquaculture activities from extensive to semi-intensive and intensive activities. Intensification of aquaculture practices in terms of high stocking density, intensive feeding, use of chemo-therapeutics and antibiotics etc. for getting higher productivity results in stress leading to immuno-suppression, which further increases the susceptibility of organism under culture to infectious diseases. Moreover, along with creating stressful conditions for the fish under culture, use of antibiotics and biocides may lead to detrimental effects like development of antibiotic resistant bacteria, leading to risk for human as well as environmental health. To alleviate all these problems, increasing attention is being paid towards the use of alternative natural health supplements or nutraceuticals in the form of medicinal herbs (Citarasu, 2010).

In recent years, there has been a growing demand for the use of nutraceuticals, derived from renewable and natural sources, for sustainable aquaculture production (Gomez et al., 2019). The term "nutraceutical" is used to describe food/feed ingredients that demonstrate various "health-beneficial" effects in addition to their "basic nutritional" effects (Encarnacao, 2016). Using medicinal herbs as nutraceuticals in aquaculture is an environmentally safe disease control strategy, as they enhance resistance towards infectious diseases by positively affecting the non-specific and specific immune mechanisms (El-Sayed et al., 2014). Further, multi-functionality of these nutraceuticals for aquaculture species along with easy access, cost effectiveness and ecofriendly nature are some of the added advantages, which has encouraged their use in commercial level aquaculture, which can be used as whole plant or its parts (leaf, root or seed) or in extract form (Hai, 2015). Both terrestrial and aquatic plants represent an untapped resource for biotechnological multi-product

development as they contain a diverse array of bioactive components with potential nutritional and health-benefiting biological properties e.g. growth promotion, antioxidant, prooxidant, antimicrobial, immune stimulation etc. (Baruah et al., 2017). Inclusion of phytobiotic nutrients from number of herbal medicinal plants like garlic, ginger, onion, tulsi, ashwagandha, aloe, amla, neem, fenugreek, turmeric etc. have been reported to improve palatability and digestibility of feed along with health improvement and enhanced product quality of monogastrics including fish/shellfish (Kiczorowska et al., 2017).

Among number of promising terrestrial medicinal herbs with nutraceutical properties, Ginger (*Zingiber officinale*) belonging to family *Zingiberaceae,* also named as *"The Great Medicament"* in Ayurvedic medicines (Shakya, 2015) is a creeping perennial underground rhizome. Ginger is indigenous to tropical Asia mainly to Southern China and India. Ginger powder/extract with wide variety of prophylactic and therapeutic properties, have positive impact on immune system and also proved to be potent appetizer and growth promoter resulting from enhanced activity of digestive enzymes. Pharmacological studies revealed that in addition to anti-inflammatory and anti-diabetic properties, ginger has anti-cancerous, chemo-preventive and chemotherapeutic effects on a variety of tumor cell lines. The effectiveness of ginger in regulation of innate and adaptive immunity of fish along with regulation of expression levels of immune related genes (IL-1ß, TNF-α, IL-10, TGFß, iNOS, COX-2, NF-kB etc.) further confirms the strong immune-stimulatory effects of this herbal ingredient (Vallejos-Vidal et al., 2016). The powerful effect of ginger (powder and extract) has been demonstrated in various fish species fed at different levels with enhaced immune (phagocytic, respiratory burst, lysozyme, bactericidal and antiprotease activities) and growth parameters along with increased resistance against pathogenic bacteria (Awad & Awaad, 2017). Ghadikolaei et al., (2017) reported 2g of ginger powder as best dose for enhanced growth, survival and biochemical composition of common carp (*Cyprinus carpio*), whereas, according to El-Sayed et al., (2014) supplementation of ginger @ 1% improved growth performance and immune status of *Oreochromis niloticus*. Ginger contains natural organic material beneficial to health, which enhances resistance to infectious disease by increasing non-specific and specific immune mechanisms. Ginger showed great influence on the immune system of Asian seabass (Talpur et al., 2013) with improvement in the defence mechanism of fish against pathogenic bacteria, *Vibrio harveyi*. The immuno-modulatory effect of ginger extract has been demonstrated in rainbow trout (Dugenci

et al., 2003) and Mozambique tilapia (Immanuel et al., 2009), where 1% of ginger extract showed significant improvement in most of the tested humoral (lysozyme activity, total protein and globulins) and cellular (phagocytic and respiratory burst activity) immune parameters. Moreover, challenge with *V. vulnificus* showed an increase in survival rate of Mozambique tilapia fed ginger extract.

So far, most of the studies showing positive impact on aquaculture species were conducted under conventional environmental conditions (xenic), in which there are present wide variety of known and unknown microbial communities, often changing in terms of composition and activities. Under such xenic (germ-associated) conditions, the native microbiota that remained associated with the host interacts in a complicated manner with the host e.g. by directly influencing the metabolism of the host and/or by interfering with the tested nutraceutical, and this markedly alters the true outcomes of such studies (Baruah et al., 2015). From mechanistic perspectives, it is, therefore, unrealistic to give a logical conclusion to the outcome of the experiments. Additionally, studies carried out under xenic conditions often suffer from high variability and/or a lack of reproducibility, due to the fact that the microbiota is different for different batches of experimental animals and even for different animals of the same batch.

A key experimental strategy to study these interactions is to first define the functioning of the host in the absence of microbes and then to evaluate the effects of adding a single or defined population of microbes, and/or certain nutraceutical (i.e., under gnotobiotic/axenic conditions). In the germ-free animal, any nutraceutical effect can be investigated without interference from the microorganisms commonly inhabiting the gut in a conventional environment. The extent to which that process is modified by microbial action can then be examined by introducing a known microflora. Such kind of gnotobiotic tools is now recognized as essential in the advancement of knowledge on various aspects of nutrition and health. Previous studies have shown that organic solvent extracts of ginger possess antibacterial properties (Nya & Austin, 2009; Jagetia et al., 2003). Nevertheless, the critical problem in studying the anti-pathogenic effects of ginger *in vivo* is the difficulty to eliminate the effect of the microbial communities that occur naturally in the system (Baruah et al., 2017). In addition, in germ-associated conditions, the compound of interest is either metabolized by microbial communities thereby masking any biological effect of the compound or influences the physiology of host-associated

microbes, thereby making it difficult to understand the host response towards tested compound (Baruah et al., 2015). Hence, selection of an appropriate animal model system that portray the biological effects of ginger on the induction of resistance against disease is therefore of high importance. In this context, the brine shrimp (*Artemia franciscana*) an aquatic invertebrate, a crustacean represent an excellent (aquaculture) model organism for studying the health-benefiting effects of nutraceuticals, because it can be reared under gnotobiotic conditions (i.e. a germ-free system that allows full control over the host-associated microbial communities). Apart from its unusual life history, relatively less space and cost requirement for culture, rapid generation cycle (cyst to adult in 20–30 days), and established molecular techniques like RT-PCR makes it an exceptional experimental system for carrying out such studies, as it allows to distinguish direct effects on the host (by pre exposing axenic shrimp to the compound for a certain duration) from indirect effects. It also eliminates any possible microbial interference during the exposure period and hence facilitates the interpretation of the results in terms of a cause-effect relationship (Baruah et al., 2014).

So far, nutraceutical properties of ginger powder/extract as immunostimulant has not been studied in brine shrimp, however, number of other purified compounds, extracted from different natural sources are being used to understand their mechanism of action and their beneficial effects in gnotobiotic environment (Han et al., 2020). Thus, the holistic approach would be to compare the nutraceutical properties of ginger under conventional environmental conditions as well as in gnotobiotic conditions, which will help in developing health promoting diets for most promising aquaculture species i.e. rohu, *Labeo rohita*, along with elucidating health benefiting effects of ginger in germ free *Artemia* and *Vibrio parahemolyticus* as host pathogen model. Hence, it is proposed to conduct the research study to compare the nutraceutical properties of ginger in rohu, *L. rohita* under traditional semi-intensive culture system and in *Artemia* under germ free gnotobiotic conditions with the following objectives:

Objectives

1. To study the nutraceutical properties of dietary supplementation of ginger powder on growth, health status and disease resistance in rohu
2. To develop ginger extract enriched *Artemia* with nutraceutical properties for aquaculture feed formulation

CHAPTER II

REVIEW OF LITERATURE

Review of literature with respect to application of medicinal herbs with special reference to **ginger** in aquaculture is hereby presented under following heads

2.1 Medicinal herbs as growth promoting immune-stimulants in aquaculture

2.2 Ginger and its bioactive components

2.3 Nutraceutical properties of ginger

2.4 Ginger as nutritional and health supplement in aquaculture

2.5 Bioactve compunds with nutraceutical properties for *Artemia* enrichment

2.1 Medicinal herbs as growth promoting immune-stimulants in aquaculture

Disease outbreaks has increased proportionally with intensification of aquaculture practices, which has led to use of chemicals/drugs/antibiotics etc. resulting in series of problems including antibiotic-resistance, human health issues and environmental pollution (Cabello, 2006). In the present scenario, medicinal plants/herbs are emerging as promising remedy for various diseases, especially to reduce the use of synthetic drugs and associated harmful side effects in aquaculture. Traditional medicines are mainly depended on medicinal plants, and more than 3.3 billion people in less developed countries rely on them (Ajlan et al., 2016; Tsabang et al., 2016). Medicinal plants perform multiple biological activities including growth promotion, appetite stimulation, immune stimulation, antimicrobial, and anti-stress in aquaculture species (Chakraborty & Hancz, 2011; Chitmanat et al., 2003). Further, the mode of action of these plants and their derivatives are attributed to the presence of many active principle components such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Harikrishnan et al., 2011; Sivaram et al., 2004). Moreover, these medicinal plants/herbs have a wide range of applications without any hazardous effect on environment as these are non-toxic, biodegradable and biocompatible (Citarasu, 2010). Easy access and cheap price for many plants are also encouraging factors for their large-scale use in aquaculture to provide better growth and protection at the same time. These herbs have been used in several forms, either in crude form, or in the form of extracts or purified active compounds.

Most importantly, in addition to growth promotion, herbal plants possess immune-modulatory properties and act by stimulating both specific and non-specific immunities. The unique characteristic of bioactive components of number of medicinal plants has resulted in increased interest for their use as immunostimulants in aquaculture (Singh et al., 2016). It is worth mentioning here that medicinal plants can be used as immunostimulants before a disease outbreak to reduce the losses in finfish/shellfish production. Recently, global interest towards using plants extracts (herbs) for therapeutic purposes has increased as an alternative source to chemical compound in animal and human drugs, since the active ingredients are present in concentrated form. Moreover, adding herbal nutrients, enhance the palatability and digestibility (Shanthi et al., 2012; Bhavan et al., 2011) of the feed.

A wide variety of immune-stimulating herbs, including *Zingiber officinale* (Ginger), *Curcuma longa* (Turmeric), *Allium sativum* (Garlic), *Allium cepa* (Onion), *Trigonella foenum-graecum* (Fenugreek), *Aloe vera* (Aloe), *Ocimum sanctum* (Tulsi), *Withania somnifer*a (Ashwaganda or Indian ginseng), *Azadirachta indica* (Neem), *Piper longum* (Indian long pepper), *Cinnamomum verum* (Cinnamon), *Asparagus racemose* (Satavari), *Glycyrrhiza glabra* (Mulethi), *Mangifera indica* (Mango Kernel), *Phyllanthus niruri* (Jaramla/Niruri) etc. have been tested successfully in number of finfish/shellfish species (Table 1).

Table 1. Summary of work done on different herbs by various researchers in aquaculture species

Fish species	**Herb**	**References**
Rohu *(Labeo rohita)*	Ginger (*Z. officinale)*	Sukumaran et al. (2016)
	Amla (*Phyllanthus emblica*)	Srivastava et al. (2019)
	Turmeric (*C. longa)*	Kaur et al. (2020) Behera et al. (2011) Sahu et al. (2008)
	Green chirayta (*Andrographis paniculate*)	Basha et al. (2013)
	Ashwagandha (*W. somnifera*)	Sharma et al. (2010)
	Garlic *(A. sativum)*	Sahu et al. (2007a)
	Mango Kernel *(M. indica)*	Sahu et al. (2007b)

Common carp (*Cyprinus carpio*)	Ginger (*Z. officinale)*	Jafarinejad et al. (2020) Mohammadi et al. (2020) Ghadikolaei et al. (2017) Ajeel & Al-Farangi (2013)
	Neem leaf extract (*A. indica*)	Kaur et al. (2019)
	Aloe (*A. vera*)	Khan et al. (2018) Mahadavi et al. (2013)
	Garlic (*A. sativum*)	Muzaffar et al. (2017) Monoppo et al. (2016)
Silver carp (*Hypopthalmichthys molitrix*)	Aloe (*A. vera)*	Syed Ali Fathima et al. (2014)
Catla (*Gibelion catla*)	Neem (*A. indica*) Garlic (*A. sativum*) and Turmeric (*C. longa*)	Dey & Chandra (1995)
Mrigal (*Cirrhinus mrigala)*	Mulethi (*G. glabra*)	Kumar et al. (2007)
Gold fish (*Carassius auratus)*	Garlic (*A. sativum*)	Santra et al. (2016)
	Aloe (*A. vera*)	Palmero et al. (2012) Ahilan et al. (2010)
Rainbow trout (*Oncorhynchus mykiss*)	Ginger (*Z. officinale*)	Fadeifard et al. (2018) Shaluei et al. (2017) Haghighi & Rohani (2013) Nya & Austin (2009) Dugenci et al. (2003)
Nile Tilapia (*Oreochromis niloticus*)		Mohammed et al. (2020) Alsaiad & Al-Zayat (2019) Payung et al. (2017)
Striped catfish (*Pangasianodon hypophthalmus*)		Swain et al. (2018)
Nile catfish (*Clarias gariepinus*)		Abo-Esa et al. (2008)
Shellfish species	**Herbs**	**References**
Tiger shrimp (*Penaeus monodon*)	Ginger (*Z. officinale*)	Venkataramalingam et al. (2007)

Freshwater prawn (*Macrobrachium rosenbergii*)	Ginger (*Z. officinale*)	El-Desouky et al. (2012)
	Ginger (Z. *officinale*) Garlic (*A. sativum*) and Turmeric (*C. longa*)	Poongodi et al. (2012)
Pacific White shrimp (*Litopenaeus vannamei*)	Ginger (*Z. officinale)*	Soowannayan et al. (2019)
	Garlic (*A. sativum*) Echinacea (*E. purpurea*) Ginger (*Z. officinale*) and Basil (*O. sanctum*)	Fierro-Coronado et al. (2019)
	Garlic (*A. sativum*)	Labrador et al. (2016)
	Ginger (*Z. officinale)*	Chang et al. (2012)
Mud crab (*Scylla ranquebarica*)	Ginger (*Z. officinale)*	Hatai et al. (2018)

2.2 Ginger and its bioactive components

Ginger (*Z. officinale* Roscoe) has been commonly consumed as a spice and an herbal medicine, since ancient time due to availability of abundant active compounds, which include phenolic and terpene compounds (Prasad & Tyagi, 2015). The pungent property of ginger is due to phenolic compounds (non-volatile), which include mainly gingerols, shogaols, and paradols. In fresh ginger, gingerols are the major polyphenols, such as 6-gingerol, 8-gingerol, and 10-gingerol, which are transformed to corresponding shogaols with heat treatment or after long-time storage. Further, shogaols can be transformed into paradols after hydrogenation (Nile & Park, 2015). In addition to gingerol, there are many other phenolic compounds in ginger, such as quercetin, zingerone, gingerenone-A, and 6 dehydrogingerdione (Ji et al., 2017; Schadich et al., 2016) and several terpene (volatile) components, including β -bisabolene, α -curcumene, zingiberene, α-farnesene, and β -sesquiphellandrene, which are considered to be the main constituents of ginger essential oil (Yeh et al., 2014). Ginger contains up to 3% essential oil, accounting for 20–25% of the oleoresin. Major constituents of ginger oil are monoterpenes, sesquiterpenes, aldehydes, esters and alcohols. The taste of ginger is mainly affected by monoterpenes. Besides these, fresh ginger contains 80.9% moisture, 2.3% protein, 0.9% fat, 2.4% fibre, 12.3% carbohydrates and 1.2% minerals including iron, calcium and phosphorous along with polysaccharides and organic acids. It is also rich source of vitamins such as thiamine, riboflavin, niacin and vitamin C. The nutrient composition of ginger varies with the

type, variety, agronomic conditions, curing methods, drying and storage conditions (Prasad & Tyagi, 2015; Shakya, 2015).

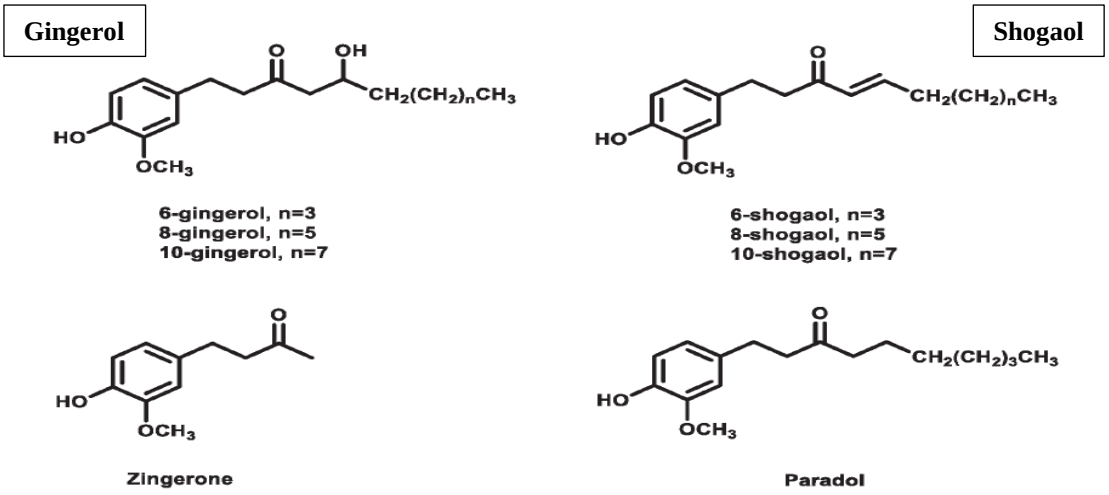


Figure 1. Chemical structure of active compounds of ginger

2.3 Nutraceutical properties of ginger

In Ayruveda, ginger is known as "The Great Medicament" as it is generally used to treat a variety of ailments. Known to be a classic gastric tonic, this tropical rhizome is recommended for gastric pain, vomiting, diarrhoea, cough and cold (Chen et al., 1997). In recent years, ginger has been found to perform number of biological activities, such as antioxidant (Nile & Park, 2015), anti-inflammatory (Chrubasik et al., 2005; Grzanna et al., 2005), antifungal (Agarwal et al., 2001), antibacterial (Jagetia et al., 2003), anticancerous (Citronberg et al., 2013) and immune-modulatory agent for many finfish (Talpur et al., 2013; Immanuel et al., 2009; Nya & Austin 2009) and shellfish species (Chang et al., 2012; El-Desouky et al., 2012). In addition, ginger is also considered as one of the potential agent to prevent and manage several diseases, such as neurodegenerative diseases (Ho et al., 2013), cardiovascular diseases (Akinyemi et al., 2015), obesity (Suk et al., 2017), diabetes mellitus (Wei et al., 2017), chemotherapy-induced nausea and emesis (Walstab et al., 2013) and respiratory disorders (Townsend et al., 2013). Ginger has also been reported to possess a broad spectrum of prophylactic and therapeutic activities (Ernst & Pittler, 2000).

Ginger enhances resistance to infectious diseases by improving non-specific and specific immune mechanisms (Harikrishnan et al., 2011). The active organic

constituents present in ginger facilitate growth along with acting as anti-stress and antimicrobial agent in fish (Maqsood et al., 2011) in an environmentally friendly manner. Owing to its diverse biological properties, ginger is used as prophylactic as well as remedial agent in treating diseases in humans, poultry and livestock including fish.

2.3.1 Ginger - Antioxidant Properties

Ginger, due to its antioxidant properties may either mitigate or prevent generation of free radicals (Kim et al., 2007). All major active ingredients of ginger, such as gingerols, shogaols, zingerone, gingerdiol etc. are known to possess anti-oxidant activities (Chrubasik et al., 2005). Bioactive compounds of ginger such as 6-shogaol exhibited antioxidant activity via the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway (Mao et al., 2019). The antioxidant activity of ginger has been evaluated in vitro and the results revealed that dried ginger exhibited the strongest antioxidant activity than that of fresh, fried and carbonized form. However, the 6-gingerol-rich fraction from ginger could also reduce the levels of H_2O_2 and enhance antioxidant enzyme activity in rats with oxidative damage induced by chlorpyrifos (Abolaji et al., 2017). The supplementation of dietary ginger in fish feed can enhance the antioxidant capacity in response to oxidative stress in enzymatic and molecular functional levels in the tissue, which results in the increase of stress resistance in fish (Jafarinejad et al., 2020).

2.3.2 Ginger - Antimicrobial Properties

Ginger is effective in controlling wide range of bacterial, viral, fungal and parasitic diseases (Agrawal et al., 2001; Martins et al., 2001; Endo et al., 1990) due to presence of most effective antimicrobial constituent i.e citral. As cultured fish is exposed to a wide variety of disease causing agents, therefore the application of ginger in aquaculture is an innovative approach to prevent disease conditions and thus achieving healthy stock. Ethanol extracts of ginger are able to inhibit both gram positive and gram-negative bacteria. Moreover, γ-terpinene, citral and dehydrozingerone in ginger showed potent antifungal properties against *Aspergillus flavus* and reduced the expression of some of the genes related to aflatoxin biosynthesis (Moon et al., 2018; Tan & Vanitha 2004). Ginger also inhibits the growth of pathogenic bacteria like *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus*

aureus, *Streptococcus pyogenes* and *Salmonella* (Ernst & Pittler, 2000). The benzene extract of *Z. officinale* showed highest antibacterial activity against drug resistant *P. aeruginosa* isolated from wound and pus samples. Furthermore, fresh ginger was found to inhibit plaque formation induced by human respiratory syncytial virus (HRSV) in respiratory tract cell lines, as it was found in blocking viral attachment and internalization (Chang et al., 2013). According to Levy et al., (2015), bath treatment with ethanolic extract of ginger is an effective method to control monogenean (*Gyrodactylus turnbulli*) infection in guppies.

2.3.3 Ginger - Anti-inflammatory Properties

A series of studies showed that ginger and its active constituents possess anti-inflammatory properties. The anti-inflammatory effects were mainly related to phoshatidylinositol-3-kinase (PI3K), protein kinase B (Akt), and the nuclear factor kappa light chain-enhancer of activated B cells (NF-kB). In addition, 6-shogaol showed protective effects against tumor necrosis factor α (TNF- α) induced intestinal barrier dysfunction in human intestinal cell models. However, 6-dehydroshogaol was more potent than 6-shogaol and 6-gingerol in reducing the generation of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2) in mouse macrophage RAW 264.7 cells. Moreover, nanoparticles derived from edible ginger could prevent intestinal inflammation by increasing the levels of anti-inflammatory cytokines such as interleukin-10 (IL-10) and IL-22 and decreasing the levels of proinflammatory cytokines such as TNF- α, IL-6, and IL-1 -β in mice with acute colitis and chronic colitis (Chang et al., 2013). It has been reported by Sukumaran et al., (2016) that dietary administration of ginger exert anti-inflammatory effects in *L. rohita* by upregulating IL-10 and TGF-ß and down regulating TNF-α and IL-1ß.

2.3.4 Ginger - Biochemical Properties

Diabetes mellitus is known as a severe metabolic disorder caused by insulin deficiency resulting in an abnormal increase in blood glucose. Prolonged hyperglycemia could accelerate protein glycation and the formation of advanced glycation end products (AGEs). An in vitro experiment revealed that both 6-shogaol and 6-gingerol prevent the progression of diabetic complications, as they inhibited the production of AGEs by trapping methylglyoxal (MGO), the precursor of AGEs (Sampath et al., 2017). Chronic hyperglycemia increases circulating levels of

inflammatory biomarkers such as IL-6, TNF- α, and C-reactive protein (CRP). Oral ginger supplementation ameliorated inflammation through reduction in levels of TNF-α and CRP concentrations in blood samples of the patients with type 2 diabetes mellitus (Nishiyama et al., 2005; Spranger et al., 2003). It has been observed in a study that feeding ginger reduced the body weight and lipid profile of high-fat diet rats and had a greater effect on increasing the level of HDL-C (Mahmoud & Elnour, 2013). Ginger and its bioactive constituents, including gingerenone A, 6-shogaol, and 6-gingerol, have shown antiobesity activity, with the mechanisms mainly related to the inhibition of adipogenesis and the enhancement of fatty acid catabolism.

2.3.5 Ginger - Anti-cancer Properties

The anticancer properties of ginger is attributed to the presence of pungent phenolic compounds viz., 6-gingerol, 6-paradol, shogaols and zingerone. 6-gingerol has been studied for its cytotoxic effects in various cancer cell lines and was shown to induce cell death in cervical cancer cell line, HeLa, by caspase 3-dependent apoptosis and autophagy (Chakraborty et al., 2012). According to Radhakrishnan et al., (2014), inhibition of ERK1/2/JNK/AP-1 pathway is the possible mechanism behind the anticancer as well as chemopreventive efficacy of 6- gingerol against colon cancer.

2.4 Ginger as nutritional and health supplement in aquaculture

Ginger, a promising medicinal plant is used in aquaculture not only as chemotherapeutics but also as feed additive, as it contains a wide variety of nutrients and chemical compounds. The powerful effect of ginger (crude form and extract) has been demonstrated in various finfish and shellfish species fed at different levels with enhaced immune (phagocytic, respiratory burst, lysozyme, bactericidal and antiprotease activities) and growth parameters along with increased resistance against pathogenic bacteria (Awad & Awaad, 2017).

2.4.1 Effect of ginger on growth and survival

Ghadikolaei et al., (2017) studied the effects of ginger powder on growth, survival and biochemical composition in juvenile common carp. Fishes were fed with commercial diets supplemented with ginger powder at different doses i.e. 0.25g, 0.5g, 1g and 2g along with control (without ginger). Fish fed with 2g of ginger powder resulted in enhanced growth and biochemical composition. Similarly, Immanuel et al., (2009) reported that by supplementing diets with acetone extract (1%) of ginger for 45 days led to enhanced growth of *O. mossambicus* in terms of final weight gain and

specific growth rate. In one of the study, Mohammed et al., (2020) reported that aqueous extracts of liquorice (4 ml kg^{-1} feed) and ginger (5 ml kg^{-1} feed) either added separately or in combination to the basal diet of *O. niloticus* resulted in decreased heavy metal accumulation in the fish flesh along with positive effects on growth performance, metabolic profile and histopathology of gills and intestine. Alsaiad & Al-Zayat (2019) too evaluated the effect of ginger on growth performance, feed utilization, body composition and cost benefit analysis of the Nile tilapia. Fishes were fed with isonitrogenous and isocaloric diets supplemented with different levels of ginger extract (0, 0.5 and 1%) @ 5% of their body weight for 16 weeks. It has been concluded that 1% ginger extract exhibited the best growth performance, feed conversion, and protein efficiency ratio. Also, cost benefit analysis showed high profile index and low incidence cost with 1% ginger inclusion. Similarly, Payung et al. (2017) demonstrated that dietary ginger enhanced resistance of Nile tilapia against *A. hydrophila*. Fish was fed experimental diets supplemented with 0, 1.25, 2.5, 5, and 10 g of ginger powder kg^{-1} of feed @ 3% body weight for 30 days. Significantly highest survival rate (76.6%) was observed in fish fed with 2.5 g ginger.

In one of the study conducted on black rockfish by Lee et al. (2020), dietary inclusion of ginger (1%) for 8 weeks improved weight gain, specific growth rate, protein retention and disease resistance of black rockfish (*Sebastes schlegeli*) against *Vibrio anguillarum*, when compared to fish fed on control diet (without ginger). Likewise, Olaniyi et al. (2020) reported that inclusion of ginger @ 15% in the diet of African catfish significantly enhanced growth performance in terms of weight gain, specific growth rate, feed conversion ratio and protein efficiency ratio. Swain et al. (2018) too reported that supplementation of ginger powder @ 10 g kg^{-1} showed better growth response in terms of higher weight gain and specific growth rate in striped catfish, *P. hypophthalmus*.

The powerful effect of ginger has been demonstrated in various shellfish species. In one of the experiments by Venkataramalingam et al. (2007) ginger is used as an herbal appetizer in the tiger shrimp larviculture. *P. monodon* postlarvae were fed with ginger enriched *Artemia*, which resulted in significantly higher weight gain, specific growth rates and enhanced digestive enzyme activity (amylase, protease and lipase) in shrimp post larvae compared to control (non-enriched *Artemia*). Among the different percentages of enrichment (0%, 25%, 50%, 75% and 100%), 100% *Z.*

officinalis enriched *Artemia*-fed postlarvae performed better in the overall status. Likewise, El-Desouky et al. (2012) reported that *M. rosenbergii* juveniles fed with ginger (1.5 and 3%) showed significant increase in weight gain, length gain, specific growth rates, condition factor and feed conversion ratio compared to juveniles fed on control diet (without ginger).

2.4.2 Effect of ginger on hematological, biochemical and antioxidant parameters

Chanu et al. (2014) reported antistress potential of acetone extract of ginger on biochemical and oxidative stress parameters in *L. calbasu* subjected to acid stress. Fish were fed with four diets containing different levels of ginger extract (0, 0.05, 0.5 and 5%) for 30 days. It has been observed that 0.5% ginger extract supplementation contributed to reduction in levels of serum cortisol, serum glucose, serum cholesterol, triglycerides and a marked improvement in production of oxidative stress enzymes viz. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in experimental fish exposed to acid stress. Similarly, Jafarinejad et al. (2020) demonstrated the powerful effect of dietary ginger on hematological parameters, antioxidant capacity, growth performance and gene expression in *C. carpio*. Fish were fed with four experimental diets containing 0 (control diet), 0.5, 2 and 5% ginger powder for 56 days. Results revealed that 2 and 5% ginger supplementation significantly enhanced growth performance and hematological parameters along with antioxidant enzyme activities viz. SOD, CAT and GPX. Further, dietary ginger could partially alleviate oxidative stress by down regulation of SOD, CAT and GPX mRNA expression levels. Mohammadi et al. (2020) too studied the effects of ginger extract on growth performance, body composition, haematology, serum and mucosal immune parameters in common carp. Four experimental diets were fed to the fish containing different inclusion levels of ginger extract (0.1, 0.2 and 0.4%) for 60 days. It has been concluded that ginger extract @ 0.2% effectively improved the weight gain, feed conversion ratio, specific growth rate, erythrocytes (RBC), leucocytes (WBC), hematocrit (Ht), hemoglobin (Hb) along with significant enhancement in lysozyme, SOD, total immunoglobins and alkaline phosphatase.

Mahmoud et al. (2019) studied the influence of ginger supplementation on oxidative stress in the muscle tissues of nile tilapia. Fish was fed with isonitrogenous and isocaloric diets @ 3% body weight for 60 days. It has been concluded that there was a significant decrease in lipid peroxidation (malondialdehyde/MDA) level and

increase in SOD level in muscle tissues of fish fed with 1.5% ginger supplementation compared with control. Sahan et al. (2016) too observed a significant increase in hematological parameters (RBC, Hb and Ht values) and antioxidant enzymes (SOD and CAT) in ginger fed groups (0.5 and 1%) of *O. niloticus* compared to control along with improvement in the defence mechanism of fish against pathogenic bacteria, *A. hydrophila*.

In an another study, Jahanjoo et al. (2018) evaluated that 1% inclusion of dietary ginger in sea bream diets significantly increased RBC, WBC, total protein content, total immunoglobulins, lysozyme activity and complement activity (ACH50), while a significant decrease was observed in cholesterol levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), when compared with control. Further, Vahedi et al. (2017) studied the effect of dietary supplementation with ginger extract on growth, biochemical and hemato-immunological parameters in juvenile beluga (*Huso huso*). Fishes were fed with diets supplemented with 0.5, 1 and 1.5% ginger extract for 8 weeks and with unsupplemented commercial diet as control. Results suggested that supplementation with ginger extract @ 1.5% improved hematological, biochemical and immune function of juvenile beluga.

2.4.3 Effect of ginger on immunological parameters

According to Sukumaran et al. (2016), dietary administration of ginger @ 0.8 % modulated the growth performance, skin mucosal immune responses, anti-bacterial activities, expression of cytokine related genes along with significantly improved resistance against *A. hydrophila* infection in rohu. Further, anti-inflammatory effects of ginger were obvious from upregulation of IL-10 and TGF-ß and down regulation of TNF-α and IL-1ß genes. Similarly, Korni & Khalil (2017) reported that dietary ginger and its nanoparticles enhanced growth performance, cognition capability, immune responses (total protein, globulin, lysozyme) and prevention of Motile Aeromonas Septicaemia (MAS) in *C. carpio* fingerlings, when compared with control.

Ginger contains natural organic material beneficial to health, which enhances resistance to infectious disease by increasing non-specific and specific immune mechanisms. The indomitable effect of ginger extract has been demonstrated in rainbow trout (Dugenci et al., 2003), where using 1% aqueous extract showed significant improvement in most of the tested immune parameters including humoral (lysozyme activity total protein and globulin) and cellular (phagocytic and respiratory

burst activity). Similarly, Nya & Austin (2009) reported the use of dietary ginger (@ 0.05, 0.1, 0.5 and 1.0%) as an immunostimulant to control *A. hydrophilla* infections in rainbow trout. Incorporation of 0.5 g ginger per 100 g of feed reduced mortalities to 0% compared to control (64%), with significant improvement in growth, feed conversion and protein efficiency. Further number of neutrophils, macrophages and lymphocyte increased with enhanced phagocyte, respiratory burst, lysozyme, bacteriocidal and antiprotease activities. Likewise, Fadeifard et al. (2018) demonstrated that dietary essential oils of ginger @ 1% significantly enhanced serum lysozyme level and phagocytic activity of *O. mykiss* compared with control group. It has been concluded that herbal essential oils might strengthen the non-specific immunity of the fish. In another study, Shaluei et al. (2017) studied the effect of ethanolic extract of ginger on growth performance and mucosal immune response in rainbow trout. Fishes were fed with commercial diets supplemented with ginger extract at different doses i.e. 1g, 2.5g and 5g along with control (without ginger) for 45 days. Significantly improved weight gain, feed utilization efficiency and feed conversion along with higher skin mucus lysozyme, alkaline phosphatase, protease activity and protein level were observed in the group fed with 2.5g and 5g ginger. Similarly, Haghighi & Rohani (2013) studied the effects of ginger powder on haematological and immunological parameters of rainbow trout. It has been observed that addition of 1% ginger increased white blood cells, haematocrit, red blood cells along with respiratory burst activity and serum lysozyme activity, when compared with the control group (without ginger powder supplementation).

El- Sayed et al. (2014) evaluated the effects of graded levels (0.1, 0.2, 0.3, 0.5 and 1 %) of ginger as feed additive on growth performance and immunological status of *O. niloticus*. Fish were fed with isonitrogenous and isocaloric diets @ 5% of body weight 4 times daily for 10 weeks. It was concluded that supplementation of ginger @ 1% improved growth performance and immune status of fish. Similarly, ginger also showed considerable influence on the immune system of Asian seabass (Talpur et al., 2013) along with improvement in the defence mechanism of fish against pathogenic bacteria, *Vibrio harveyi*. Likewise, Ahmadifar et al. (2019) evaluated the effect of dietary ginger on biochemical and immunological parameters along with gene expression in zebrafish. Fishes were fed with commercial feed supplemented with ginger powder at different doses i.e. 1, 2 and 3% for 8 weeks. Best results for

biochemical and immunological parameters along with catalase and lysozyme mRNA expression representatives of antioxidant and immune genes respectively, were up-regulated in the fish fed with 3% ginger. In another study, Apines-Amar et al. (2012) observed that dietary ginger powder (20g kg^{-1}) significantly increased total immunoglobulin, lysozyme activity and ROS production in *Epinephelus fuscoguttatus*. Upon challenge with a bacterial pathogen *V. harveyi*, mortality was significantly reduced in ginger fed group. It has been concluded that ginger could positively affect the innate immune responses and protect grouper against *V. harveyi* infection. In addition to the above studies, Abo-Esa et al. (2008) studied the effects of ginger on ectoparasitic diseases of catfish, *Clarias gariepinus*. It has been found that ginger bath treatment (20mg l^{-1}) was safe and effective against ectoparasites *Trichodina* and *Epistylis spp*.

The immuno-modulatory effect of ginger has also been demonstrated in various shellfish species. Chang et al. (2012) reported that administration of zingerone @ 2.5 and 5 mg kg^{-1} in the shrimp (*L. vannamei*) diet could greatly benefit the immune status and disease resistance to *V. alginolyticus*. In addition, zingerone also stimulated appetite in shrimp, leading to enhanced weight gain and feed efficiency. Similarly, El-Desouky et al. (2012) investigated the effects of ginger, *Z. officinalis* and Bermuda grass, *Cyanodon dactylon* supplementation on growth performance and immune parameters of *M. rosenbergii* juveniles. Five experimental diets, including a control basal diet and four experimental diets with 1.5 and 3% ginger, 2 and 4% *C. dactylon* were prepared and fed to juveniles for five weeks. There was a significant increase in growth performance along with some immune parameters viz. Total Hemocytic Count (THC), Hemocyte Viability (HV), Differential Hemocytic Count (DHC), Phagocytic Index (PI) in ginger and *C. dactylon* fed groups as compared to control.

In another study, Zhang et al. (2020) studied the effect of the herb namely *Sanguisorba officinalis* in controlling acute hepatopancreatic necrosis disease caused by infection of *V. parahaemolyticus* in shrimp farming. It has been observed the ethanol-extract possessed the stronger antibiotic effect and could kill *Vibrio* at 6.4 mg ml^{-1} within 10 h. Further, extracts of *S. officinalis* can inhibit T6SS-related gene expression of *V. parahaemolyticus*, leading to the attenuated pathogenicity of *Vibrio* to the host.

Table 2. Summary of work done on ginger as nutritional and health supplement for finfish and shellfish species

Fish Species	Ginger Doses Tested	Best Dose-Observations	References
Rohu (*L. rohita)*	0.2, 0.4, 0.6, 0.8 and 1.0%	0.8% - Enhanced growth performance, immune responses, anti-bacterial activities, improved resistance against *A. hydrophila* and upregulation of IL-10 and TGF-ß and down regulation of TNF-α and IL-1ß.	Sukumaran et al. (2016)
Rohu (*L. calbasu)*	0.05, 0.5 and 5%	0.5% - Reduction in levels of serum cortisol, glucose, cholesterol, triglycerides and marked improvement in production of oxidative stress enzymes (SOD, CAT and GST)	Chanu et al. (2014)
Common carp (*C. carpio*)	0.1, 0.2 and 0.4%	0.2% - Improved weight gain, FCR, SGR, RBC, WBC, Ht, Hb, lysozyme, SOD, total Ig and alkaline phosphatase	Mohammadi et al. (2020)
	0.5, 2 and 5%	2 and 5% - Improved growth performance, hematological parameters along with antioxidant enzyme. Down	Jafarinejad et al. (2020)

		regulation of SOD, CAT and GPX mRNA expression levels	
	0.25, 0.5, 1 and 2%	2% - Improved growth performance and biochemical composition	Ghadikolaei et al. (2017)
Rainbow trout (*O. mykiss*)	1, 2.5 and 5g kg^{-1} feed	2.5 and 5g - Enhanced growth performance, skin mucus lysozyme, alkaline phosphatase, protease activity	Shaluei et al. (2017)
	1%	1% - Increased RBC, WBC, haematocrit, respiratory burst activity and serum lysozyme activity	Haghighi & Rohani (2013)
	0.05, 0.1, 0.5 and 1%	0.5% - Improved growth rate and enhanced phagocytic, respiratory burst, lysozyme and bactericidal activity after challenge with *A. hydrophila*	Nya & Austin (2009
	0.1 and 1%	1% - Enhanced phagocytic, respiratory burst activity and total protein content	Dugenci et al. (2003)
Nile Tilapia (*O. niloticus*)	2.5 and 5 ml kg^{-1}	2.5ml - Decreased heavy metal accumulation in flesh and improved growth performance, metabolic profile and	Mohammed et al. (2020)

		histopathology of gills and intestine	
	1.5%	1.5% - Decrease in lipid peroxidation level and increase in SOD level in muscle tissues of fish	Mahmoud et al. (2019)
	0.5 and 1%	1% - Improved growth performance, feed conversion and protein efficiency ratio. Also, cost benefit analysis showed high profile index and low incidence cost with 1 % ginger inclusion	Alsaiad & Al-Zayat (2019)
	1.25, 2.5, 5, and 10g kg^{-1} feed	2.5g - Enhanced resistance against *A. hydrophila*	Payung et al. (2017)
	0.5, 0.1, 1.0%	1.0% - Enhanced hematological parameters, antioxidant enzymes and protection against *A. hydrophila*	Sahan et al. (2016)
	0.1, 0.2, 0.3, 0.5 and 1 %	1% - Improved growth performance and immune status of fish	El- Sayed et al. (2014)
Zebrafish (*D. rerio*)	1, 2 and 3%	3% - Improved biochemical and immunological parameters	Ahmadifar et al. (2019)
Beluga (*H. huso*)	0.5, 1 and 1.5%	1.5% - Improved hematological,	Vahedi et al. (2017)

		biochemical and immune functions	
Asian Seabass (*L. Calcarifer)*	1, 2, 3, 5 and 10g kg^{-1} feed	5 and 10g - Strengthening of the non-specific immunity and reduce susceptibility to *V. harveyi*	Talpur et al. (2013)
Striped catfish (*P. hypophthalmus*)	5, 10, 15 and 20 g kg^{-1} feed	10g - Improved growth response in terms of higher weight gain and specific growth rate	Swain et al. (2018)
Nile catfish (*C. gariepinus*)	10 and 20 mg l^{-1}	20mg l^{-1} - Ginger bath treatment was safe and effective against ectoparasites *Trichodina* and *Epistylis spp*.	Abo-Esa et al. (2008)
Shellfish Species	**Ginger Dose Tested**	**Best Dose-Observations**	**References**
Tiger shrimp (*Penaeus monodon*)	25, 50, 75 and 100% ginger enriched *Artemia*	100% - Enhanced weight gain, SGR and digestive enzyme activity (amylase, protease and lipase)	Venkataramalingam et al. (2007)
Freshwater prawn (*M. rosenbergii*)	1.5 and 3%	1.5% - Improved weight gain, SGR, condition factor, FCR along with immune parameters viz. Total hemocytic count, Hemocyte viability, Differential hemocytic count and Phagocytic index	El-Desouky et al. (2012)

Pacific White shrimp (*L. vannamei*)	1, 2 and 4 g kg^{-1} feed	4g - Improved survival and protection against WSSV and *V. parahaemolyticus*	Fierro-Coronado et al. (2019)
	1, 2.5 and 5%	2.5% - Improved weight gain, SGR and survival	Kumar et al. (2019)
	2.5 and 5 mg kg^{-1} feed	2.5 and 5 mg - Enhanced resistance against *V. alginolyticus*, increased phenoloxidase activity, growth and immune responses	Chang et al. (2012)
Mud crab (*S. tranquebarica*)	80, 160, 320, 640, 1280, 2560, 5120 and 10240 ug ml^{-1} feed	320 and 640 ug - Ginger extract act as a promising alternative fungicide to treat marine oomycete infection in hatcheries	Hatai et al. (2018)

2.5 Bioactve compunds with nutraceutical properties for *Artemia* enrichment

The brine shrimp (*Artemia*), an aquatic invertebrate belongs to phylum Arthropoda and class Crustacea. *Artemia* are extremely euryhaline and eurythermal withstanding salinities from 3 to 300 ppt and can survive in temperatures ranging from 15 to 55 ^{0}C. Moreover, *Artemia* is an excellent live food used for the cultivation of finfish and crustacean larvae in commercial hatcheries, as it is highly nutritious containing 52% protein and 18% lipid (Leger et al., 1986).

Although highly nutritious, however, through enrichment techniques, essential nutrients as per requirement such as prophylactics and therapeutics can also be delivered to fish/shellfish larvae via *Artemia*. The application of enriched live food in the form of *Artemia* nauplii is reflected in enhanced growth, survival, stress tolerance, and microbial diversity for a variety of aquatic species (Roo et al., 2019). Undoubtedly, enriched *Artemia* nauplii have an enhanced nutritional composition

along with high energy content. Fish/shellfish larvae fed on enriched *Artemia* are indeed healthier and more resistant to stressful conditions such as infections, weaning of fish etc. Further, the brine shrimp can be reared under gnotobiotic conditions (i.e. germ-free system that allows full control over the host-associated microbial communities) represents an exceptional experimental model/system for carrying out various studies, because it eliminates any possible microbial communities (naturally present in the experimental system), which interfere during the exposure period in mechanistic studies and furthermore facilitates the interpretation of the results in terms of a cause effect relationship (Baruah et al., 2014)

Baruah et al. (2015) demonstrated the protective effects of pyrogallol against bacterial infection by using the gnotobiotically-cultured brine shrimp *A. franciscana* and pathogenic bacteria *V. harveyi* as host-pathogen model system. Brine shrimp were pre-treated with pyrogallol at different concentrations (79, 198, 396, 595 and 1185 mM) for a fixed time (2h) at 28 °C. The protective effect of pyrogallol was observed due to its prooxidant action by generation of hydrogen peroxide. Also, generation of prooxidant leads to induction of heat shock protein (Hsp70), which is involved in eliciting the prophenoloxidase and transglutaminase immune responses. Similarly, Han et al. (2019) studied that high concentration (@ 2000ppm) of sodium ascorbate (Vitamin C) acted as a prooxidant by generation of hydrogen peroxide, inducing protective effects in the gnotobiotic brine shrimp against *V. harveyi* infection and the protective effect is associated with Hsp70 induction. Further, Baruah et al. (2017) provided strong evidences that carvacrol (phenolic compound) is a potent inducer of HSP72, responsible for inducing resistance in *Artemia* larvae against abiotic and biotic stress. It has been observed that *Artemia* larvae that were pre-treated with carvacrol @ 66.4 μM concentration had significantly higher survival than the control, when exposed to lethal heat stress (41°C for 20 min) as well as when challenged with *V. harveyi.*

Kumar et al. (2018) reported that pre-treatment with phloroglucinol @ 30 μM, protects axenic brine shrimp larvae against *V. parahaemolyticus* infection and induced heat shock protein 70 (Hsp70) production (two folds) as compared with the control. It has been seen that the *Vibrio*-protective effect of phloroglucinol was caused by its prooxidant effect and is linked to the induction of Hsp70. In addition, RNA interference (RNAi) confirms that phloroglucinol-induced Hsp70 mediates the

survival of brine shrimp larvae against *V. parahaemolyticus* infection. Likewise, Baruah et al. (2017) reported the protective effect of poly-ß-hydroxybutyrate (PHB) against bacterial disease using gnotobiotically-cultured brine shrimp *A. franciscana* and pathogenic *V. campbellii* as host-pathogen model. There were clear evidences indicating that PHB conferred protection to *Artemia* against *V. campbellii* by a mechanism of inducing heat shock protein (Hsp) 70. Also, PHB was associated with the generation of protective innate immune responses, especially the prophenoloxidase and transglutaminase immune systems.

Putative probiotic effect of *Bacillus* sp. LT3 was evaluated by Niu et al. (2014) in a model system with gnotobiotic brine shrimp *A. franciscana* larvae. It has been observed that administration of LT3 can improve the survival of brine shrimp larvae, when challenged with pathogenic *V. campbellii*, both by decreasing the in vivo activity of the pathogen and by priming the innate immune response through activating the prophenoloxidase system. A decreased mRNA level of transglutaminase and Hsp 70 further suggests that pretreatment with LT3 results in less stress and tissue damage in the brine shrimp larvae upon *V. campbellii* challenge. Further, Baruah et al. (2014) provided experimental evidence that underlies the mechanism of Hsp70 -induction using plant-based product Tex-OE in gnotobiotically (germ-free) cultured brine shrimp model system challenged with pathogenic *V. campbellii and V. harveyi*. *Artemia* were pre-treated with increasing concentrations of Tex-OE (2.5, 5, 10 and 50 mgl^{-1}) for a period of 2 h. During the pre-treatment period, there was generation of reactive oxygen species in rearing water, which finally triggers the induction of Hsp70 in *Artemia* enhancing innate immune system. It has been observed that brine shrimp larvae given a pretreatment with Tex-OE @ 5 mgl^{-1} were best protected upon challenge with pathogenic vibrios, with survival augmenting by 12% and 37%, in case of *V. campbellii* and *V. harveyi*, respectively, as compared to the control animals. Similarly, Han et al. (2020) studied the immunomodulatory properties of ß-glucan extracted from wild type strain of baker's yeast (*Saccharomyces cerevisiae*). Significantly higher survival of *A. franciscana* upon challenge with *V. harvyei* along with upregulation of innate immune genes such as *lgbp, hmgb, proPO, sod* and *gst* indicated strong immunomodulatory functions of ß-glucan.

Giarma et al. (2017) studied the alterations in immune responses of *Artemia* nauplii after challenging with the pathogen i.e *V. anguillarum* using probiotics. It has been concluded that administration of probiotics protected *Artemia* against *V. anguillarum* by enhancing its immune responses and phenoloxidase activity thus contributing to reduced oxidative damage, reduced lipid peroxidation and increased survival. In the same way, Roy et al. (2019) reported that phloroglucinol treatment can increase resistance in *Artemia* in 3 subsequent generations against biotic (*V. parahaemolyticus* and *V. harveyi*) and abiotic stress (lethal heat shock). Further, there was a significant ($p<0.05$) enhancement in the expression of a core set of innate immune genes viz. *dscam, propo, pxn, hsp90, hsp70, lgbp* in *Artemia*.

CHAPTER III

MATERIALS AND METHODS

3.1 Site of the research study

The research study was conducted under following two experiments (I and II)

Experiment I: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *Labeo rohita* (Ham.)

Place/Location of Work:

- **Research Trial** - Fish Farm, College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana (Punjab), India.
- **Analysis work** - Aquaculture Nutrition Lab, Water Quality Lab and Aquatic Health Management Lab of the College of Fisheries, GADVASU, Ludhiana.

Experiment II: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia*

Place/Location of Work: *Artemia* Lab, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

3.2 Experiment I - "To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *Labeo rohita* (Ham.)''

3.2.1 Experimental Layout

3.2.1.1 Preparation and maintenance of experimental tanks

- The experiment was carried out in $20m^2$ outdoor cemented tanks (0.002 ha). Approximately 2-inch-thick layer of soil was spread at the bottom of each tank to hasten the decomposition process. During the experimental period, bore well water was used for filling, exchanging and maintaining the water level in the tanks. Initial liming was done with limestone @ 300kg/ha for disinfection and as per requirement (pH balance) throughout the experiment. $1/4^{th}$ of water from experimental tanks was exchanged with fresh water once every fortnight.

3.2.1.2 Procurement and acclimatization of experimental fishes

- Equal sized, active and healthy fingerlings of *Labeo rohita* (average body weight 20.55 ± 1.06 g, average total body length 11.92 ± 0.16 cm) reared at Fish Farm, College of Fisheries, GADVASU were used for the experiment.
- Fingerlings were transported in plastic tubs with well oxygenated water, covered with net and kept for acclimatization (15 days) in 500 L fibre reinforced plastic (FRP) pools under indoor conditions.
- Fish were fed @ 3% of their body weight once daily with control diet during the acclimatization period.
- The water quality parameters of all the experimental tanks were analyzed before stocking the fish.

3.2.1.3 Preparation of ginger powder and experimental diets/treatments

Fresh rhizomes of ginger (*Z. officinale*) were procured from the local market of district Ludhiana, Punjab (India). The ginger rhizomes were washed, peeled, grated and dried for 48 h in shade, and ground into fine powder using a mixer grinder and sieved using a household sifter (2mm). Ginger powder (GP) was mixed with the previously prepared fine powder of other feed ingredients, and five experimental diets were prepared. One with no GP (control, GP0), and the other four experimental diets (treatments) with 5 g (GP5), 10 g (GP10), 15 g (GP15) and 20 g (GP20) of GP kg^{-1} of GP0 (Table 3), respectively. The diets were prepared by mixing all the ingredients homogeneously followed by the addition of water to prepare the feed pellets through a hand pelletizer, which were dried at 60^0C in hot air oven for 24-48 hours and packed in air-tight containers.

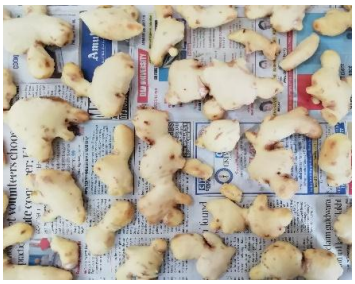
Table 3. Details of control and experimental diets (treatments)

Treatments				
Control (GP0)	**GP5**	**GP10**	**GP15**	**GP20**
Control Diet* without GP	Experimental Diet with GP @ 5g kg^{-1} GP0 (0.5%)	Experimental Diet with GP @ 10g kg^{-1} GP0 (1.0%)	Experimental Diet with GP @ 15g kg^{-1} GP0 (1.5%)	Experimental Diet with GP @ 20g kg^{-1} GP0 (2.0%)

*Rice bran[1] (49%) + Mustard meal[1] (49%) + Vit-Min. mixture (1.5 %) + Salt (0.5%)
[1]Solvent extracted; GP= Ginger powder.

Each 250 g vitamin mineral mixture contain Vitamin A - 500,000 IU., Vitamin DS- 100,000 IU, Vitamin B2- 0.2 g, Vitamin E - 75 units, Vitamin K- 0.1 g, Calcium pentathonate - 0.25 g, Nicotinamide - 0.1 g, Vitamin BI2 -0.6 mg, Choline chloride -15 g, Calcium -75 g, Manganese -2.75 g, Iodine -0.1 g, Iron -0.75 g, Zinc -1.5 g, Copper -0.2 g and Cobalt -0.045 g

Ginger Rhizome



Peeled Ginger Rhizome

Dried Ginger

Ginger Powder (GP)

Mixing of Ingredients

Preparation of Pelleted Feed

Drying of pelleted Feed

Dried Experimental Pelleted Feed

Figure 2. Preparation of Experimental Diets

3.2.1.4 Stocking of fish

Fingerlings (acclimatized) of rohu, *L. rohita* (Ham.) were stocked in prepared outdoor cemented tanks by following complete randomized design (CRD). Three replicates were maintained for each treatment and control. 30 fingerlings were stocked in all the replicates in the month of May 2019.

3.3 Proximate analysis of feed ingredients and experimental formulated diets

- The proximate analysis of different feed ingredients and experimental diets (Table 4) with respect to crude protein (CP), ether extract (EE), crude fiber (CF), ash and nitrogen-free extract (NFE) was done on dry matter (DM) basis by following the methods of AOAC (2012).

3.3.1 Crude protein (CP)

The nitrogen content of the sample was estimated quantitatively by following the Kjeldahl method. Sample digestion was done with KEL PLUS and distillation with KEL PLUS- Classic DX Model (Pelican Equipment).

Principle

The sample is digested in sulphuric acid with a catalyst, which results in conversion of nitrogen to ammonia. Then, distillation of ammonia into a trapping solution (boric acid) and trapped ammonia (ammonium borate) is quantified by titration with a standard acid solution. The crude protein percentage was obtained by multiplying the nitrogen percentage by a factor of 6.25.

Reagents for digestion

- Concentrated sulphuric acid (H_2SO_4)
- Catalyst/Digestion mixture [Cupric sulfate ($CuSO_4.5H_2O$) and potassium sulfate (K_2SO_4) in the ratio 1:9]

Reagents for distillation

- 40% sodium hydroxide solution [40 g NaOH dissolved in distilled water to make a total volume of 100 ml]
- 15% sodium hydroxide solution [15 g NaOH dissolved in distilled water to make a total volume of 100 ml]
- 4% boric acid-indicator solution [40 g of boric acid in 900 ml of distilled water + 10 ml of indicator solution* to make the final volume to 1000 ml]

  *Mixed indicator solution [0.1 g methyl red and 0.5 g bromocresol green dissolved in 100 ml of 95% alcohol]

Reagents for titration

- Standard 0.1 N H_2SO_4 solution [2.6 ml of conc. H_2SO_4 dissolved in distilled water to make a total volume of 100 ml]

Procedure for digestion

- Preheat the digestion system to 320°C.
- 0.1 g of sample was added in the digestion tube, with 3 g of catalyst mixture and 10 ml of conc. H_2SO_4.
- The temperature of the digestion tubes was increased to 420°C after the contents started boiling, continue heating until the color of the digestion mixture turned blue and finally green.
- Digested samples were cooled by keeping in rack undisturbed overnight.

Procedure for distillation

- 10 ml of distilled water was added in cooled digestion tube having digested sample.
- Digestion tube and 250 ml conical flask (having boric acid) were kept in distillation unit for pre-programmed automatic distillation.
- Ammonia was collected in conical flask having boric acid indicator solution in the form of ammonium borate (blue colored solution).

Procedure for titration

- Titrate the conical flask content with 0.1 N H_2SO_4 till wine colorend end point and volume of H_2SO_4 used for titration was recorded.

  Note: A blank was run simultaneously to detect nitrogen present in the reagents and absorbed from the atmosphere, if any.

Observations

Weight of the sample	-	W g (0.1 g)
Normality of the standard H_2SO_4 used	-	0.1 N
Initial burette reading	-	X_1 ml
Final burette reading	-	X_2 ml
Acid used in titration for sample	-	(X_2-X_1) ml
Acid used in titration for blank	-	Y ml

Actual acid used - (X_2-X_1) - Y = Z ml

$$N_2 (\%) \text{ in the sample} = \frac{0.014 \times Z \times 0.1\ N}{W}$$

Calculations - Crude protein (%) = N_2 (%) x 6.25

3.3.2 Ether Extract (EE) or Crude fat

The ether extract was estimated by Soxhlet apparatus (SOCS PLUS, SCS 08 AS, Pelican Equipment).

Principle

Dried and ground sample is extracted with petroleum ether (organic solvent) which dissolves fats, oils, pigments and other fat-soluble substances. The ether is then evaporated from the fat solution. The resulting residue is weighed and referred to as ether extract or crude fat.

Reagent

- Petroleum ether (Boiling point 60-80 °C)

Procedure

- Empty dried soxhlet beakers were weighed.
- Thimble preparation: Place 1g oven dried sample inside the thimble and plugged from both ends with clean cotton plugs. 80 ml petroleum ether was added into each beaker.
- Thimbles (with sample) were fixed in the thimble holder, placed in the beakers, which were loaded in soxhlet apparatus.
- Distillation cycle I (Extraction cycle) – Distillation at a temperature of 100 °C for 1 hour.
- Distillation cycle II (Collection cycle) – Distillation at temperature 140 - 160 °C for 30 minutes.
- Beakers removed from the apparatus during cycle II (with 1-2 ml of petroleum ether left in beaker) and dried in a hot air oven.
- Beakers were kept in a desiccator for cooling after complete evaporation of the organic solvent. Beakers along with ether extract (EE) were weighed.

Observations

Sample weight - W g

Weight of empty beaker - W_1 g

Weight of beaker + EE - W_2 g

Weight of EE or crude fat - $W_2 - W_1$

Calculations - $$\% \text{ EE} = \frac{W_2 - W_1}{W} \times 100$$

3.3.3 Crude fiber (CF)

The crude fiber content in the sample was determined by FIBRA-PLUS, FES 4 (Pelican Equipment).

Principle

The samples were treated successively with boiling solutions of sulphuric acid and sodium hydroxide of specified concentrations. The residue is filtered, dried, weighed and ashed. The loss of weight resulting from ashing corresponds to the crude fibre present in the sample.

Reagents

- 1.25 % H_2S0_4 - 0.7 ml conc. H_2SO_4 dissolved in distilled water to make a final volume of 100 ml.
- 1.25 % NaOH - 1.25 g NaOH pellets dissolved in distilled water to make a final volume of 100 ml.

Procedure

- 1 g of sample (W g) was taken in an oven dried sintered glass crucible. Crucibles were placed into the metal adapters of FIBRA PLUS hot extraction unit.
- Acid wash - 150 ml of 1.25 % H_2S0_4 was poured into each extractor from the top. The instrument was switched on and the initial temperature was set at 500°C. When boiling started, the temperature was reduced to 400°C. The sample was allowed to boil for 40 minutes in acid and after this, acid was drained and the sample was washed twice or thrice with distilled water.
- Alkali wash - 150 ml of 1.25 % NaOH was poured into each extractor from the top. The instrument was switched on and the initial temperature was set at

500°C. When boiling started, the temperature was reduced to 400°C. The sample was allowed to boil for 40 minutes in alkali and after this, alkali was drained and the sample was washed twice or thrice with distilled water. After alkali wash, crucibles were taken out and dried in a hot air oven to make the samples moistures free. Hot crucibles were cooled down to room temperature using a desiccator. Crucibles were weighed (W_1). All the crucibles were placed in the muffle furnace at 330±10°C for 3 hours for ashing. Hot crucibles were cooled down to room temperature after ashing using a desiccator and weighed (W_2).

Calculations - $$\% CF = \frac{W_1 - W_2}{W} \times 100$$

3.3.4 Ash

Principle

The dried and burnt (carbon free) samples were ignited to know the mineral content. The loss of weight resulting after ignition corresponds to the ash present in the sample.

Procedure

- Cleaned, dried and empty silica crucibles were weighed (W_1) accurately.
- 1g of feed sample (W) was added in the crucible and heated till completely burned (smokeless).
- Crucibles were placed in a muffle furnace at 550^oC±10°C for 3 hours.
- Crucibles were cooled overnight and weighed (W_2)
- Ash % was calculated by following formula.

Observations

W_1 = Weight of empty crucible

W_2 = Weight of crucible and Ash

W = Weight of the sample

Calculations - $$\% Ash = \frac{W_2 - W_1}{W} \times 100$$

3.3.5 Nitrogen Free Extract (NFE)

It was obtained by subtracting the sum of the percentage of CP, EE, CF and ash from 100.

Calculations

NFE (%) = 100 - (% CP + % EE + % CF + % ash)

Table 4. Proximate composition (on % dry matter basis) of different feed ingredients and experimental diets

Ingredients/ Diets	CP	EE	CF	Ash	NFE	Moisture	GE (Kcal $100g^{-1}$)
Rice Bran*	15.41	2.10	23.60	11.91	46.98	3.10	299.52
Mustard Meal*	37.13	2.87	25.55	8.93	25.52	2.26	341.53
Ginger Powder (GP)	5.57	2.53	5.02	5.22	81.66	4.60	390.18
GP0	24.03	2.32	23.36	9.86	40.43	2.96	323.45
GP5	24.90	2.39	24.08	10.23	38.40	3.02	320.71
GP10	25.02	2.41	24.18	10.33	38.06	3.16	320.18
GP15	26.73	2.46	24.53	10.41	35.87	3.87	321.33
GP20	27.28	2.49	25.11	10.57	34.55	4.13	319.31

*Solvent extracted; CP = crude protein; EE = ether extract; CF = crude fat; NFE = nitrogen free extract; GE = gross energy

3.3.6 Gross Energy

It was calculated on the basis of gross energy values of crude protein, soluble carbohydrates (nitrogen-free extract) and total lipids (ether extract) of respective diets in terms of Kcal 100 g^{-1} by using energy factor 5.65 for proteins, 9.45 for fats and 4.10 for carbohydrates (Hepher et al., 1983).

Gross energy (Kcal 100 g^{-1}) = Protein (%) x 5.65 + Lipid (%) x 9.45 + Carbohydrate (%) x 4.10

3.4 Feeding of fish

Fish were fed with control and experimental diets (GP0 - G20) @ 5% (1-60 days) and 3% (61-120 days) of fish body weight (BW) twice daily (10:00 and

16:00 h) until 120 days (May – August 2019). Amount of feed was adjusted after each sampling according to increase in fish weight.

3.5 Observations recorded

- **Fortnightly observations**
  - Physico-chemical parameters of water (temperature, pH, dissolved oxygen, total hardness, total alkalinity and ammonical-nitrogen).
- **Monthly observations**
  - Fish growth (length and weight of fish).
- **Observations at 60 day of experiment**
  - **Haematological parameters** - Red Blood Cells (RBC), White Blood Cells (WBC), Haemoglobin (Hb), Hematocrit (Ht), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC)
  - **Growth hormones -** Triiodothyronine (T3) and Thyroxine (T4)
  - **Non-specific immune responses -** Respiratory burst activity (RBA), Lysozyme activity and Total Immunoglobulins (Total Ig)
- **Observations at the completion (120 day) of experiment**
  - **Fish survival**
  - **Growth parameters** - Net Weight Gain (NWG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER) and Condition Factor (K)
  - **Hematological parameters** – RBC, WBC, Hb, Ht value, MCV, MCH and MCHC
  - **Growth hormone -** Triiodothyronine (T3) and Thyroxine (T4)
  - **Non-specific immune responses** - RBA, Lysozyme activity and Total Ig
  - **Blood metabolic profile**-
    - **Lipid profile** – High density lipids (HDL), Triglycerides and Cholesterol

- **Anti-oxidant parameters** - Superoxide dismutase (SOD) and Lipid peroxidation level (LPO)
- **Liver enzymes** - Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)
- **Immunological parameters** - Total proteins, Albumin (Alb), Globulins (Glb), Alb/Glb ratio and Glucose

- **Challenge study against fish pathogen** - fish fed with control (GP0) and different experimental diets (GP5-GP20) for 120 days were challenged with pathogenic bacteria i.e. *Aeromonas hydrophila* to study the disease resistance

3.5.1 Physico-chemical parameters of water

Water samples were collected at fortnightly intervals in the morning hours for the analysis of various physico-chemical parameters mentioned below:

3.5.1.1 Temperature - Water temperature (°C) was recorded by using digital thermometer (0 to 50 °C).

3.5.1.2 pH - pH was recorded by using a digital pH meter (Metler Toledo- FE 20-1).

3.5.1.3 Dissolved oxygen (D.O.) - Dissolved oxygen of water was estimated by modified Winkler's method (APHA, 2012).

Principle

It is based on the addition of divalent manganese solution, followed by a strong alkali to a glass-stoppered bottle. DO rapidly oxidizes an equivalent amount of the dispersed divalent manganous hydroxide precipitate to hydroxides of higher valency states. In the presence of iodide ions in an acidic solution, the oxidized manganese reverse to the divalent state, with the liberation of iodine equivalent to the original DO content. The iodine is then titrated against standard sodium thiosulphate, using starch indicator from blue to colourless end point.

Reagents

a) N/40 sodium thiosulphate [6.205 g sodium thiosulphate in one litre water]

b) Manganous sulphate [480 g $MnSO_4.4H_2O$ in one litre water]

c) Alkaline iodide-azide solution [350 g KOH and 75 g KI dissolved separately in distilled water. Both were mixed and the volume was made up to 500 ml. 5 g sodium azide (NaN_3) was mixed in 20 ml distilled water separately. This azide solution was added to the alkaline-iodide reagent].

d) Starch indicator: Dissolve 1g of starch (soluble) in 200 ml distilled water and few drops of toluene as preservative.

e) Concentrated sulphuric acid

Procedure

- Water sample was collected without bubbling in 250 ml BOD bottle.
- 2 ml each of manganous sulphate and alkaline iodide-azide solutions were added one after the other for the formation of brown coloured precipitates.
- The bottle was shaken upside down and the brown precipitates were allowed to settle down.
- The precipitates were dissolved by adding 2 ml concentrated sulphuric acid and by shaking the bottle.
- 50 ml of the sample was taken and titrated with sodium thiosulphate solution till colour changed to pale straw. Two drops of starch solution were added and titrated further till the colourless end point.

Calculations - $DO\ (mg\ l^{-1}) = \frac{8 \times 1000 \times N}{V} \times v$

Where,

V = Volume of sample (ml)

v = Volume of titrant (sodium thiosulphate) used (ml)

N = Normality of titrant (sodium thiosulphate)

3.5.1.4 Total Hardness (TH): Total hardness of water was determined by volumetric method (APHA, 2012).

Principle

Erichrome black T forms wine red complex compound with metal ions (Ca^{2+} and Mg^{2+}). The disodium metallation complexed as colorless chelate complex leaving a blue coloured aqueous solution of the dye.

Reagents

a) Standard EDTA titrant (0.01 M): Dissolve 3.723g of the disodium salt of EDTA in distilled water to prepare 1 liter of titrant. Store in plastic bottle.

b) Eriochrome black T indicator: Dissolve 0.5g dye in 100 ml of 80% ethyl alcohol.

c) Ammonia buffer solution: Add 114 ml concentrated NH_4OH to 13.5 g of NH_4Cl and make volume up to 200 ml.

Procedure

- 50 ml of well-mixed water sample was taken in an Erlenmeyer flask, to which 1 ml of ammonia buffer was added followed by 5 drops of Eriochrome black T indicator.
- On appearance of wine-red color, it was titrated with standard EDTA (0.01M) titrant till wine red color changed to blue as end point.
- The volume of titrant used was recorded to calculate TH.

Calculations

$$\text{TH } (CaCO_3 \text{ mg } l^{-1}) = \frac{\text{Volume of titrant used (ml)}}{\text{Volume of the sample (ml)}} \times 1000$$

3.5.1.5 Total Alkalinity (TA) (Phenolphthalein and Methyl Orange Alkalinity)

Total Alkalinity of water was estimated by volumetric method (APHA, 2012).

Principle

Alkalinity is determined by titrating the sample with a standard solution of the strong acid. Alkalinity due to hydroxide and carbonate is determined to first end point (pH 8.3) using phenolphthalein indicator and bicarbonate alkalinity is determined to the second end point (pH 4.5) using methyl orange indicator.

Reagents

a) 0.02 N sulphuric acid

b) Phenolphthalein indicator

c) Methyl orange indicator

Procedure

- 50 ml of water sample was taken in an Erlenmeyer flask and two drops of phenolphthalein indicator were added to it.
- The pink color developed and was titrated with 0.02N H_2SO_4 till disappearance of the pink color.
- The volume of H_2SO_4 (A) used was recorded to calculate phenolphthalein alkalinity (PA).
- After this, two drops of methyl orange indicator were added to the same flask, the yellow color appeared.
- The solution was further titrated with 0.02N H_2SO_4 till yellow color changed to orange.
- The volume of H_2SO_4 (B) used was recorded to calculate methyl orange alkalinity (MOA). The TA was calculated as the sum of PA and MOA.

Calculations

$$\text{PA } (CaCO_3 \text{ mg } l^{-1}) = \frac{A}{\text{Volume of the sample (ml)}} \times 1000$$

$$\text{MOA } (CaCO_3 \text{ mg } l^{-1}) = \frac{B}{\text{Volume of the sample (ml)}} \times 1000$$

$$\text{TA } (CaCO_3 \text{ mg } l^{-1}) = \text{PA} + \text{MOA}$$

3.5.1.6 Ammonical - Nitrogen (NH_3-N) - Ammonical nitrogen of water was estimated by following the method of APHA (2012).

Principle

Ammonia reacts with phenol and alkaline hypochlorite to form indophenols blue. The reaction is catalyzed by the nitroprusside or ferricyanide. The resulting absorbance is proportional to the concentration of ammonia and is measured spectrophotometrically at 635 nm.

Reagents

a) Alkaline hypochlorite reagent: Dissolve 15g phenol in 500 ml water. To this add 1 ml of freshly prepared 1.5% (w/v) nitroprusside

b) Phenol-nitroprusside reagent

c) Standard ammonium chloride solution

Procedure

- 20 ml of water sample was taken in 25 ml capacity amber colored volumetric flask.
- To this 2 ml of each; phenol-nitroprusside and alkaline hypochlorite solution was added one after another.
- Distilled water was added to make a total volume of 25 ml. It was incubated at 25°C for 1 hour. Absorbance (A) was read at 635 nm.

Result- The value of Ammonical-Nitrogen was calculated from standard Ammonium chloride solution.

3.5.2 Survival and growth of fish

3.5.2.1 Survival of fish

Survival (%) of fish in each treatment was recorded by comparing the live fish recovered at the end of the experiment with that of total fish stocked according to following formula

$$\textbf{Fish survival}\ (\%) = \frac{\text{Number of fish recovered after completion of experiment}}{\text{Number of fish stocked at initiation of experiment}} \times 100$$

3.5.2.2 Growth of fish

Fish sampling was done at monthly intervals to record fish growth in terms of total body length and body weight. Total body length gain (TBLG), Net weight gain (NWG), Specific growth rate (SGR), Feed conversion ratio (FCR), Protein efficiency ratio (PER) and Condition factor (K) of fish for each treatment were calculated as per following formulae (Halver, 1976).

TBLG = Final total body length (cm) – Initial total body length (cm)

NWG = Final body weight (g) – Initial body weight (g)

$$\textbf{SGR (\% weight gain day}^{-1}\textbf{)} = \frac{\ln \text{final BW (g)} - \ln \text{initial BW (g)}}{\text{Culture days}} \times 100$$

ln = natural logarithm

Feed conversion ratio (FCR) = Feed given (g) / Weight gain (g)

Protein efficiency ratio (PER) = Weight gain (g) / Protein intake (g)

Condition factor (K-value) = Body weight (g) / Body Length $(cm)^3 \times 100$

3.5.3. Haematological parameters

Haematological parameters were analyzed at mid (60 days) and the completion of the experiment (120 days).

Blood collection

Blood was collected by the caudal vein puncture and pooled from a random sample of three fish from each replicate after anesthetized by clove oil @ 30-50 mg l^{-1} (1-part clove oil and 9-parts 94% ethanol) (Hajek et al. 2006). The blood (heparinised 150 IU ml^{-1}) collected from each group was tested for RBC, WBC, Hb, Ht and values for MCV, MCH, MCHC were calculated.

3.5.3.1 Red Blood Cells (RBC) - Mukherjee (1988)

Principle

It is based on an accurate dilution of measured quantity of blood with a fluid, which is isotonic with the blood and prevents coagulation. The counting of RBC is done on haemocytometer having neubaur grid, on which cell counting areas are marked for the estimation and the technique is popularly known as haemocytometry.

Reagents

a) RBC diluting fluid

Procedure:

- The blood was drawn into the RBC pipette up to 0.5 mark, followed by sucking of RBC diluting fluid up to 101 mark. This gives a dilution of 1: 200 (Blood: RBC diluting fluid).
- The solution is mixed by rotating gently and allowed to settle for 2 to 3 minutes.
- The counting chamber and cover glass were properly cleaned and the cover glass was placed over the ruled area.
- The solution was mixed gently again and the stem full of solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 45^o.

- It was allowed to settle for 2 to 3 minutes, erythrocytes without air bubble under the coverslip were counted.
- The ruled counting area was focused under the microscope and the number of RBC's were counted in fine small squares of the counting area under high power lenses and number of RBC were calculated by using the following formula:

$$\text{RBC } (\times 10^{6}\ mm^{3-1}) = \frac{\text{No. of Cells counted} \times \text{dilution factor (1:200)} \times \text{depth factor (0.1 mm)}}{\text{Total No. of small squares (5)}}$$

3.5.3.2 White blood cells (WBC) - Mukherjee (1988)

Principle

It is based on an accurate dilution of measured quantity of blood with a fluid which is isotonic with the blood and prevents coagulation.

Reagents

a) WBC diluting fluid

Procedure

- The blood was drawn into the WBC pipette up to 0.5 mark and followed by sucking of WBC diluting fluid up to 11 mark.
- This gives a dilution of 1:20. For counting of WBC in Neubaur counting chamber, cells in each of 4 square millimeter area, subdivided into 16 squares were counted by using low power objective lens.
- The following formula was taken for the enumeration of WBC.

$$\text{WBC } (\times 10^{4}\ mm^{3-1}) = \frac{\text{No. of cells counted} \times \text{Volume of the square} \times \text{dilution factor (20)}}{\text{No. of Squares (4)}}$$

3.5.3.3 Haemoglobin (Hb) - Sahli (1962)

Principle

Hb is a reasonable index of the red cell population and was estimated by acid haematin method. When Hb reacts with 0.1 N HCl, it forms acid hematin, which is brown in colour.

Reagents

a) 0.1 N Hydrochloric acid (HCl)

Procedure

- 0.1 N HCl was taken up to the mark 20 in the graduated tube and a drop (0.1 ml) of blood was added.
- It was allowed to stand for 5 minutes, until it changes to dark brown colour. The solution was diluted by adding distilled water drop by drop (each time mixing the solution with a stirring rod), until it matches standard colour.
- Reading was taken from the scale on the graduated tube and the Hb concentration was expressed as gram percent (g %).

3.5.3.4 Hematocrit (Ht) or Packed Cell Volume (PCV) - Mukherjee (1988)

Principle

Ht (%) was estimated by micro-capillary method. It is based on the principle of separation of blood by centrifugation.

Procedure

- In the micro capillary method, filled and sealed capillaries are centrifuged at 10,000 rpm for 8 minutes and subsequently final observations are taken from micro-capillary scale.
- Ht value was expressed in %

3.5.3.5 Mean Corpuscular Volume (MCV)

MCV is the average volume of red blood cells (RBC). Because the size of the cell is very small, volume is expressed in cubic microns (μm^3). It is calculated by using the following formula:

$$MCV\ (\mu\ m^3) = \text{Hematocrit (\%)} / \text{RBC} \times 10$$

3.5.3.6 Mean Corpuscular Haemoglobin (MCH)

MCH is the average Hb content of the red blood cell. MCH is influenced by the size of the cell and concentration of hemoglobin. It is derived by the following formula:

$$MCH\ (g\ \%) = Haemoglobin\ (g\ \%) / RBC \times 10$$

3.5.3.7 Mean Cell Haemoglobin Concentration (MCHC)

The MCHC is an expression of the average Hb concentration per unit volume (100) of packed cells (W/V). Hence it is expressed in g dilution^{-1}, which is same as percent (%)

$$MCHC\ (\%) = MCH/MCV \times 100$$

3.5.4 Growth Hormones: Triiodothyronine - T3 and Thyroxine - T4

These parameters were analyzed from blood serum at 60 and 120 day by using Erba Manhelm Kit.

Serum collection

For serum collection, blood samples were withdrawn from the caudal vein and transferred to eppendorf tubes without anticoagulant and kept for 6 h at room temperature followed by centrifugation at 3000 rpm for 15 min. The supernatant was collected and stored at -20^{0}C until used.

3.5.4.1 Triiodothyronine (T3)

Principle

Serum sample is pipetted into streptavidin coated well, addition of the horseradish peroxidase-T_3 conjugate (HRP-T3) and of the immunological reaction starter Anti- T_3-Biotin conjugate initiates the competitive assay. During incubation, T_3 of the sample competes with the HRP-T_3 conjugate for the anti-T_3 binding sites of the biotinilated antibody which, in turn, is bound by the streptavidin coated wells through the biotin moiety. Streptavidin: Biotin-Anti- T_3: T_3-HRP is revealed by the incubation with the chromogen/substrate and develop blue colour which is stopped with sulphuric acid, turning the final solution to a yellow colour, which is measured photometrically at 450 nm.

The intensity of the colour is proportional to the bound HRP- T_3 conjugate, and therefore inversely related to the amount of T_3 in the sample. By reference to a

series of T_3 standards, assayed in the same way, the concentration of T_3 in the unknown sample is quantified.

Reagents

- HRP-Conjugate
- Anti- T_3-Biotin Conjugate
- Washing solution
- 3, 3', 5, 5'-Tetramethylbenzidine (TMB)

✓ Bring all reagents and specimens to room temperature (20°C - 30°C) before beginning the assay. Swirl gently before use

Procedure

Pipetting and Incubation:

- Pipette in duplicate 50 µl of each standard and each sample into the appointed wells.
- Pipette 50 µl of the HRP-Conjugate (HRP- T_3 conjugate) into all the wells
- Pipette 50 µl of the Anti- T_3-Biotin Conjugate into all the wells
- Shake the plate for 10 seconds on an orbital shaker or manually, by gently hitting the side of the microplate against your index finger; the movement must be sideways to avoid spilling the well content.
- Incubate the plate at room temperature for 1 hour.
- At the end of the incubation period, fill the wells with 300 µl washing solution contained in the wash-bottle and wash the strips 3 times.
- Add 100 µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate.
- Incubate at room temperature for 15 min.
- Stop the reaction by adding 100 µl of stop solution to each well in the same order followed for dispensing the substrate.
- Measure the absorbance within 30 min. with microtiter reader at the wavelength of 450 nm (reference filter at 620 nm)

Calculation of Results - Plot the ODs of standards versus the respective T3 concentration (use a linear or a semi-logarithmic scale). Determine the T3 concentration of the sample by interpolation of the sample ODs on the calibration curve.

3.5.4.2 Thyroxine (T4)

Principle

Serum sample is pipetted into the well coated with Streptavidin. The addition of the Biotin-T_4 conjugate (Biotin-T4) and of the immunological reaction starter Anti-T_4 -HRP conjugate initiates the competitive assay. During the immunological incubation, the T_4 of the sample competes with the Biotin- T_4 conjugate for the anti-T_4 binding sites of the HRP Anti T_4 conjugate which, in turn, is bound by the streptavidin coated wells through the biotin moiety. After washing off the non-reacted species, the amount of the immunological complex remained bound to the wells. Streptavidin: Biotin- T_4: Anti- T_4-HRP is revealed by the incubation with the chromogen/substrate. The blue colour development is then stopped with sulphuric acid, turning the final solution to a yellow colour which is measured photometrically at 450 nm. The intensity of the colour is proportional to the bound HRP- T_4 conjugate, and therefore inversely related to the amount of T_4 in the sample. By reference to a series of T_4 standards, assayed in the same way, the concentration of T_4 in the unknown sample is quantified.

Reagents

- HRP-Conjugate
- Anti-T_4-Biotin Conjugate
- Washing solution
- 3, 3', 5, 5'-Tetramethylbenzidine (TMB)
    - ✓ Bring all reagents and specimens to room temperature (20°C - 30°C) before beginning the assay. Swirl gently before use.

Procedure

Pipetting and Incubation:

- Pipette in duplicate 50 µl of each standard and 50 µl of each sample into the appointed wells.
- Pipette 50 µl of the Biotin-Conjugate (Biotin- T_4 conjugate) into all the wells

- Pipette 50 µl of the Anti-T_4-HRP Conjugate into all the wells
- Shake the plate for 10 seconds on an orbital shaker or manually, by gently hitting the side of the microplate against your index finger; the movement must be sideways to avoid spilling the well content.
- Incubate the plate at room temperature for 1 hour.
- At the end of the incubation period, fill all the wells with 300 µl of the washing solution contained in the wash-bottle and wash the strips 3 times.
- Add 100 µl of TMB Substrate.
- Incubate at room temperature for 15 min.
- Stop the reaction by adding 100 µl of stop solution to each well in the same order followed for dispensing the substrate.
- Measure the absorbance within 30 min. with microtiter reader at the wavelength of 450 nm (reference filter at 620 nm).

Calculation of Results - A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens. Plot the ODs of standards versus the respective T4 concentration (use a linear or a semi-logarithmic scale). Determine the T4 concentration of the sample by interpolation of the sample ODs on the calibration curve.

3.6 Non-specific immune responses

These parameters were analyzed from blood serum at 60 and 120 day of experiment

3.6.1 Respiratory burst activity (RBA)

Principle

The RBA of phagocytes was carried out following the protocol of Anderson & Siwicki (1995) using the nitroblue tetrazolium (NBT).

Reagents

- Nitro blue tetrazolium solution - 0.2%
- N, N-dimethyl formamide (DMF)

Procedure

- 100 µl of the heparinized blood sample of each treatment in triplicate was placed into 96 well U shaped bottomed microtitre plates and 100 µl of 0.2% NBT was added into it, homogenized and incubated for 30 min at 25 °C.
- After homogenization, 50 µl of solution was mixed with 1 ml of N, N-dimethyl formamide (DMF). Mixture was homogenized and centrifuged at 300 x g for 5 min.

Result

- The optical density (OD) of the supernatant was measured at 540 nm in the microplate reader (Model: Infinite M200 PRO, Tecan, Switzerland). For blank, blood was replaced with distilled water.

3.6.2 Lysozyme activity

Principle

The lysozyme activity was measured using the turbidimetric assay (Parry et al., 1965; Sankaran & Gurnani, 1972) with partial modification.

Reagents

- Phosphate buffer saline (PBS) pH 5.8
- Young culture of *Micrococcus lueteus*

Procedure

- 50 µl of serum sample was placed in triplicate in a 96 well plate with 50 µl of PBS (pH 5.8). After mixing, the serum was serially diluted until the last well.
- Finally, 50 µl of sample was discarded in the last well. 24 h grown culture of *M. lueteus* was centrifuged and pelleted cell washed two times with PBS **(**pH 7.4).
- The cell concentration was adjusted at 0.5-0.7 spectrophotometrically at 450 nm. To each well, 125 µl of *M. lueteus* was added.
- The reduction in the absorbance at 450 nm was measured from 0 to 5 min with 30 sec interval at room temperature in ELlSA microplate reader (Model: Infinite M200 PRO, Tecan, Switzerland).

Results

- Lysozyme activity was expressed as units ml^{-1}, where one unit was defined as the reduction in absorbance of 0.001 min^{-1}. Lyophilized hen egg white lysozyme was used to develop a standard curve.

3.6.3 Total Immunoglobulins (Ig)

Modified Anderson & Siwicki (1995) method was followed for the estimation of total immunoglobulins in serum.

Reagents

- Polyethylene glycol

Procedure

- 0.1 ml of serum was placed into a plastic serum vial and added 0.1 ml of 12% polyethylene glycol added to vial, that had been suspended in deionised water.
- Incubation was done at room temperature for 2 h under constant mixing.
- After that, centrifugation was done at 5000 rpm for 10 min, supernatant was taken out and protein concentration was determined.
- Protein reading from supernatant was the amount of protein taken out by absorption to polyethylene glycol. Total immunoglobulins were alculated using following formula

Total immunoglobulins (g dl^{-1}) = Total protein in individual sample plasma - Total protein taken out by absorption to polyethylene glycol

3.7 Blood metabolic profile

3.7.1 Lipid profile

3.7.1.1 High Density Lipids (HDL)

HDL was estimated with Erba Diagnostic Mannheim GmbH kits (International Federation of Clinical chemistry) at completion of experiment (120 days).

Principle

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol methyl ether (PEGME) coupled classic precipitation method with

the improvements in using optimized quantities of PVS/PEGME and selected detergents. LDL, VLDL and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER), whereas HDL reacts with the enzymes. Addition of R2 containing a specific detergent releases LDL from the PVS/PEGME complex. The released LDL reacts with the enzymes to produce H_2O_2 which is quantified by the Trinder reaction.

$$\text{HDL+LDL+VLDL+CM} \xrightarrow[\text{PEGME}]{\text{PVS}} \text{HDL+(LDL+VLDL+CM). PVS/PEGME}$$

$$\text{HDL} \xrightarrow{\text{CHOD, CHER}} \text{Fatty Acid} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AA} + \text{TODB} \xrightarrow{\text{Peroxidase}} \text{Quinone} + 5\,H_2O$$

Procedure: Assay procedure for HDL estimation

Components	**Reagent Blank**	**Sample / Calibrator**
Reagent 1	375 µl	375 µl
Distilled water	5 µl	---
Sample / Calibrator	---	5 µl
Mix and incubate at 37°C for 5 minutes		
Add Reagent 2	125 µl	125 µl
Mix and incubate at 37°C for 5 minutes		

Read final absorbances at the specified wavelength against reagent blank

Calculation:

$$\text{HDL} = \frac{(\text{Abs. of Sample} - \text{Abs. of Sample blank}) \times \text{Concentration of Calibrator}}{(\text{Abs. of Cal.} - \text{Abs. of Cal. blank})}$$

3.7.1.2 Triglycerides

Triglyceride was estimated by following the method of Fossati et al. (1983).

Principle

The triglycerides are converted into glycerol and free fatty acids in the presence of lipase. The glycerol and adenosine triphosphate (ATP) in the presence of glycerol kinase forms glycerol-3-phosphate. The glycerol-3-phosphate is oxidized to dihydroxy acetone phosphate (DHAP) and H_2O_2 in the presence of glycerol phosphate oxidase (GPO). The H_2O_2, 4-aminoantipyrine (4-AAP) and 3, 5-Dichloro-2-hydroxybenzene sulfonate (DHBS) forms quinoneimine dye in the presence of peroxidase. The intensity of dye is proportional to the triglycerides concentration in the sample.

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase, } Mg^{++}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DHAP} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{3, 5-DHBS} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2H_20$$

Procedure: Assay procedure for serum triglyceride estimation

Components	Blank	Standard	Test
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	----	----
Standard	----	10 µl	----
Sample (Blood serum)	----	----	10 µl

All the reagents were mixed well and incubated at 37°C for 10 minutes. Blank was aspirated followed by standard and tests.

3.7.1.3 Cholesterol

Total cholesterol was estimated by modified Roeschlau et al. (1974) method.

Principle

The estimation is based on the conversion of cholesterol ester to cholesterol and fatty acid in the presence of cholesterol esterase (CE). Cholesterol is oxidized to Cholest-4-en-3-one and hydrogen peroxide (H_2O_2) in the presence of cholesterol oxidase. The phenol and 4-amino antipyrine (4-AAP) present in the cholesterol reagent kit interacts with the hydrogen peroxide in the presence of peroxidase (POD) forms a red dyestuff Quinoneimine. The absorbance of Quinoneimine so formed is directly proportional to cholesterol concentration.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{phenol} \xrightarrow{\text{Peroxidase}} H_2O + \text{Quinoneimine dye}$$

Procedure: Assay procedure for serum cholesterol estimation

Components	Blank	Standard	Test
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	---	---
Standard	---	20 µl	---
Sample (Blood serum)	---	---	20 µl

All the components were mixed well and incubated at 37°C for 10 minutes. Blank was aspirated followed by standard and tests.

3.7.2 Antioxidant parameters

Antioxidant parameters were analyzed after completion of experiment (120 day) from blood hemolysate (RBC lysate).

Preparation of haemolysate: Blood haemolysate was prepared before proceeding for different markers to determine erythrocyte oxidative damage**.** Blood samples were centrifuged at 3000 rpm for 15 minutes and supernatant was separated out. The sedimented cells were washed thrice with chilled 0.85% NaCl solution. Washed erythrocytes were lysed with nine parts of distilled water to prepare 10% haemolysate.

Haemolysate was stored in aliquots at -20^{0}C for determination of oxidative stress markers.

3.7.2.1 Superoxide dismutase (SOD) - Nishikimi et al. (1972)

Principle

The assay is based on the principle that the nitroblue tetrazolium inhibits superoxide dismutase with reduced nicotinamide adenine dinucleotide (NADH) mediated by phenazonium methosulphate under aerobic conditions.

Reagents

a) 0.017 M sodium phosphate buffer (pH 8.3)

- Solution 1: 2.052 g of sodium dihydrogen phosphate (NaH_2PO_4) l^{-1} distilled water
- Solution 2: 2.413 g of disodium hydrogen phosphate (Na_2HPO_4) l^{-1} distilled water
- Solution 1 (7.36 ml) and solution 2 (92.64 ml) were mixed and diluted to 200 ml with distilled water after adjusting the pH to 8.3

b) 1.5 mM Nitroblue tetrazolium chloride (NBT): 132.26 mg of nitroblue tetrazolium per 100 ml distilled water

c) 2.34 mM Nicotinamide adenine dinucleotide - disodium salt (NADH): 16.6 mg of NADH per 10 ml distilled water

d) 0.093 mM Phenazine methosulphate (PMS): 2.85 mg of phenazonium methosulphate per 100ml distilled water

Procedure

- To 2.6 ml of phosphate buffer at 20^{0}C in the cuvette, 100 µl each of PMS, NBT and haemolysate (1:100 v/v) were added.
- The reaction was initiated by adding 100 µl of NADH and increase in absorbance was recorded at 560 nm for 2 minutes at 30 seconds interval using UV/VIS spectrophotometer.
- Unit of SOD was defined as activity of enzyme concentration required to inhibit chromogen production by 50% in 1 min under assay conditions.

- All determinations were performed in triplicate.

Calculations

$$\text{Erythrocytic SOD activity (U mgl}^{-1}\text{ Hb)} = \frac{\Delta T \times 100}{\Delta C/2 \quad \curlyvee}$$

ΔT = Change in optical density of test at 30 sec intervals

ΔC = Change in optical density of control at 30 sec intervals

$\curlyvee$ = Haemoglobin concentration in haemolysate (in mg)

3.7.2.1 Lipid peroxidation - Placer et al. (1966)

Principle

The assay is based on the reaction of malondialdehyde (MDA), an end product of lipid peroxidation with thiobarbituric acid to yield a pink coloured trimethine complex exhibiting an absorption maximum at 548 nm wavelength.

Reagents

a) 0.2 M Tris-0.16 M Kcl buffer (pH-7.4): 2.422 g Tris and 1.192 g Kcl per 100 ml distilled water

b) 7% Perchloric acid

c) 1N Sodium hydroxide (NaOH): 4g NaOH per 100 ml distilled water

d) Thiobarbituric acid reagent:

   - TBA solution: 0.8 g TBA per 100 ml 1N NaOH
   - TBA reagent: Two volumes of TBA solution per one volume of 7% perchloric acid

e) Pyridine –n-butanol reagent (3:1, v/v)

Procedure

- 0.1ml RBC lysate was taken in a test tube and 1.4 ml tris buffer was added to it.
- The contents were incubated for 30 minutes followed by addition of 1.5 ml TBA reagent.
- Mixture was heated in boiling water bath for 10 minutes. 3 ml pyridine-n-butanol reagent and 1 ml NaOH was added to test tube after cooling and mixed properly by shaking.

- Control was neither incubated nor heated.
- Absorbance was recorded at 548 nm using UV/VIS spectrophotometer against distilled water blank. All determinations were performed in triplicate.

Calculation

Erythrocytic lipid peroxidation (nmol MDAg Hb^{-1}) $$\frac{(A_{test} - A_{control}) \times 46 \times 1000}{\gamma}$$

γ = Haemoglobin concentration in g 0.1^{-1} ml haemolysate

3.7.3 Liver Profile - Aminotransferases

The aminotransferases including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicators of hepatocyte damage. These enzymes are present in hepatocyte cytosol and during episodes of altered plasma membrane permeability, they leak into the extracellular fluid. ALT and AST were analyzed after completion of experiment (120 day) from blood serum

3.7.3.1 Aspartate aminotransferase (AST) - AST was estimated with Erba Diagnostic Mannheim GmbH kits (International Federation of Clinical chemistry-IFCC).

Principle

The principle involved in the estimation of AST involves the interactions of L-aspartate and 2-oxoglutarate in the presence of aspartate aminotransferase (AST) to form oxaloacetate. The oxaloacetate in the presence of malate dehydrogenase (MDH) forms malate. The pyruvate present in the sample reacts with lactate dehydrogenase (LDH) to form L-lactate.

$$\text{L-Aspartate + 2-Oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate + L-Glutamate}$$

$$\text{Oxaloacetate + NADH} \xrightarrow{\text{MDH}} \text{Malate + NAD}$$

$$\text{Sample pyruvate + NADH} \xrightarrow{\text{LDH}} \text{L-lactate + NAD}$$

Procedure

Components	Sample
Working reagent	1000 µl
Sample (Blood serum)	100 µl

The serum was mixed well with the working reagent and the absorbance of the sample was read at 340 nm after mixing.

3.7.3.2 Alanine aminotransferase (ALT) - ALT was estimated with ERBA Diagnostic Mannheim GmbH kits (International Federation of Clinical chemistry-IFCC).

Principle

The principle involved in the estimation of ALT involves the interactions of L-Alanine and 2-Oxoglutarate in the presence of alanine aminotransferase (ALT) to form pyruvate, which further interacts with lactate dehydrogenase (LDH) and forms L-lactate.

$$\text{L-Alanine} + \text{2-Oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

Procedure

Components	Sample
Working reagent	1000 µl
Sample (Blood serum)	100 µl

The serum was mixed well with the working reagent and the absorbance of the sample was read at 340 nm after mixing.

3.7.4 Immunological parameters

Immunological status of fish in terms of serum total proteins, albumins, globulins, albumin/globulin ratio and glucose concentration was estimated after

completion (120 days) of the experiment. These parameters were analyzed from blood serum by using Erba Manhelm Kit.

3.7.4.1 Total proteins

Total proteins (TP) in blood serum was analyzed by following the principle of Biuret reaction (Gornall et al., 1949)

Principle

The peptide bonds of protein react with copper (Cu^{2+}) ions in alkaline solution to form a blue-violet ion complex, (biuret reaction); each copper ion complexing with 5 or 6 peptide bonds. Tartrate is added as a stabilizer, whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm (520-560 nm).

Reagents

- Copper II sulphate - 19 mmol l^{-1}
- Potassium sodium tartrate - 43 mmol l^{-1}
- Potassium iodide - 30.0 mmol l^{-1}
- Sodium hydroxide - 600 mmol l^{-1}

Procedure

Reagent blank, standard and test samples were prepared as follows

	Reagent blank	**Standard**	**Sample (Test)**
Reagent (R1)	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	–	–
Standard Reagent (R2)	–	20 µl	–
Sample (Blood serum)	–	–	20 µl

- Reagent 1, Standard Reagent 2 and sample (blood serum) were mixed and incubated for 10 minutes at 37^{o} C.
- Absorbance of the standard and each sample was read at 546 nm (520 – 560 nm) against reagent blank

Calculations

TP (gdl^{-1}) = Absorbance of sample/Absorbance of standard x Concentration of standard

3.7.4.2 Albumin

Principle

Albumin binds with Bromo Cresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin, when measured photometrically between 540–630 nm with maximum absorbance at 625 nm.

Reagent

- Bromocresol green - 0.08 mmol l^{-1}
- Succinate buffer - 50 mmol l^{-1}
- Sodium azide - 1.0 g l^{-1}

Procedure

Reagent blank, standard and test samples were prepared as follows

	Reagent blank	**Standard**	**Sample (Test)**
Reagent (R1)	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	–	–
Standard Reagent (R2)	–	10 µl	–
Sample (Blood serum)	–	–	10 l

Reagent 1, Standard Reagent 2 and Test Sample were mixed and the absorbance of the standard and each test sample was read at 630 nm (580 – 630 nm) against reagent blank, after one minute of incubation at 37^0C.

Calculation

Albumin (gdl^{-1}) = Absorbance of test sample/Absorbance of Standard x Concentration of standard

3.7.4.3 Globulins

Globulins were calculated as

Globulin (gdl^{-1}) = Total protein (gdl^{-1}) – Albumin (gdl^{-1})

3.7.4.4 Albumin/Globulin ratio (Alb/Glb)

Alb/Glb ratio = Albumin (gdl^{-1}) / Globulin (gdl^{-1})

3.7.4.5 Glucose

Glucose concentration in blood serum was analyzed after completion of experiment (120 day) using Erba Diagnostic Mannheim GmbH kits (Trinder, 1969)

Principle

The principle involved in the estimation of glucose involves the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) in the presence of glucose oxidase. The formed hydrogen peroxide interacts with 4-aminoantipyrine (4AAP) and 4-hydroxy benzoic acid (4HBA) in the presence of peroxidase to form quinoneimine dye. The intensity of the pink color formed is proportional to the glucose concentration and absorbance was measured at 510 nm.

Glucose oxidase

Glucose + O_2+H_2O ----------► Gluconic acid + H_2O_2

Peroxidase

H_2O_2 +4HBA + 4AAP ----------►Quinoneimine Dye +2H_2O

Procedure

Pipette into tubes marked	Blank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	----	----
Standard	----	10 µl	----
Sample (Blood serum)	----	----	10 µl

The serum was mixed well with the working reagent and was incubated at 37°C for 15 min. The blank was aspirated followed by standard and tests for glucose estimation. The absorbance of the test and standard was noted at 510 nm against reagent blank.

3.8 Challenge study: Challenge study against fish pathogen to know the immunity level of control and experimental fish

- After the completion of experiment-I (120 days), fish were challenged with *Aeromonas hydrophilla*
- In the challenge study trial, there were 6 treatments including negative control (GN), in which fish fed on control diet (GP0) without supplementation of ginger in feed and injected only sterile phosphate buffered saline (PBS).
- Total 15 fish in each group (5 from each replicate) were netted out, kept (5 in each replicate) in separate aquarium (50 L capacity) and were given intra-peritoneal injections of *A. hydrophilla* (100 µL of 10^7 $CFUml^{-1}$ in PBS solution).
- Observation was made for a period of 15 days (during and after the challenge trial) to calculate fish mortality (%), level of protection (%) according to the Amend (1981) along with behavioural changes (loss of balance, rejection of feed etc.), abnormalities and post mortem changes (haemorrhages, inflammation etc.).

3.8.1 Fish mortality (%) – Fish mortality was calculated by comparing the dead fish at the end of challenge study with that of total fish stocked.

3.8.2 Level of protection (%) against *A. hydrophilla*, calculated by the formula

$$\% \text{ Level of protection} = 1 - \frac{\text{Mortality in treated group (\%)}}{\text{Mortality in control group (\%)}} \times 100$$

3.9 Statistical analysis

Statistical analysis of the data was performed with a Statistical Package for the Social Science (SPSS v.25.0). One-way ANOVA was applied to analyze the data (all parameters) at 0.05 level of significance ($p \leq 0.05$), followed by Duncan's multiple comparison to determine significant differences among the treatments (Duncan, 1955).

3.10 Experiment II: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia*

3.10.1 Preparation of Ginger Extract

Ginger extract (GE) was prepared from ginger powder [from ginger rhizome (used in Experiment-I) procured from local vegetable market of district Ludhiana, Punjab, India] from Nutri Biotech Services Ltd., Malta (Europe). GE was prepared by immersing 100g of ginger powder in 2 L of acetone for 6 weeks, with daily stirring. At every 2-week interval, acetone was decanted and stored, with the addition of fresh acetone to the GP. The acetone of three extractions (3 times at 2-week interval) was pooled, mixed and placed with the previously collected acetone. All the collected acetone was mixed and placed in a rotary evaporator to recover the GE in concentrated form. The concentrated GE was removed from the evaporator and diluted into 200 ml of acetone for storage and transportation. The concentration of extract is equivalent to 0.5 g of dry ginger ml^{-1} of diluted extract.

3.10.2 Preparation of Filtered Artificial Seawater (32ppt)

Filtered artificial seawater (FASW) containing 32 g l^{-1} of Instant Ocean® synthetic sea salt (Aquarium Systems, Sarrebourg, France) was prepared as per manufacture guidelines and autoclaved at 121 °C for 15 minutes.

3.10.3 Preparation of Marine broth

Dissolve 37.4 g of marine broth powder 2216 (Difco Laboratories, Detroit, MI. USA) in 1L of distilled water. Marine broth powder was mixed thoroughly followed by filtering it using 110 mm whatman paper and autoclaved at 121 °C for 15 minutes.

3.10.4 Growth of *Vibrio parahaemolyticus* for challenge assay

Vibrio parahaemolyticus 20130629002S01 strain was used for *Artemia* challenge assays. The strain was stored in 40% glycerol at -80^0C and was grown in marine broth 2216 (Difco Laboratories, Detroit, MI. USA) by incubation at 28^0C for 24 h with continuous shaking for use in challenge experiments. Bacterial cell numbers were determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), with an OD of 1.000 corresponding to 1.2×10^9 cells ml^{-1}.

3.10.5 Hatching of *Artemia* Larvae under axenic conditions

Axenic *Artemia* larvae were obtained following decapsulation and hatching procedure (Sorgeloos et al., 1986) as described below

Hydration

50-60 mg of *Artemia franciscana* cysts (EG®, INVE Aquaculture, Belgium) were hydrated in 10 ml autoclaved distilled water in sterile falcon tube (50ml) provided with aeration for 1 hour under sterilized (axenic) condition

Decapsulation

After 1hour, add 330ul of 32% Sodium hydroxide (NaOH) in falcon tube followed by 5ml of sodium hypochlorite (NaOCl). Falcon tube was kept for 1-2 minutes (colour changes to orange). The decapsulation process was stopped by addition of sterile 5ml sodium thiosulphate ($Na_2S_2O_3$)

Rinsing

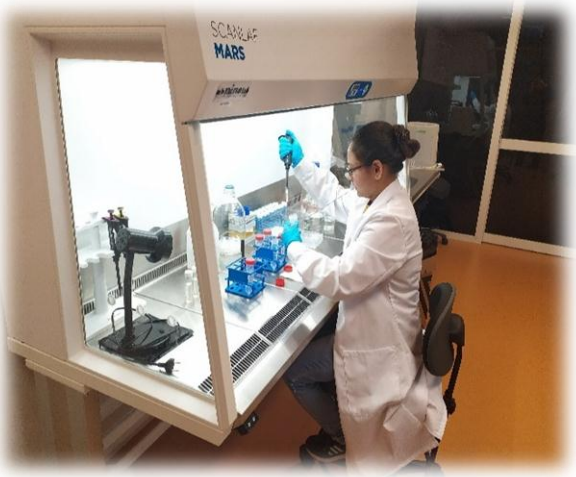
The aeration was stopped and decapsulated cysts were filtered through a sterile sieve (0.2 um) and rinsed with autoclaved FASW (around 1 L) until rinsing water is clear. After that, all cysts were removed with the help of sterile spatula and put in three falcon tubes (50ml) containing 30 ml sterile sea water

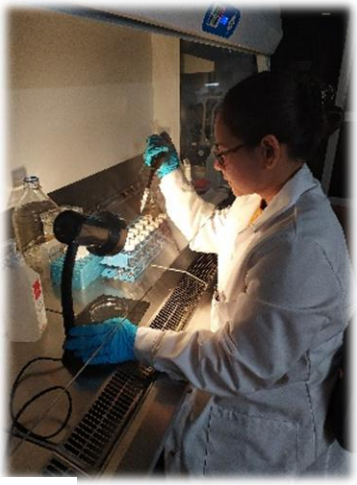
Hatching

The falcon tubes were incubated for 28 h on a rotor at 4 rpm at 28 °C with constant illumination (approximately 27 µE m^2s^{-1}). The hatched *Artemia* larvae reaching (instar II stage - mouth is opened to ingest particles) were collected and used for further experiments

***A. franciscana* cyst**

Hydration





Decapsulation and Rinsing

Incubation on rotor

Hatched *Artemia* larvae

Figure 3. Hatching of *Artemia* under axenic conditions

3.10.6 *Artemia* Toxicity Assay

Cytotoxic effect of the GE was determined in the axenic *Artemia* larvae as described by Baruah et al. (2015).

- Hatched *Artemia* larvae at developmental stage II were transferred to a 40 ml sterile glass tube. The axenic larvae were pre-treated for 2 h at 28°C with increasing concentrations of GE (83.3, 125, 166.6, 208.3, 250, 333.3 ug ml^{-1}) and with acetone alone as a positive control (C2). The final acetone concentration in the positive control or GE pre-treatment groups corresponds to the amount added in the pre-treatment group with the highest concentration of the extract.
- Negative control (C1) was also maintained without the addition of GE and acetone.
- After 2-h of pre-treatment, the larvae were repeatedly rinsed with FASW to wash away the GE and then *Artemia* larvae allowed to recover for 2 h at 28°C.
- Following the recovery period, toxicity assay was performed in sterile 40-ml glass tubes with 20 larvae in 10ml FASW per tube.
- The toxicity of the GE was determined after 48 h by scoring the number of survivors as described by Baruah et al. (2015).
- Five replicates were maintained for each treatment and control (negative and positive) group (**Fig. 4**)

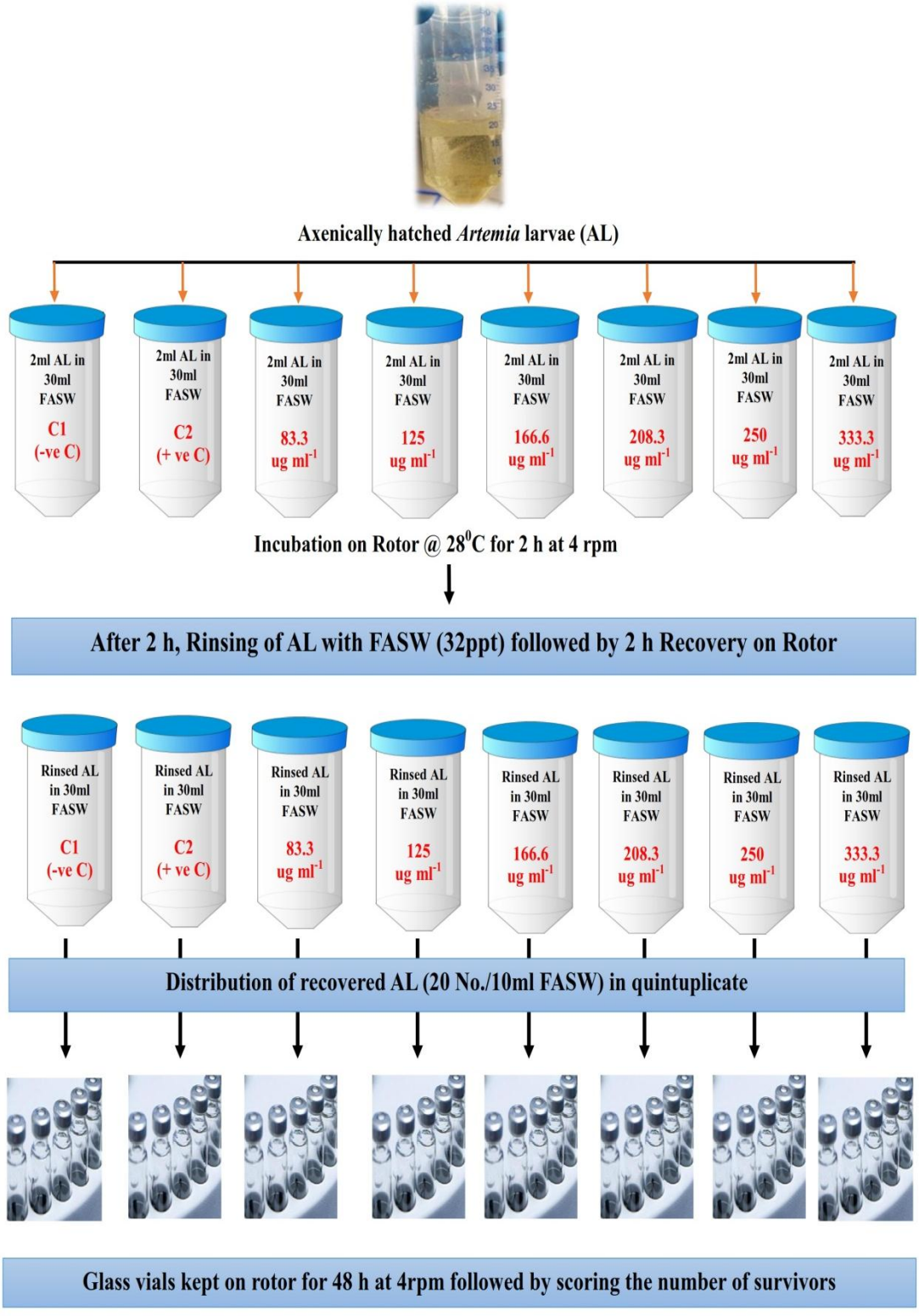


Fig. 4 *Artemia* Toxicity Assay (Pre-treatment study)

3.10.7 *Artemia* Challenge Assay

The putative prophylactic effect of the ginger extract against *V. parahemolyticus* was determined by carrying out *Artemia* challenge assay as previously described (in 3.10.6). During challenge assay, germ-free larvae (instar II stage) were pretreated with increasing concentrations of GE, selected based on the outcome of the toxicity assay. Following the recovery period, a group of 20 larvae was transferred in sterile 40-ml glass tubes containing 10-ml FASW. Afterward, it was verified whether the pretreatment of the GE could protect the larvae against subsequent challenge with *V. parahaemolyticus* at 10^7 cells ml^{-1}. The survival of *Artemia* larvae was scored 48 h after the addition of the pathogen. Negative (C1) and positive controls (C2) were maintained as described in the toxicity assay (3.10.6). Challenge assay (each treatment and control) was carried out in quintuplicate. The dose(s) of GE that gave maximum protection to larvae against *V. parahemolyticus* challenge in the dose-response assay were further taken to carry out the mechanistic studies as described below.

3.10.8 Bulk Sampling and Analysis

Bulk sampling was done to collect the samples for performing immunological assay. After axenic hatching (bulk) of *Artemia* cyst (15g), fully swimming artemia larvae were collected, counted volumetrically and transferred to 1L sterile glass bottles. There were 3 experimental groups: negative control (C1: no pre-treatment of larvae with GE and/or acetone), positive control (C2: pre-treatment with acetone only), and T1 (pre-treated with an optimized dose of the GE). Each of these experimental groups was challenged with *V. parahaemolyticus* as described in 3.10.7. Bulk sampling (each treatment and control) was performed in triplicate. Samples containing 40 to 80 mg of live larvae were collected from all the experimental groups before challenge (0 h) and at 6, 12, 24, and 48 h post challenge, rinsed in distilled water, immediately frozen in liquid nitrogen, and stored in −80°C for further analysis of gene expressions.

3.10.9 RNA Extraction

Total RNA was extracted from *Artemia* samples in triplicate with Qiagen RNeasy Plus Mini Kit (Qiagen, Sweden) according to the manufacturer's instructions. The quantity and quality of RNA were confirmed by NanoDrop™ 8000 (Thermo Scientific, USA).

3.10.9.1 RNA Isolation: using RNeasy Mini Kit from Qiagen, Sweden

Principle

The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNAses to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl of water.

Protocol

- Disrupt the sample (≤ 30 mg) and homogenize the lysate in the appropriate volume of buffer RLT (Table 5). Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting, and use it in next step
- Add 1 volume of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to next step.
- Transfer up to 700 µl of the sample, including any precipitate, to an RNeasy mini spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
- Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
- Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.
- Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 2 min at ≥8000 x *g*.
- Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid and centrifuge for 1 min at ≥8000 x *g* to elute the RNA.
- The quality and quantity of RNA were confirmed by NanoDrop™ 2000 (Thermo Scientific, USA).

Table 5. Volumes of Buffer RLT for sample disruption and homogenization

Sample	Amount	Buffer RLT	Disruption and Homogenization
Animal tissue (*Artemia* larvae sample)	<20 mg	350 µl	Mortar and pestle followed by QIA shredder or needle and syringe
	≤30 mg	600 µl	

3.10.9.2 cDNA synthesis

Reverse transcription was done from 1 µg total RNA samples with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Sweden) according to the manufacturer's guidelines.

Protocol

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Particulars	Nucleic acid	Amount
Template RNA	total RNA *or* poly(A) mRNA *or* specific RNA	0.1 ng – 5 ug 10 pg - 0.5 ug 0.01 pg - 0.5 ug
Primer	oligo (dT)18 primer *or* random hexamer primer *or* gene-specific primer	1ul 1ul 15-20 pmol
Water, nuclease-free		Make up volume upto 12 ul
Total volume		12 ul

2. Add the following components in the indicated order:

5X Reaction Buffer	4 ul
RiboLock RNase Inhibitor (20 U uL^{-1})	1 ul
10 mM dNTP Mix	2 ul
RevertAid H Minus M-MuLV Reverse Transcriptase (200 U uL^{-1})	1 ul
Total volume	20 ul

3. All the components of reaction mixture were mixed gently followed by vortex
4. For oligo (dT)18 or gene-specific primed cDNA synthesis - incubate for 60 min at 42°C, for random hexamer primed synthesis - incubate for 5 min at 25°C followed by 60 min at 42°C.
5. Terminate the reaction by heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

3.11 Preparation of Primers

For preparation of primer stock, lyophilized primers (Eurofins Genomics, Denmark) were diluted by adding nuclease free water (as per company's instructions). From this stock, 100 ul was taken in new eppendorf tube to prepare working solution, from which, final qPCR primers were made (50 times diluted). Thereafter, in a new eppendorf 4ul of working solution and 196 ul nuclease free water was added. In this way, all forward and reverse primers were prepared.

3.12 Assay of defense-related gene expression by quantitative real-time PCR (RT-qPCR) analysis

The expressions of key genes associated with the defense system of *Artemia* were measured by RT-qPCR analysis using a pair of specific primers **(Table 6):** heat shock protein 70 (*hsp70*), heat shock protein 90 (*hsp90*), down syndrome cell adhesion molecule (*dscam*), lipopolysaccharide and β-1,3-glucan-binding protein (*lgbp*), prophenoloxidase (*proPO*), high mobility group box (*hmgb*), peroxinectin (*pxn*), superoxide dismutase (*sod*), transglutaminase 1 (*tgase1*) and transglutaminase 2 (*tgase2*): elongation factor (*ef-1*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) were used as the internal control.

Table 6. Specific Primers used in RT-PCR

Gene	Primer sequences (5′-3′)
ef-1	F: TCGACAAGAGAACCATTGAAAA R: ACGCTCAGCTTTAAGTTTGTCC
gapdh	F: GTTGATGGCAAACTCGTCATA R: CCACCTTCCAAGTGAGCATTA
Sod	F: CAATCAGCATTGGGGTTTGTC R: GAATCTCTTCGTTGGTTGTAGGG

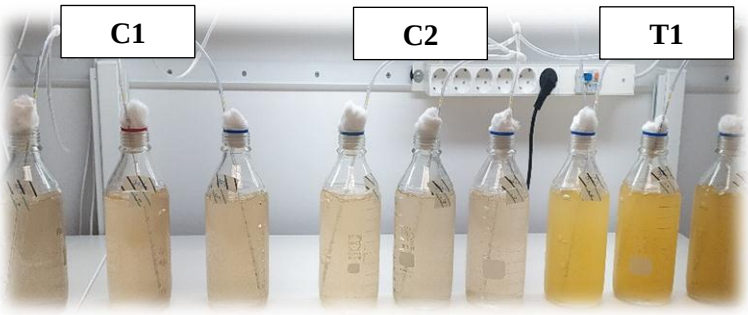
dscam	F: TCAAGAGGCTGAAAGAGAAGAAAT R: CAGTAGAAGCAGTGACCCAGAAAT
lgbp	F: CCGTGAAGATCCCAACGAAC R: GGAGGAGGTAATTGGGAGTTTCAAGG
hmgb	F: AGAGGCGGGAAAGGAAGC R: CCCACACCAAGACCAGGTTG
proPO	F: TCTGCAAGGAGGATTTAAGGA R: TGACTGACAAAGGAGATGGGAC
pxn	F: TTGGTGCTGCTGCTTTTCG R: CCCCATCGCTTGTCTTCGT
hsp70	F: CGATAAAGGCCGTCTCTCCA R: CAGCTTCAGGTAACTTGTCCTTG
hsp90	F: GCTGACCGTGTTGTTGTCAC R: ACGATCTTGGTTCCACGTCC
tgase1	F: GCAAGGAGCTGGAATGGGT R: TGTTTGGGAGTTAATCGGACTGT
tgase2	F: TTCTTTACACAGGCATTCCGTC R: GTTACATCAAATCCCAGCTCCA

The RT- qPCR assay was performed on Step One Plus Real-Time System (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, Sweden) according to the manufacturer's guidelines described below. The comparative CT method ($2^{-\Delta\Delta Ct}$ method) following Livak & Schmittgen (2001) was used to analyze the expression level of the target genes.

Protocol

1. Gently vortex all solutions of the kit after thawing.
2. Prepare a reaction master mix by adding the following components (except template DNA) for each 25 µl reaction to a tube at room temperature:

Particulars of Master mix	**Volume**
Maxima SYBR Green/ROX qPCR Master Mix (2X)	12.5 µl
Forward Primer	0.4 µl
Reverse Primer	0.4 µl
Template DNA	2 µl
Water (nuclease-free)	9.7 µl
Total volume	25 µl

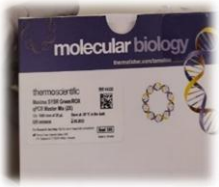


Bulk Sampling

Hatched *Artemia* (in bulk)

***Artemia* Challenge Assay Preparation**

Ginger Extract



Primers & Molecular Biology Kits

Nano Drop Spectrophotometer

Step One Plus Real-Time System

Figure 5. Bulk Sampling of *Artemia* and Immunological Assay Preparation

3. Mix the master mix thoroughly and dispense appropriate volumes (23 ul) into PCR plates.
4. Add template DNA (2 ul) to the individual PCR wells containing the master mix.
5. Gently mix the reactions without creating bubbles (do not vortex). Centrifuge briefly, if required (to avoid bubbles that will interfere with fluorescence detection).
6. Place the samples in the thermal cycler and start the program as per following recommended procedure

Thermal cycling conditions

Thermal cycling was performed using a three-step cycling protocol.

Three step cycling protocol

Step	**Temperature (^{0}C)**	**Time**	**No. of cycles**
Initial Denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

3.13 Statistical analysis

Survival data were subjected to one-way analysis of variances (ANOVA) followed by Duncan's multiple range tests using the IBM statistical software SPSS version 25.0. to determine significant differences among treatments. Results for gene expression are presented as fold-changes relative to the geometrical mean of two internal controls: *ef-1 and gapdh*. The expression level in the control group was regarded as 1.0 and thereby the expression ratio of the treatments was expressed in relation to the control. Analysis for significant differences in expression levels between the control and treatment groups was performed using one-way ANOVA. P values ≤ 0.05 were considered significant.

CHAPTER IV

RESULTS AND DISCUSSION

Results of the present study, with respect to experiment I and II, are presented under the following heads

4.1 Experiment I: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *L. rohita* (Ham.)

4.1.1 Physico-chemical parameters of water

4.1.2 Survival and growth of fish

4.1.3 Hematological parameters

4.1.4 Non-specific immune responses

4.1.5 Blood metabolic profile

4.1.6 Growth hormones

4.1.7 Challenge study against *A. hydrophilla*

4.2 Experiment II: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia*

4.2.1 *Artemia* toxicity assay (Pre-treatment study)

4.2.2 *Artemia* challenge assay against *V. parahemolyticus*

4.2.3 Immunological assay (Expression of defense-related genes in *Artemia*)

4.1 Experiment-I: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *L. rohita* (Ham.)

4.1.1 Physico-chemical parameters of water

Physico-chemical parameters of experimental tanks were analyzed every fortnight during 120 days of experimental period.

4.1.1.1 Temperature

During the culture period (May to August), temperature (°C) of water ranged between 26.10-29.10, 26.23-28.40, 26.46-28.56, 26.30-28.40 and 25.63-28.50 in GP0, GP5, GP10, GP15 and GP20 respectively (Table 7 and Fig. 6). Differences with respect to mean temperature (°C) values (GP0 = 27.34, GP5 = 27.32, GP10 = 27.63,

GP15 = 27.40 and GP20 = 26.98) were insignificant ($p \leq 0.05$) among all the treatments.

Table 7. Water temperature (°C) in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	26.43±0.33	26.23±0.12	26.76±0.20	26.46±0.17	26.36±0.20
	15	27.96±0.16	28.06±0.26	28.16±0.20	28.13±0.23	27.53±0.26
	30	26.60±0.41	26.46±0.21	26.80±0.20	26.73±0.37	26.50±0.15
June	45	27.73±0.06	27.66±0.18	28.10±0.50	28.26±0.12	27.80±0.05
	60	27.90±0.25	28.13±0.17	28.30±0.35	28.40±0.11	28.03±0.06
July	75	26.50±0.32	26.46±0.46	26.46±0.23	26.30±0.30	25.63±0.31
	90	26.10±0.60^{b}	26.43±0.20^{ab}	27.43±0.20^{a}	26.40±0.23^{ab}	26.03±0.37^{b}
August	105	27.80±0.05^{a}	28.10±0.51^{a}	28.10±0.28^{a}	27.53±0.26^{a}	26.46±0.23^{b}
	120	29.10±0.36	28.40±0.20	28.56±0.03	28.40±0.50	28.50±0.36
Mean values		27.34±0.30	27.32±0.25	27.63±0.24	27.40±0.25	26.98±0.22

*GP0 = Feed without ginger powder (GP), GP5 = 0.5% ginger supplementation (5 g kg^{-1} GP0), GP10 = 1% ginger supplementation (10 g kg^{-1} GP0), GP15 = 1.5% ginger supplementation (15 g kg^{-1} GP0), GP20 = 2% ginger supplementation (20 g kg^{-1} GP0)
Values are Mean ± S.E., ($p \leq 0.05$), n=3
Values with same superscript (a, b,…..d) in a row do not differ significantly ($p \leq 0.05$)

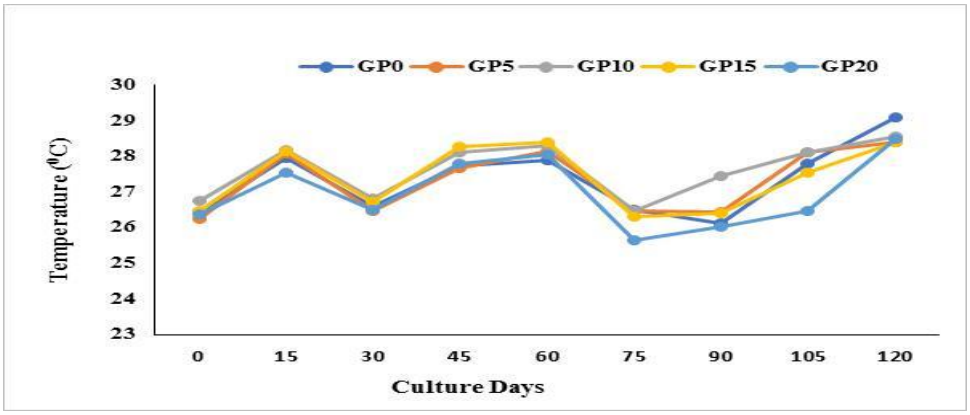


Figure 6. Changes in water temperature (^{o}C) in different treatments during the experimental period

Being cold blooded, temperature plays an important role in fish growth, as basal metabolic rate (BMR) and feed intake of fish increases with increase in temperature. Species suitable for culture under semi-intensive culture system might tolerate wide temperature range, but the temperature range for optimum growth remained narrow. Water temperature range of 25.63-29.10°C during the present study

agreed well with the optimum range of 24-32^{0}C documented by Boyd & Tucker (1998) and Jhingran (1991) for warm water fish with special reference to Indian major carps.

4.1.1.2 pH

During the culture period, the pH of water ranged between 7.22-8.34, 7.42-8.62, 7.48-8.37, 7.53-8.49 and 7.31-8.55 in GP0, GP5, GP10, GP15 and GP20 respectively (Table 8 and Fig. 7). Differences were insignificant ($p \leq 0.05$) for mean pH values for all the treatments except GP5 (GP0 = 7.72, GP5 = 7.67, GP10 = 7.71, GP15 = 7.79 and GP20 = 7.75), with significantly low value (7.67) as compared to control and other treatments.

Table 8. Water pH in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	7.77±0.16	7.77±0.17	7.63±0.14	7.63±0.18	7.94±0.04
	15	8.34±0.03	8.62±0.14	8.37±0.11	8.49±0.04	8.55±0.06
	30	7.22±0.06^{c}	7.51±0.08^{b}	7.81±0.11^{a}	8.01±0.02^{a}	7.83±0.06^{a}
June	45	7.69±0.13	7.62±0.14	7.66±0.13	7.68±0.18	7.78±0.008
	60	7.71±0.14	7.42±0.11	7.53±0.11	7.69±0.08	7.64±0.11
July	75	7.79±0.15	7.57±0.11	7.82±0.11	7.58±0.16	7.62±0.12
	90	7.73±0.19	7.47±0.24	7.48±0.06	7.53±0.20	7.31±0.29
August	105	7.78±0.01^{a}	7.69±0.11^{ab}	7.52±0.06^{ab}	7.68±0.18^{ab}	7.40±0.05^{b}
	120	7.49±0.14	7.42±0.08	7.63±0.20	7.84±0.07	7.71±0.17
Mean values		7.72±0.11^{a}	7.67±0.12^{b}	7.71±0.11^{a}	7.79±0.12^{a}	7.75±0.10^{a}

* See Table 7 for legends

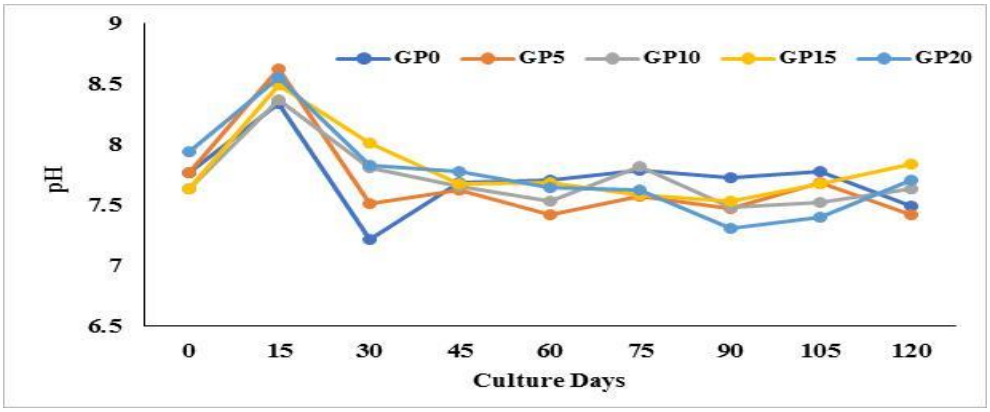


Figure 7. Changes in water pH in different treatments during the experimental period

Water pH plays an important role in optimum physiological activities of fish, decomposition of dead organic matter and release of nutrients from bottom soil and hence, fish growth and productivity. The most suitable pH range for optimum growth in Indian major carps is 6.5 to 9.6 as reported by Jhingran (1991) and Parameswaran et al. (1971). The pH of the water (7.22 to 8.62) remained well within range during the entire experimental period, which indicates that supplementation of fish feed with ginger had no undesirable effect on the water quality with respect to pH.

4.1.1.3 Dissolved Oxygen (DO)

During the culture period, the DO content of water ranged between 7.33-10.13, 8.16-10.36, 6.13-11.00, 7.43-10.73 and 7.63-9.66 mg l^{-1}in GP0, GP5, GP10, GP15 and GP20 respectively (Table 9 and Fig. 8). Differences were insignificant ($p \leq 0.05$) among the treatments for mean DO values (GP0 = 8.82, GP5 = 9.26, GP10 = 8.72, GP15 = 8.95 and GP20 = 8.81) except GP5, with significantly higher ($p \leq 0.05$) mean DO value (9.26 mg l^{-1}) as compared to all other treatments and control.

Table 9. Dissolved oxygen (mg l^{-1}) of water in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	10.13±0.96	9.13±0.94	7.16±0.85	8.20±0.75	9.66±0.88
	15	10.06±0.63	9.93±0.52	10.00±0.57	10.06±0.52	8.66±0.33
	30	7.33 ± 0.88^{ab}	9.13 ± 0.10^{a}	6.13 ± 0.59^{b}	7.43 ± 0.38^{ab}	9.00 ± 0.57^{a}
June	45	8.16 ± 0.44^{c}	10.36 ± 0.68^{ab}	9.30 ± 0.35^{bc}	10.73 ± 0.17^{a}	8.46 ± 0.26^{c}
	60	8.33±0.56	10.33±0.88	8.33±0.88	9.33±0.35	9.43±0.90
July	75	9.73±0.35	9.62±0.68	11.00±0.57	9.56±0.26	9.33±0.88
	90	8.46±0.76	8.16±0.12	8.90±0.23	7.66±0.33	9.00±0.57
August	105	9.56 ± 0.26^{a}	8.46 ± 0.26^{ab}	9.30 ± 0.35^{ab}	9.33 ± 0.35^{ab}	8.20 ± 0.43^{b}
	120	7.70±0.25	8.30±0.35	8.40±0.23	8.30±0.26	7.63±0.31
Mean values		8.82 ± 0.56^{b}	9.26 ± 0.50^{a}	8.72 ± 0.51^{b}	8.95 ± 0.37^{b}	8.81 ± 0.57^{b}

* See Table 7 for legends

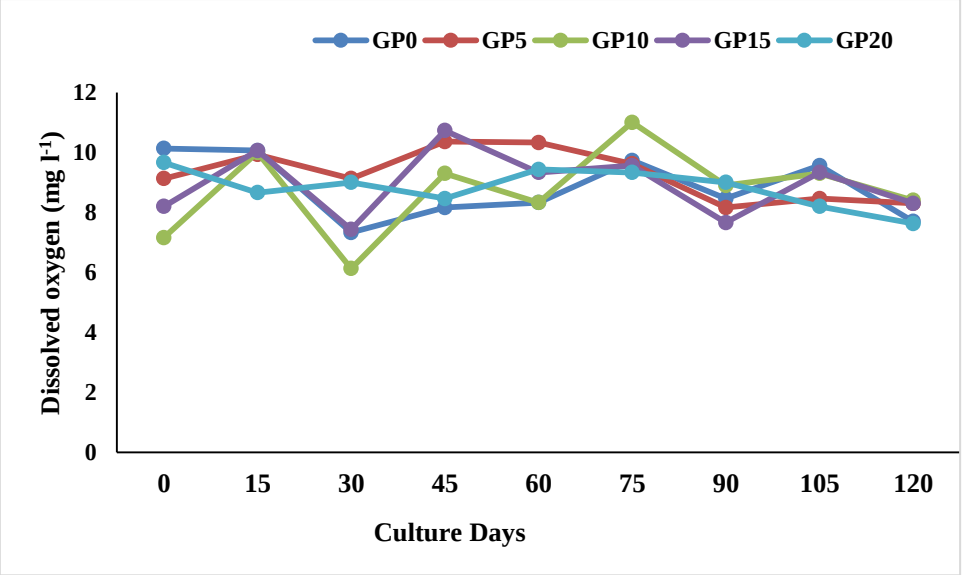


Figure 8. Changes in dissolved oxygen (mg l^{-1}) of water in different treatments during the experimental period

DO is one of the most critical water quality parameter affecting survival and growth of aquatic organisms, as it regulates all the metabolic activities of aquatic organisms and is a very good indicator of water health. For optimum productivity of carps, >5.0 mg l^{-1} of DO of water is to be maintained throughout the culture period (Swingle, 1967). Huet (1975) too suggested that the optimum dissolved oxygen content for better growth and survival of cyprinids range between 6 to 7 mgl^{-1}. DO levels lower than 3.0 mg l^{-1} are lethal for carps, which cause increased respiration rates, stress, immune-suppression, increased susceptibility to diseases, poor feed conversion efficiency, retarded growth and even death, as fish stop feeding.

In the present study, DO content remained well above (6.13 to 11.00 mg l^{-1}) the optimum limit of 5.0 mg l^{-1} throughout the culture period in all the treatments, which reveals that ginger supplementation in fish feed had no adverse effect on the water quality with respect to DO.

4.1.1.4 Total alkalinity (TA)

During the culture period, TA of water ranged between 158.66-238.00, 198.00-249.33, 178.66-248.66, 201.33-238.33 and 208.66-242.66 $CaCO_3$ mg l^{-1} in GP0, GP5, GP10, GP15 and GP20 respectively (Table 10 and Fig. 9). Significant changes ($p \leq 0.05$) were recorded among the treatments with respect to mean TA

content (GP0 = 209.96, GP5 = 258.33, GP10 = 220.14, GP15 =223.18 and GP20 = 226.84 $CaCO_3$ mg l^{-1}), with significantly higher value in GP5.

Alkalinity refers to assemblage of three ionic components i.e. carbonates, bicarbonates and hydroxyl in the water, which provides a good buffering effect to the diurnal pH swings, that occur in aquaculture ponds due to respiration (continuous day-night process) of aquatic organisms (fauna and flora) and the photosynthetic activity occurring in the pond only during the day time. In case of poor buffering capacity of water, extreme pH fluctuations occur in the pond, which affect the health and growth of fish adversely and may cause mortality under severe conditions. In the present study, TA of water in the different treatments remained well within the recommended range (158.66 to 249.33 $CaCO_3$ mg l^{-1}) throughout the culture period (Jena & Das, 2006; Boyd & Tucker, 1998) which reveals that ginger supplementation in fish feed had no adverse effect on the water quality with respect to TA of water.

Table 10. Total Alkalinity ($CaCO_3$ mg l^{-1}) of water in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	229.33±6.56	207.33±15.37	210.66±7.85	213.33±9.61	221.33±15.76
	15	238.00±21.16	204.00±7.02	248.66±11.56	223.33±15.24	242.66±15.71
	30	194.00±1.15^{b}	209.33±5.45^{ab}	208.66±10.72^{ab}	217.66±5.36^{a}	213.66±7.88^{ab}
June	45	208.66±13.13	198.00±1.15	235.33±13.38	201.33±16.90	224.66±9.33
	60	158.66±20.66	214.66±8.66	178.66±10.70	219.33±7.68	231.33±23.36
July	75	193.33±6.66^{b}	209.33±5.81^{ab}	196.00±4.00^{b}	224.00±10.58^{a}	226.66±6.66^{a}
	90	225.33±16.38	233.33±16.22	230.66±8.74	233.34±11.39	235.31±15.76
August	105	215.00±11.70	208.00±20.29	241.33±18.44	238.00±11.71	208.66±13.13
	120	227.33±11.56	249.33±8.74	231.33±4.66	238.33±11.46	237.33±8.41
Mean values		209.96±5.44^{c}	258.33±7.44^{a}	220.14±10.11^{b}	223.18±8.90^{b}	226.84±10.11^{b}

* See Table 7 for legends

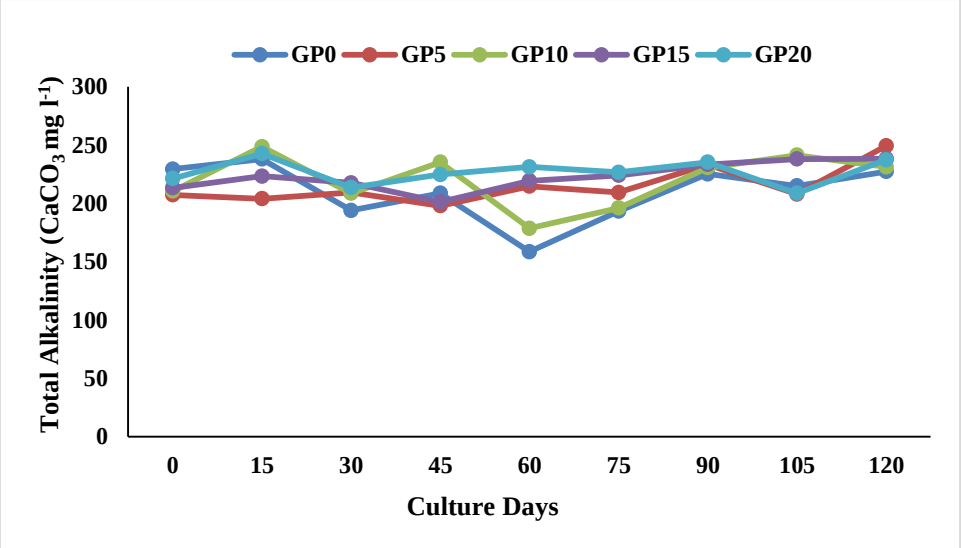


Figure 9. Changes in total alkalinity ($CaCO_3$ mg l^{-1}) of water in different treatments during the experimental period

4.1.1.5 Total Hardness (TH)

During the culture period, TH of water ranged between 204.00-246.66, 201.33-246.66, 210.66-241.00, 198.00-250.66 and 180.00-238.00 $CaCO_3$ mg l^{-1} in GP0, GP5, GP10, GP15 and GP20 respectively (Table 11 and Fig. 10). Significant changes ($p \leq 0.05$), were recorded among the treatments with respect to mean TH content (GP0 = 220.88, GP5 = 224.03, GP10 = 230.07, GP15 =224.03 and GP20 = 218.55 $CaCO_3$ mg l^{-1}) with significantly higher value of mean TH in GP10.

Table 11. Total Hardness ($CaCO_3$ mg l^{-1}) of water in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	225.33±16.38	233.33±16.22	230.66±8.74	233.33±11.39	235.33±15.76
	15	214.0±20.23	215.66±14.24	240.00±11.54	217.66±13.86	230.66±13.28
	30	226.66±13.33	246.66±19.22	218.66±14.84	244.00±16.00	238.00±14.00
June	45	206.66±17.63	238.00±11.71	241.00±17.89	208.00±20.29	215.00±11.78
	60	204.00±16.0	210.66±25.95	226.66±16.22	250.66±7.05	216.66±6.76
July	75	246.66±13.33^{a}	221.33±1.33^{a}	229.33±17.33^{a}	213.66±7.83^{ab}	180.00±11.54^{b}
	90	229.33±6.51	207.03±15.37	210.66±7.85	213.33±9.61	221.33±15.76
August	105	224.66±9.33	201.33±16.90	235.33±13.38	198.00±1.15	206.66±17.63
	120	210.66±13.01^{b}	242.33±4.63^{a}	238.33±9.83^{ab}	237.66±3.92^{ab}	223.33±7.31^{ab}
Mean values		220.88±13.97^{b}	224.03±13.95^{b}	230.07±12.19^{a}	224.03±10.12^{b}	218.55±12.64^{c}

* See Table 7 for legends

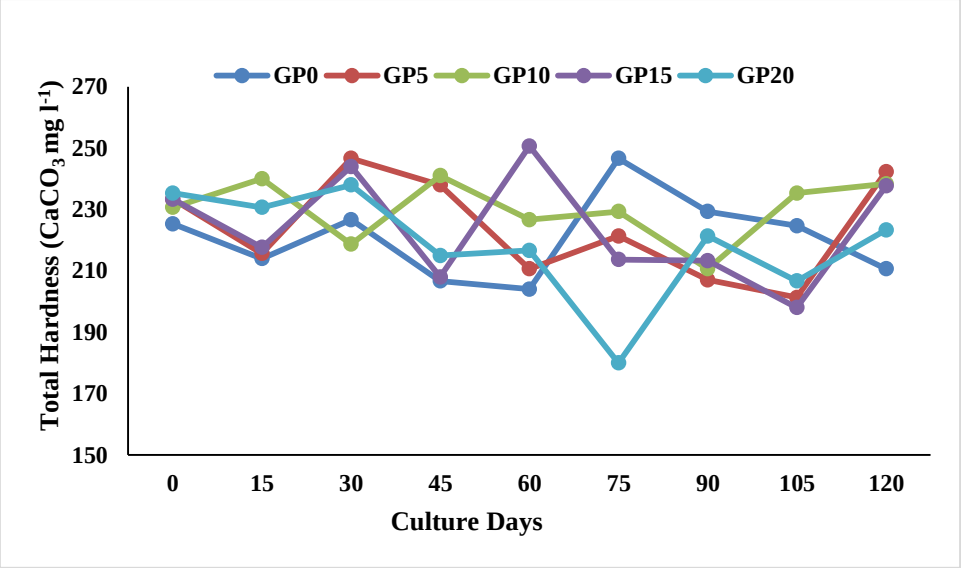


Figure 10. Changes in total hardness ($CaCO_3$ mg l^{-1}) of water in different treatments during the experimental period

Total hardness is sum of concentration of calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions in water (expressed as mg l^{-1} equivalent $CaCO_3$), required for optimum growth of aquatic animals, which are mainly absorbed from water. Although, fish can survive and grow in waters of very low calcium and magnesium content, if sufficient quantity of these two minerals are present in their diet. According to Bhatnagar et al., (2004), hardness values less than 20 ppm causes stress, 75 -150 ppm is optimum for fish culture and > 300 ppm is lethal to fish life, as it increases pH, resulting in non-availability of nutrients. In the present study, TH of water in the different treatments was in optimum range (180.00 to 250.66 mg l^{-1}) throughout the culture period (Bhatnagar et al., 2004; Boyd & Tucker, 1998) indicating that ginger supplementation in fish feed had no adverse effect on the water quality with respect to TH of water.

4.1.1.6 Ammonical-nitrogen (NH_3-N)

During the culture period the NH_3-N of water ranged between 0.01-0.08, 0.003-0.08, 0.01-0.21, 0.005-0.04 and 0.009-0.07 in GP0, GP5, GP10, GP15 and GP20 respectively (Table 12 and Fig. 11). In the present study, significant changes were observed for mean NH_3-N levels ($p \leq 0.05$), among the treatments (GP0 = 0.04, GP5 = 0.03, GP10 = 0.04, GP15 = 0.02 and GP20 = 0.03) with significantly lower value of mean NH_3-N in GP15.

Table 12. Ammonical nitrogen (mgl^{-1}) of water in different treatments during the experimental period

Month	Culture Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
May	0	0.03±0.005	0.05±0.005	0.02±0.003	0.03±0.01	0.02±0.006
	15	0.06±0.01	0.03±0.006	0.07±0.01	0.02±0.01	0.07±0.02
	30	0.05±0.01^{ab}	0.08±0.01^{a}	0.01±0.001^{b}	0.009±0.005^{b}	0.04±0.02^{ab}
June	45	0.05±0.01^{ab}	0.03±0.006^{bc}	0.21±0.002^{ac}	0.01±0.002^{c}	0.06±0.01^{a}
	60	0.03±0.005	0.07±0.01	0.02±0.003	0.04±0.02	0.02±0.006
July	75	0.03±0.008^{ab}	0.01±0.003^{b}	0.06±0.01^{a}	0.02±0.006^{b}	0.06±0.01^{a}
	90	0.08±0.02^{a}	0.04±0.005^{ab}	0.02±0.005^{b}	0.02±0.01^{ab}	0.04±0.02^{ab}
August	105	0.01±0.002	0.03±0.02	0.01±0.004	0.005±0.0008	0.009±0.002
	120	0.02±0.001	0.003±0.01	0.01±0.004	0.03±0.007	0.01±0.002
Mean values		0.04±0.01^{a}	0.03±0.008^{b}	0.04±0.004^{a}	0.02±0.007^{c}	0.03±0.01^{b}

*See Table 7 for legends

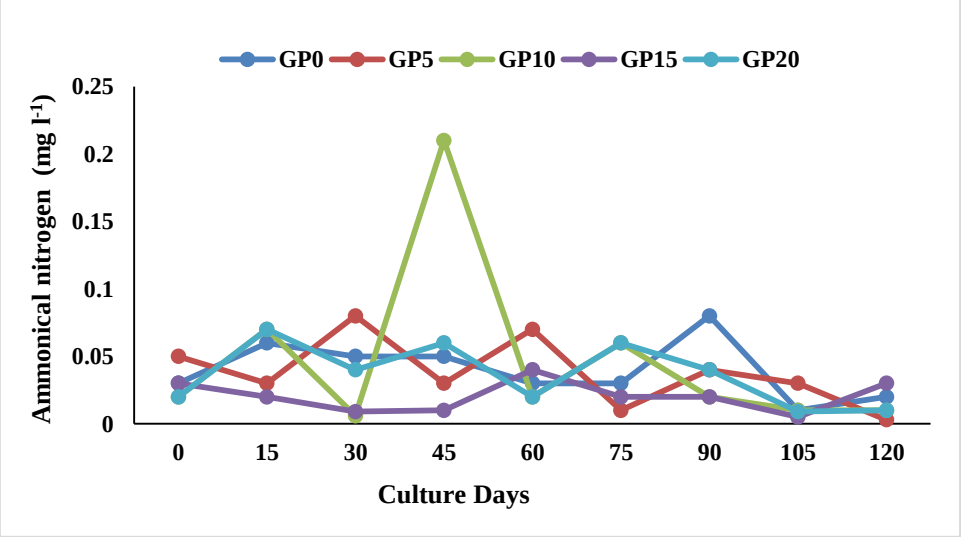


Figure 11. Changes in ammonical nitrogen (mg l^{-1}) of water in different treatments during the experimental period

Ammonia is excreted as waste product by most fishes and also produced by the decomposing dead organic matter in the culture system. Fish is very sensitive to unionized ammonia (NH_3) and its acceptable range for aquaculture is 0.02-0.05 mg l^{-1} with permissible upper limit of 0.1 mg l^{-1} (Boyd & Tucker, 1998). In the present study, mean NH_3-N of water remained within the permissible limit in all the treatments througout the experimental period. Moreover, during the 120 days culture

period, DO content remained above 6 mg l^{-1}, which indicates that there was sufficient DO in all the treatments to reduce the undesirable impact of ammonia.

During 120 days of experimental period, there were significant differences for mean values of the water quality parameters (except temperature), however, the values reamin in optimum range for carp culture for all these parameters. The possible reasons for these variations can be expalined in terms of outdoor experimental set up and completely randomized design of the experiment.

4.1.2. Survival and growth of fish

4.1.2.1 Survival of fish

At the end of experiment, all the experimental tanks were drained out completely to harvest all the fish for calculating the % survival of fish in different treatments (Table 13, Fig. 12). Average survival of fish in GP0, GP5, GP10, GP15 and GP20 was 89.39, 92.42, 93.93, 96.96 and 92.42%, respectively. Although, the highest fish survival was found in GP15 and lowest in GP0, however there was no significant difference ($p \leq 0.05$) among different treatments, which revealed no adverse effect of ginger supplementation on fish survival. Moreover, experimental fishes were healthy with shining body and without any sign of disease or parasitic infection.

4.1.2.2 Growth of fish

The growth performance of fish was assessed in terms of total body length (TBL) and body weight (BW) at monthly intervals during the culture period of 120 days. At the end of the experiment, total body length gain (TBLG), net weight gain (NWG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and condition factor (K-value) of fish were calculated for each treatment and control.

4.1.2.2.1 Length parameters

TBL (cm) of fish increased from 12.23 to 21.75 in GP0, 11.84 to 21.57 in GP5, 11.63 to 21.28 in GP10, 12.14 to 23.78 in GP15 and 11.80 to 20.39 in GP20 (Table 13). At the end of culture period (May to August), mean final TBL and TBLG was maximum (23.78 and 11.64 cm) in GP15 and minimum in GP20 (20.39 and 8.58 cm) respectively (Table 13, Fig. 13 A-B) and the differences ($p \leq 0.05$) were significant for both the parameters among the treatments and control.

Table 13. Growth parameters of *L. rohita* in different treatments during and after completion of the experimental period

Month	Culture Days	Treatments*				
		GP0	**GP5**	**GP10**	**GP15**	**GP20**
Length Parameters						
May	0	12.23±0.06	11.84±0.06	11.63±0.27	12.14±0.28	11.80±0.41
	30	17.78±3.29	14.34±0.75	14.37±0.45	14.19±0.23	13.39±0.9
June	60	17.07±1.18	17.40±0.54	17.60±0.90	16.95±0.42	16.23±0.79
July	90	19.49±1.03	19.49±0.44	19.33±1.25	19.66±0.56	17.86±1.25
August	120	21.75 ± 0.91^{ab}	21.57 ± 0.29^{ab}	21.28 ± 0.74^{ab}	23.78 ± 0.21^{a}	20.39 ± 0.94^{b}
TBLG		9.51 ± 0.91^{ab}	9.73 ± 0.27^{ab}	10.31 ± 0.96^{ab}	11.64 ± 0.11^{a}	8.58 ± 0.53^{b}
Weight Parameters						
May	0	18.87±3.38	21.27±0.27	20.37±0.22	20.63±0.64	21.63±0.82
	30	43.86±7.36	40.47±2.68	37.90±4.9	34.14±1.1	34.03±4.19
June	60	62.82±2.60	63.53±4.95	67.06±2.27	56.79±5.84	50.78±2.25
July	90	95.18±7.27	90.61±4.89	99.91±8.01	97.16±8.43	69.97±6.44
August	120	130.14 ± 5.75^{b}	136.50 ± 9.54^{b}	132.40 ± 3.99^{b}	157.04 ± 2.33^{a}	110.43 ± 5.51^{c}
NWG (g)		111.27 ± 6.64^{b}	115.23 ± 9.6^{b}	112.03 ± 4.16^{b}	136.40 ± 2.31^{a}	88.79 ± 5.62^{c}
SGR (%weight gain day^{-1})		2.45 ± 0.17^{ab}	2.36 ± 0.07^{ab}	2.37 ± 0.03^{ab}	2.53 ± 0.02^{a}	2.14 ± 0.06^{b}
PER		1.57 ± 0.19^{b}	1.62 ± 0.07^{ab}	1.61 ± 0.21^{ab}	2.00 ± 0.18^{a}	1.41 ± 0.13^{b}
FCR		1.96 ± 0.28^{ab}	1.74 ± 0.17^{ab}	1.90 ± 0.13^{ab}	1.55 ± 0.18^{b}	2.26 ± 0.20^{a}
K-value		1.27±0.10	1.35±0.04	1.38±0.12	1.16±0.02	1.32±0.11
Survival		89.39±1.51	92.42±4.00	93.93±1.51	96.96±3.03	92.42±1.51

***** See Table 7 for legends; n=10

4.1.2.2.2 Weight parameters

In different treatments, BW (g) of fish increased from 18.87 to 130.14 in GP0, 21.27 to 136.50 in GP5, 20.37 to 132.40 in GP10, 20.63 to 157.04 in GP15 and 21.63 to 110.43 in GP20 (Table 13). At the end of culture period (May to August), mean final BW and NWG was maximum (157.04 and 136.40g) in GP15 and minimum (110.43 and 88.79 g) in GP20 (Table 13, Fig. 13 C-D). The difference for final BW and NWG were significant (GP15 > GP5= GP10 = GP0 > GP20). Likewise, SGR and PER of fish was significantly higher in GP15 (2.53, 2.00) and lower in GP20 (2.14, 1.41). In comparison to control (GP0), FCR improved in all the treatments except GP20 with maximum value of FCR (2.26) and the differences were significant (GP20 $\geq$ GP0 = GP10 = GP5 $\geq$ GP15) with minimum value in GP15 (1.55). Condition factor (K) was recorded highest in GP10 (1.38), followed by GP5 (1.35), GP20 (1.32), GP0 (1.27) and GP15 (1.16) respectively (Table 13, Fig. 14 A-D), however the differences were insignificant ($p \leq 0.05$) among all the treatments and control.

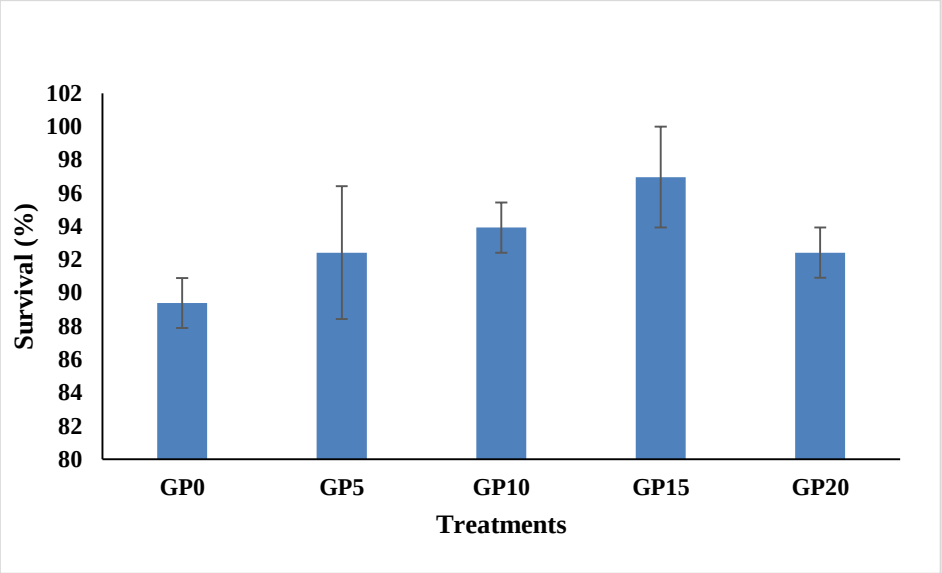


Figure 12. Survival (%) of *L. rohita* in different treatments after the completion of the experiment

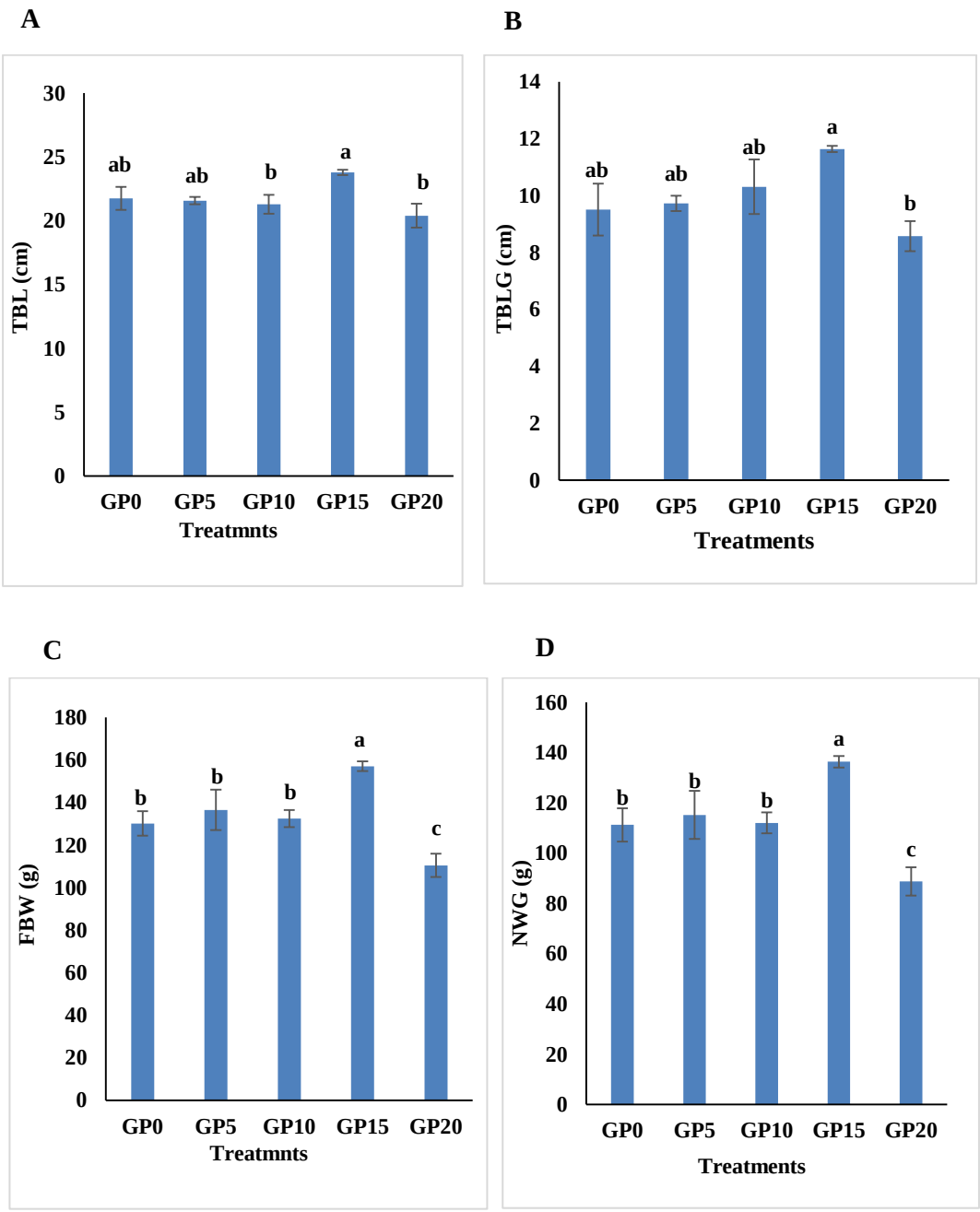


Figure 13. Changes in A) Total body length (TBL), B) Total body length gain (TBLG), C) Final body weight (FBW) and D) Net weight gain (NWG) of *L. rohita* in different treatments after the completion of the experiment

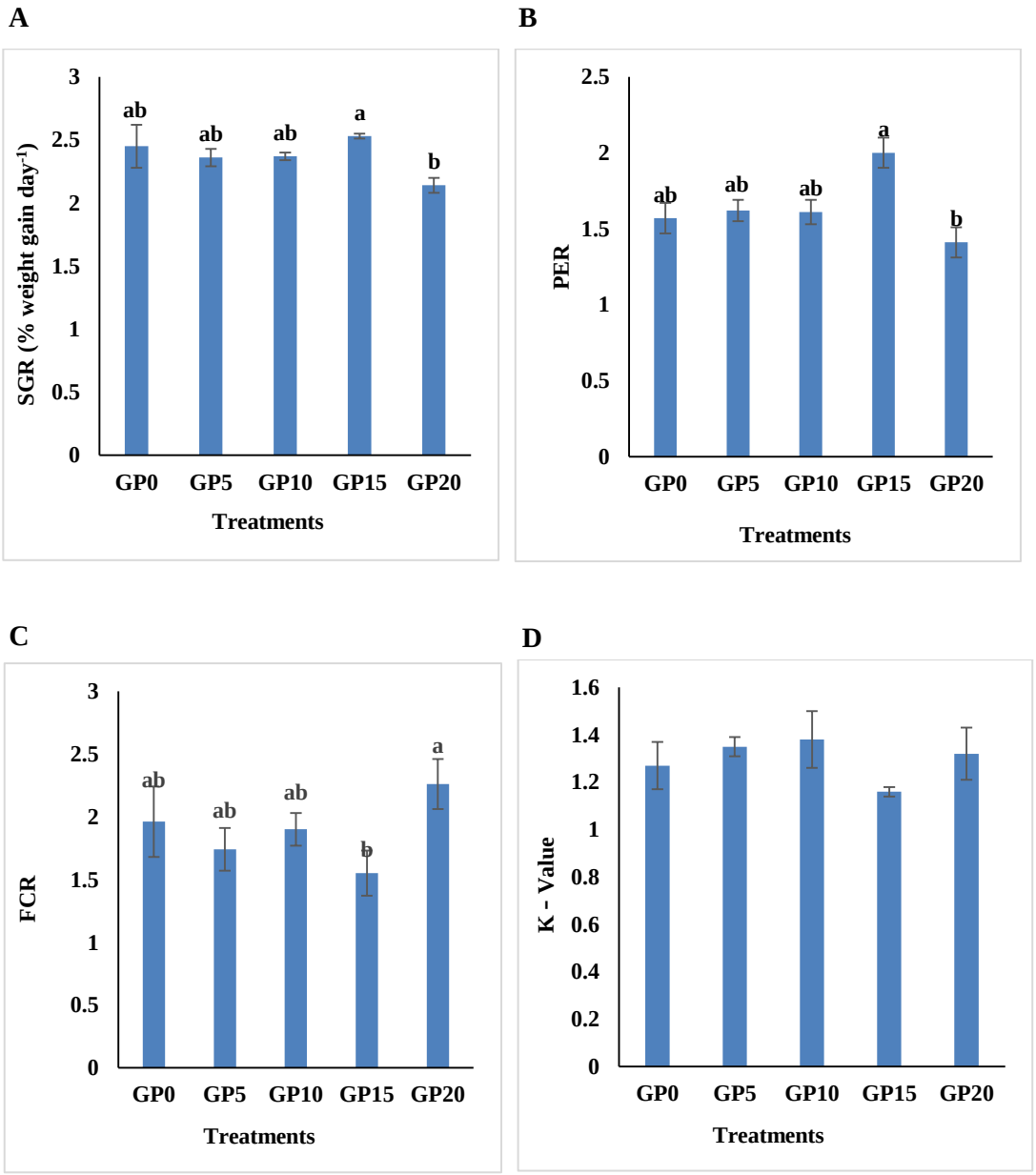


Figure 14. Changes in A) Specific Growth Rate (SGR), B) Protein Efficiency Ratio (PER), C) Feed Conversion Ratio (FCR) and D) Condition Factor (K - value) of *L. rohita* in different treatments after the completion of the experiment

The results of the present study revealed that feeding 1.5% ginger powder (15 g kg^{-1} GP0) significantly improved the growth performance of rohu, *L. rohita* in terms of final length, TBLG, FBW, NWG, SGR, FCR and PER. The enhanced fish growth in the present study may be explained as ginger might have stimulated the feeding behavior of rohu due to more palatability of experimental diets, which, must

have resulted in improved nutrient digestibility (Hassanin et al., 2014; Talpur et al., 2013). Moreover, the stimulated secretion of intestine protease enzyme in ginger fed rohu, might have resulted in improved digestion and absorption of protein components of the feed. Further, being rich source of proteinase, ginger must have enhanced in particular protein digestion and amino acid absorption in the gastrointestinal tract (Hashim et al., 2011). Most of the previous studies also revealed positive effect of ginger (powder/extract) supplementation on growth of several fish/shellfish species like *L. rohita* (Sukumaran et al., 2016), *C. carpio* (Jafarinejad et al., 2020), *O. mykiss* (Nya & Austin, 2009), *M. rosenbergii* (El-Desouky et al., 2012) and *L. calcarifer* (Talpur et al., 2013).

It has been observed by Immanuel et al. (2009) that ginger extract supplementation @ 1% enhanced the growth performance of *Oreochromis mossambicus* in terms of final weight gain and specific growth rate. Similarly, Ghadikolaei et al. (2017) too reported that 2g dietary ginger powder per 100 g of feed improved final weight and final length of *C. carpio*. Lee et al. (2020) reported that dietary inclusion of ginger (1%) for 8 weeks improved weight gain, specific growth rate and protein retention in black rockfish (*Sebastes schlegeli*), when compared to control (without ginger). Medicinal plants including ginger reported to have stimulating appetite characteristics, growth-promoting ability and tonic to improve the FCR, when they are administered to cultured fish (Reverter et al., 2014). Besides appetite stimulators, ginger and other plant extracts have been shown to improve digestive activity and metabolic processes (Nya & Austin, 2009), leading to improved protein and fat metabolism (Platel & Srinivasan, 2000). Additionally, ginger is reported to have positive effect on beneficial instestinal bacterial population, which aid in utilization of nutrients (Hoseini et al., 2018) and hence improved growth rate.

4.1.3 Hematological parameters

4.1.3.1 Red Blood Cells (RBC)

Among different treatments, mean RBC ($x10^6$ mm^{3-1}) was 1.44, 1.37, 1.59, 1.83 and 1.21 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period and the differences were significant among treatments (GP15 $\geq$ GP10 $\geq$ GP0 = GP5 $\geq$ GP20). At the completion of the experiment (after 120 days), mean RBC ($x10^6$ mm^{3-1}) was 1.69, 1.51, 1.64, 2.31 and 1.69, respectively (Table 14, Fig. 15-A) with significant improvement in RBC in GP15 as compared to all other

treatments and control. As compared to control (GP0), significant increase ($p \leq 0.05$) in RBC was observed in GP15 at day 60 and 120.

4.1.3.2 White Blood Cells (WBC)

Among different treatments, mean WBC (x 10^4 mm^{3-1}) was 5.21, 6.43, 5.65, 6.95 and 5.05 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period with significantly higher ($p \leq 0.05$) value of WBC in GP15 (6.95) and GP5 (6.43) as compared to other treatments and control. At the completion of the experiment, mean WBC (x 10^4 mm^{3-1}) was 5.70, 6.79, 5.86, 8.51 and 5.64 in GP0, GP5, GP10, GP15 and GP20 respectively (Table 14, Fig 15-B) with significantly higher ($p \leq 0.05$) value in GP15 followed by GP5 and the differences for WBC were insignificant for GP0, GP10 and GP20. As compared to control (GP0), significant increase ($p \leq 0.05$) in WBC was observed in GP5 and GP15 at day 60 and in GP15 at day 120.

4.1.3.3 Haemoglobin (Hb)

Among different treatments, mean Hb (g%) was 7.87, 8.03, 7.94, 8.33 and 7.46 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period and the differences for Hb were significant ($p \leq 0.05$) among different treatments (GP15 = GP5$\geq$ GP10 = GP0 $\geq$ GP20) with highest value in GP15. At the completion of the experiment, mean Hb (g%) was 8.11, 8.28, 8.44, 10.30 and 7.88 in GP0, GP5, GP10, GP15 and GP20 respectively (Table 14, Fig. 16-A) with significantly higher ($p \leq 0.05$) value in GP15 (10.30) as compared to all other treatments and control (GP0).

4.1.3.4 Hematocrit or Packed Cell Volume (Ht or PCV)

Among different treatments, mean Ht/PCV (%) was 19.20, 33.83, 27.40, 28.06 and 19.96 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period. Mean Ht improved significantly in all the treatments as compared to control except in GP20. At the completion of the experiment, mean Ht (%) was 23.30, 32.76, 33.30, 41.06 and 21.63 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 14, Fig. 16-B) with significant improvement in GP15, followed by GP10 and GP5 as compared to GP20 and control (GP0). As compared to control (GP0), significant increase ($p \leq 0.05$) in Ht was observed in GP5, GP10 and GP15 at day 60 and in GP15 at day 120.

Table 14. Haematological parameters of *L. rohita* in different treatments after 60 and 120 days of the experimental period

Parameters	Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
RBC (x 10^6 mm^{3-1})	60	1.44±0.07^{bc}	1.37±0.10^{bc}	1.59±0.05^{ab}	1.83±0.09^{a}	1.21±0.09^{c}
	120	1.69±0.06^{b}	1.51±0.05^{b}	1.64±0.05^{b}	2.31±0.12^{a}	1.69±0.06^{b}
WBC (x 10^4 mm^{3-1})	60	5.21±0.15^{b}	6.43±0.22^{a}	5.65±0.18^{b}	6.95±0.27^{a}	5.05±0.03^{b}
	120	5.70±0.11^{c}	6.79±0.09^{b}	5.86±0.23^{c}	8.51±0.25^{a}	5.64±0.11^{c}
Hb (g %)	60	7.87±0.23^{ab}	8.03±0.10^{a}	7.94±0.22^{ab}	8.33±0.09^{a}	7.46±0.08^{b}
	120	8.11±0.30^{b}	8.28±0.10^{b}	8.44±0.33^{b}	10.30±0.52^{a}	7.88±0.14^{b}
Ht/PCV (%)	60	19.20±0.49^{b}	33.83±3.99^{a}	27.40±1.60^{a}	28.06±0.78^{a}	19.96±0.95^{b}
	120	23.30±0.98^{c}	32.76±1.11^{b}	33.30±1.90^{b}	41.06±0.53^{a}	21.63±0.95^{c}
MCV (µ m^3)	60	133.09±3.92^{b}	246.76±19.30^{a}	172.70±14.00^{b}	154.43±10.60^{b}	167.90±19.40^{b}
	120	138.11±5.94^{c}	216.10±1.26^{a}	202.41±6.28^{ab}	178.44±11.30^{b}	128.69±9.80^{c}
MCH (g %)	60	54.56±4.66^{ab}	59.21±3.89^{a}	50.02±2.95^{ab}	45.79±2.67^{b}	62.33±4.19^{a}
	120	48.28±3.70^{ab}	54.81±2.40^{a}	51.37±1.13^{ab}	44.62±2.77^{b}	46.75±1.55^{ab}
MCHC (%)	60	40.87±2.33^{a}	24.33±2.60^{b}	29.11±1.36^{b}	29.70±0.50^{b}	37.59±2.08^{a}
	120	35.01±2.57^{a}	25.35±1.06^{b}	25.40±0.43^{b}	25.11±1.55^{b}	36.55±1.48^{a}

*See Table 7 for legends

4.1.3.5 Mean Corpuscular Volume (MCV)

Among different treatments, mean MCV (μm^3) was 133.09, 246.76, 172.70, 154.43 and 167.90 in GP0, GP5, GP10, GP15 and GP20, respectively after 60 days of culture period with significantly higher value ($p \leq 0.05$) in GP5 as compared to all other treatments and control. At the completion of the experiment, MCV (μm^3) was 138.11, 216.10, 202.41, 178.44 and 128.69 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 14, Fig. 17-A) with significantly ($p \leq 0.05$) higher values in GP5, followed by GP10 and GP15 as compared to control (GP0) and GP20. Significantly higher ($p \leq 0.05$) MCV was observed in GP5 at day 60 and 120.

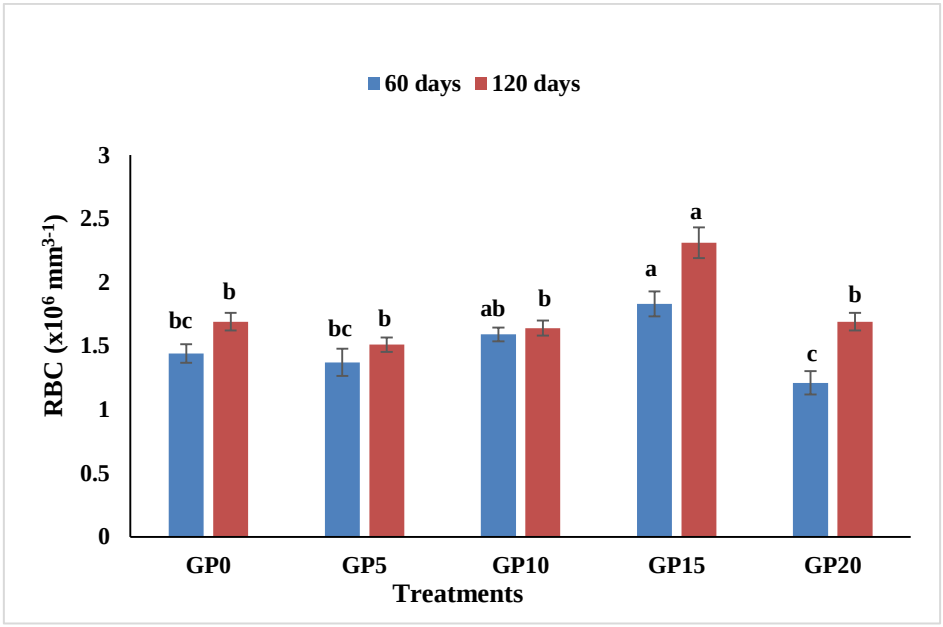
4.1.3.6 Mean Corpuscular Haemoglobin (MCH)

Among different treatments, mean MCH (g%) was 54.56, 59.21, 50.02, 45.79 and 62.33 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period and the differences for MCH were significant ($p \leq 0.05$) among different treatments (GP20 = GP5 $\geq$ GP0 = GP10 $\geq$ GP15). At the completion of the experiment, mean MCH (g %) was 48.28, 54.81, 51.37, 44.62 and 46.75 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 14, Fig. 17-B) with significantly ($p \leq 0.05$) higher values in GP5 as compared to GP15 and insignificantly from all other treatments and control (GP5 $\geq$ GP10 = GP0 = GP20 $\geq$ GP15). Significantly higher value of MCH was observed in GP20 at day 60 and in GP5 at day 120.

4.1.3.7 Mean Corpuscular Haemoglobin Content (MCHC)

Among different treatments, mean MCHC (%) was 40.87, 24.33, 29.11, 29.70 and 37.59 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period and the differences were significant for MCHC with significantly higher ($p \leq 0.05$) values in GP0 and GP20 as compared to all other treatments. At the completion of the experiment, MCHC (%) was 35.01, 25.35, 25.40, 25.11 and 36.55 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 14, Fig. 17-C) with significantly higher values in GP20 and GP0 as compared to other treatments. Significantly higher ($p \leq 0.05$) value of MCHC was observed in GP0 and GP20 at day 60 and day 120.

A



B

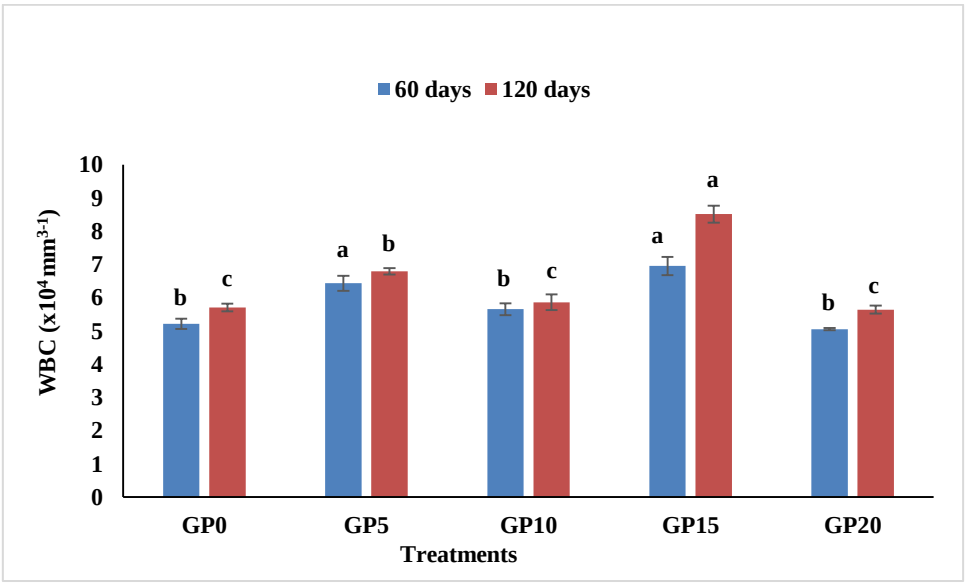
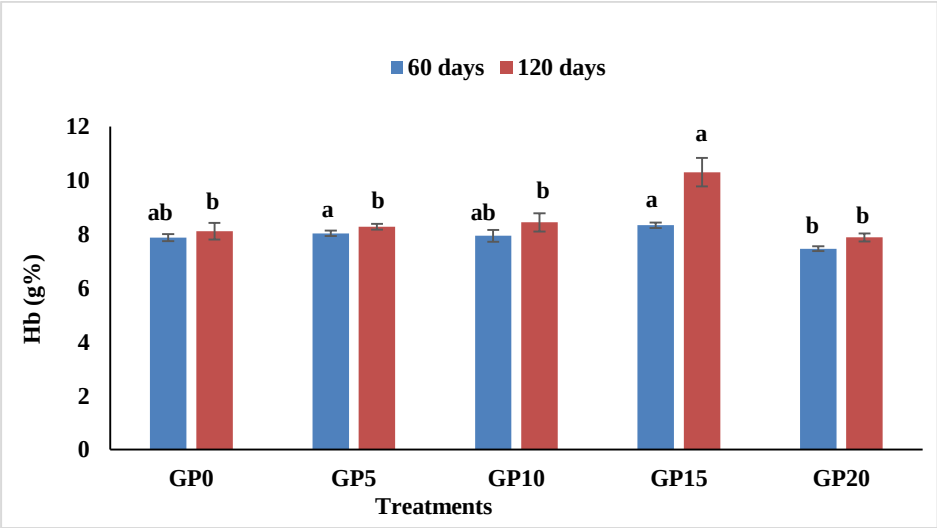


Figure 15. Comparative A) Red Blood Cells (x10^{6} mm$^{3-1}$) and B) White Blood Cells (x10^{4} mm$^{3-1}$) of *L. rohita* in different treatments after 60 and 120 days of the experiment

A



B

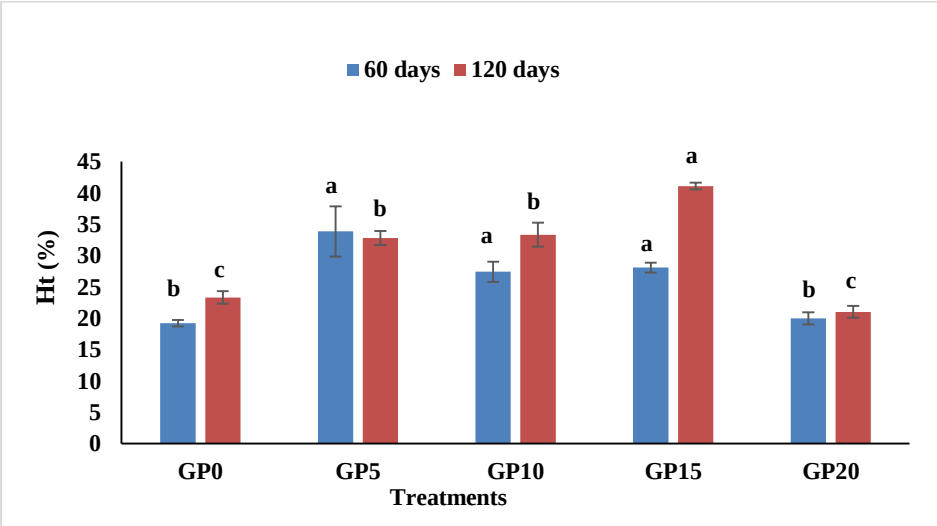


Figure 16. Comparative A) Hemoglobin (g %) and B) Hematoctrit (%) of *L. rohita* in different treatments after 60 and 120 days of the experiment

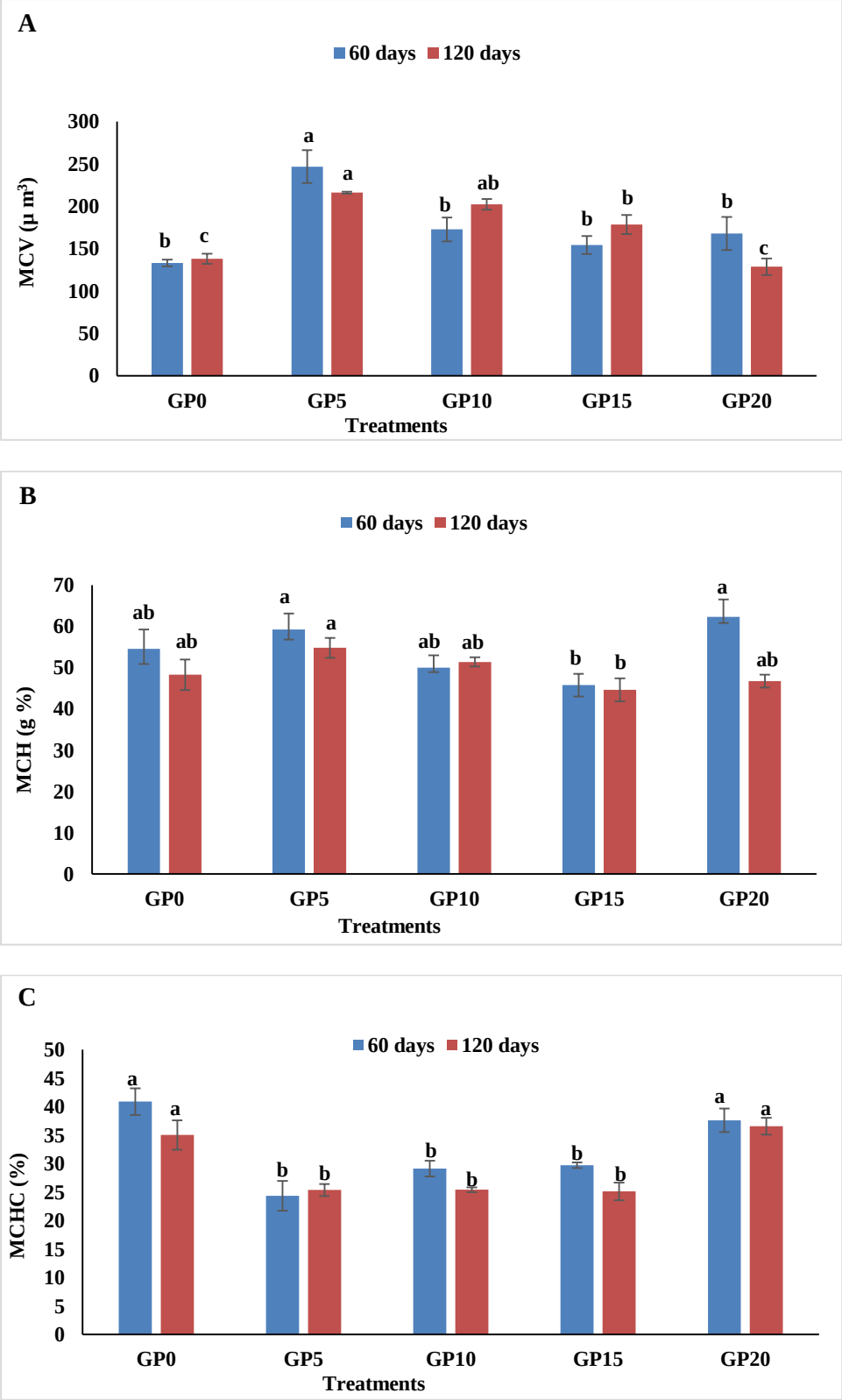


Figure 17. Comparative A) MCV (µ m^3), B) MCH (g %) and C) MCHC (%) of *L. rohita* in different treatments after 60 and 120 days of the experiment

The results of present study revealed improved haematological parameters w.r.t. RBC, WBC, Hb and Ht of rohu fed with 1 (GP10) and 1.5 % (GP15) ginger powder as compared to GP0 (without ginger supplementation).

Hematological parameters are important physiological indicator in prognosis of any problem in fish, and are affected by a range of factors including species, size, age, diets, physiological status and environmental conditions (Parma et al., 2007). In the present study, dietary factors played an important role, as fish fed with 1.5 % ginger powder supplemented diet showed significantly higher values of all the blood parameters. The Hb content in the blood plays a vital role and serves as transportation element of oxygen to body tissues. The increase in Hb level may be attributed due to increased synthesis of enzymes required either for biosynthesis of them (El-Tahir et al., 1993) or increase in size of RBC (El-Feki et al., 1993), which inturn could enhance oxygen supply. The improved Hb content in GP15 as compared to GP0, demonstrates increased oxygen supply consequently, reflecting beneficial health effect on fish. Vahedi et al., (2017) also reported beluga fed with diet supplemented with ginger (1.5%) showed higher values of Hb and WBC compare to control. On the contrary, Gholipour kanani et al., (2014) found that the level of Hb was not affected by ginger supplementation in the basal diet of juvenile beluga.

In previous studies, it has been reported that immunostimulant herbal plants could increase immune functions by affecting the blood cells (Talpur et al., 2013; Sahu et al., 2007a). In the present study too, increased RBC count in GP15 exhibitited the positive effect on fish health, because of bioactive compounds in ginger. The findings of this study are also comparable to those of Nya & Austin (2009), who reported that the number of RBC count was significantly ($p \leq 0.05$) higher in rainbow trout after feeding with ginger (1g $100g^{-1}$) supplemented diet. The WBC (leucocytes) serves as one of the first lines of body defence and their numbers increase sharply when infections arise. The increase in WBC counts along with other hematological parameters following feeding of ginger diet might reveal the immunostimulatory effects and anti-infection properties of ginger (Nya & Austin, 2009).

The hematocrit index is the volumetric percentage of erythrocytes in systemic circulation and depends on the number and size of erythrocytes. Further, the higher Ht level also reveal blood's ability to transport oxygen, which in turns indicate healthy

condition of fish (Birchard, 1997). The increased number and size of erythrocytes or Hb in the blood thus is accompanied by elevated hematocrit in GP15. The beneficial effects of ginger supplementation on RBC, Hb, and Ht have also been reported in previous studies on *Lates calcarifer* (Talpur et al., 2013) and *O. mykiss* (Nya & Austin, 2009). Variations in hematological indices (MCV, MCH and MCHC) are in accordance with the hematological parameters i.e. RBC, Hb and Ht.

4.1.4 Non-specific immune responses

Non specific immune responses play an important role to combat with infections and diseases at primary stages in fishes and help to overcome the stresses. The non-specific immune parameters i.e. Respitary Burst Activity (RBA), Lysozyme activity and Total Immunoglobulins of blood serum were assesed after 60 and 120 day of feeding with different levels of ginger powder.

4.1.4.1 Respiratory burst activity (RBA)

Among different treatments mean RBA (OD at 620nm) was 0.10, 0.12, 0.28, 0.26 and 0.24 in GP0, GP5, GP10, GP15 and GP20, respectively after 60 days of culture period. At the completion of the experiment, mean RBA was 0.14, 0.15, 0.27, 0.31 and 0.26 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 15, Fig. 18-A). The RBA improved in all the treatments except GP5 as compared to control after 60 day (GP10 = GP15 > GP20 > GP5 = GP0) and 120 day (GP15 = GP10 = GP20 > GP5 = GP0). As compared to control (GP0), significantly highest ($p \leq 0.05$) RBA was observed in GP10 and GP15 at day 60 and 120, respectively.

Respiratory bursts are produced by phagocytes in order to attack invasive pathogens during phagocytosis and have been widely used to evaluate the defense ability against pathogens. However, the excessive accumulation of reactive oxygen intermediates (ROIs) is extremely toxic to host cells (Dalmo et al., 1997). The present study revealed that feeding ginger to rohu significantly enhanced the immune responses in terms of RBA. Although RBA was significantly higher in all the treatments except GP5 as compared to control, however maximum value of RBA was reported in GP10 and GP15 at day 60 and 120 respectively, however there was no significant differences in both the treatments. The superoxide anion produced during the respiratory burst is important in the bactericidal activity of macrophages (Secombes & Olivier, 1997). The higher respiratory burst activity suggests higher

production of microbicidal reactive oxygen (ROS) and free radicals without adverse effects on host cells and tissues. Ginger seemed to be the more effective stimulator of ROS, while at the same time promoter of antioxidant defences. The immunomodulatory effects of ginger in rohu can presumably be attributed to its antioxidant scavenging properties (Apines-Amar et al., 2012). The results of this study are in consistent with the findings of Dugenci et al. (2003), who reported that extracellular and intracellular respiratory burst activity of leukocytes were enhanced by feeding with aqueous extract ginger (1%) in rainbow trout. Similarly, Nya & Austin (2009) too demonstrated significant increase in the RBA of rainbow trout, when ginger powder was fed @ 0.1%. Talpur et al. (2013) reported a significant increase in the respiratory burst in sea bass fed with ginger diet as compared to control with the highest level in fish fed with ginger @ 3 g kg^{-1} feed.

Table 15. Non-specific immune responses of *L. rohita* in different treatments after 60 and 120 days of the experimental period

Parameters	Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
RBA (OD at 620 nm)	60	0.10 ± 0.01^{c}	0.12 ± 0.01^{c}	0.28 ± 0.00^{a}	0.26 ± 0.00^{a}	0.24 ± 0.00^{b}
	120	0.14 ± 0.02^{b}	0.15 ± 0.02^{b}	0.27 ± 0.01^{a}	0.31 ± 0.01^{a}	0.26 ± 0.01^{a}
Lysozyme activity (U min^{-1} mg^{-1} $protein^{-1}$)	60	0.01 ± 0.0007^{b}	0.02 ± 0.002^{b}	0.06 ± 0.003^{a}	0.04 ± 0.001^{ab}	0.02 ± 0.001^{b}
	120	0.01 ± 0.002^{c}	0.03 ± 0.005^{bc}	0.05 ± 0.006^{ab}	0.07 ± 0.006^{a}	0.04 ± 0.001^{abc}
Total Ig (gdl^{-1})	60	0.58 ± 0.01^{b}	0.63 ± 0.03^{b}	0.71 ± 0.08^{a}	0.76 ± 0.05^{a}	0.60 ± 0.04^{b}
	120	0.64 ± 0.03^{c}	0.73 ± 0.04^{bc}	0.86 ± 0.07^{b}	1.04 ± 0.07^{a}	0.65 ± 0.008^{c}

*See Table 7 for legends

4.1.4.2 Lysosyme activity

Among different treatments mean lysozyme (U min^{-1} mg^{-1} $protein^{-1}$) activity was 0.01, 0.02, 0.06, 0.04 and 0.02 in GP0, GP5, GP10, GP15 and GP20, respectively after 60 days of culture period with significant improved lysosyme activity in GP10 and GP15. At the completion of the experiment, mean lysozyme was 0.01, 0.03, 0.05, 0.07 and 0.04 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 15, Fig. 18-B). The lysozyme activity improved in all the treatments with significant ($p\leq0.05$) improvement in GP15 after 120 days. As compared to control (GP0), significantly

highest ($p \leq 0.05$) value of lysosyme activity was observed in GP10 at day 60 and in GP15 at day 120.

Lysozyme serve as the first line of defense, when a pathogen enters the body of a host animal. It is an important enzyme in the blood that actively lyses bacterial cell wall peptidoglycans and is also known to act as opsonin, because it activate the complement system and phagocytes (Magnadottir, 2006). Lysozyme prevent adhesion and colonization of bacterial pathogens, thus resulting in the reduction of disease (Misra et al., 2004) and this might be the probable reason that at the end of the present study, lysozyme activitiy showed significant increase in all the ginger fed groups compared to control. The results of present study are consistent with the findings of Haghighi & Rohani (2013) and Talpur et al. (2013), in which dietary ginger significantly affected serum lysozyme activity in *L. calcrifer* and *O. mykiss* respectively, when fed with 1% ginger in both the studies. Likewise, Vahedi et al. (2017) too demonstrated significant ($p \leq 0.05$) increased lysozymal activity, when ginger extract was fed to *Huso huso* @ 1.5%. Administration of ginger @ 2% in *Epinephelus fuscoguttatus*, led to an increased lysozyme activity (Apines-Amar et al., 2012). Similarly, Antache et al. (2014) too observed 1% ginger incorporation increased lysozyme activity of *O. niloticus*, but there was no significant difference among the treatments. Hassanin et al. (2014) demonstrated that lysozyme activity of nile tilapia was significantly increased in ginger (1%) fed group compared to control. In contrary, lysozyme activity was not influenced in juvenile beluga fed with ginger (Gholipour kanani et al., 2014).

4.1.4.3 Total Immunoglobulins (Total Ig)

Among different treatments mean Total Ig (gdl^{-1}) was 0.58, 0.63, 0.71, 0.76 and 0.60 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 days of culture period and there was significant improvement in GP10 and GP15 as compared to control and other treatments. At the completion of the experiment, mean Total Ig was 0.64, 0.73, 0.86, 1.04 and 0.65 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 15, Fig. 18-C) and the differences were significant ($p \leq 0.05$) among different treatments (GP15 > GP10 $\geq$ GP5 $\geq$ GP20 = GP0). Although at day 60, significantly highest ($p \leq 0.05$) value of Total Ig was observed in GP10 and GP15, however, the maximum value was reported in GP15. At day 120, GP15 revealed significantly higher Total Ig as compared to all the treatments and control (GP0).

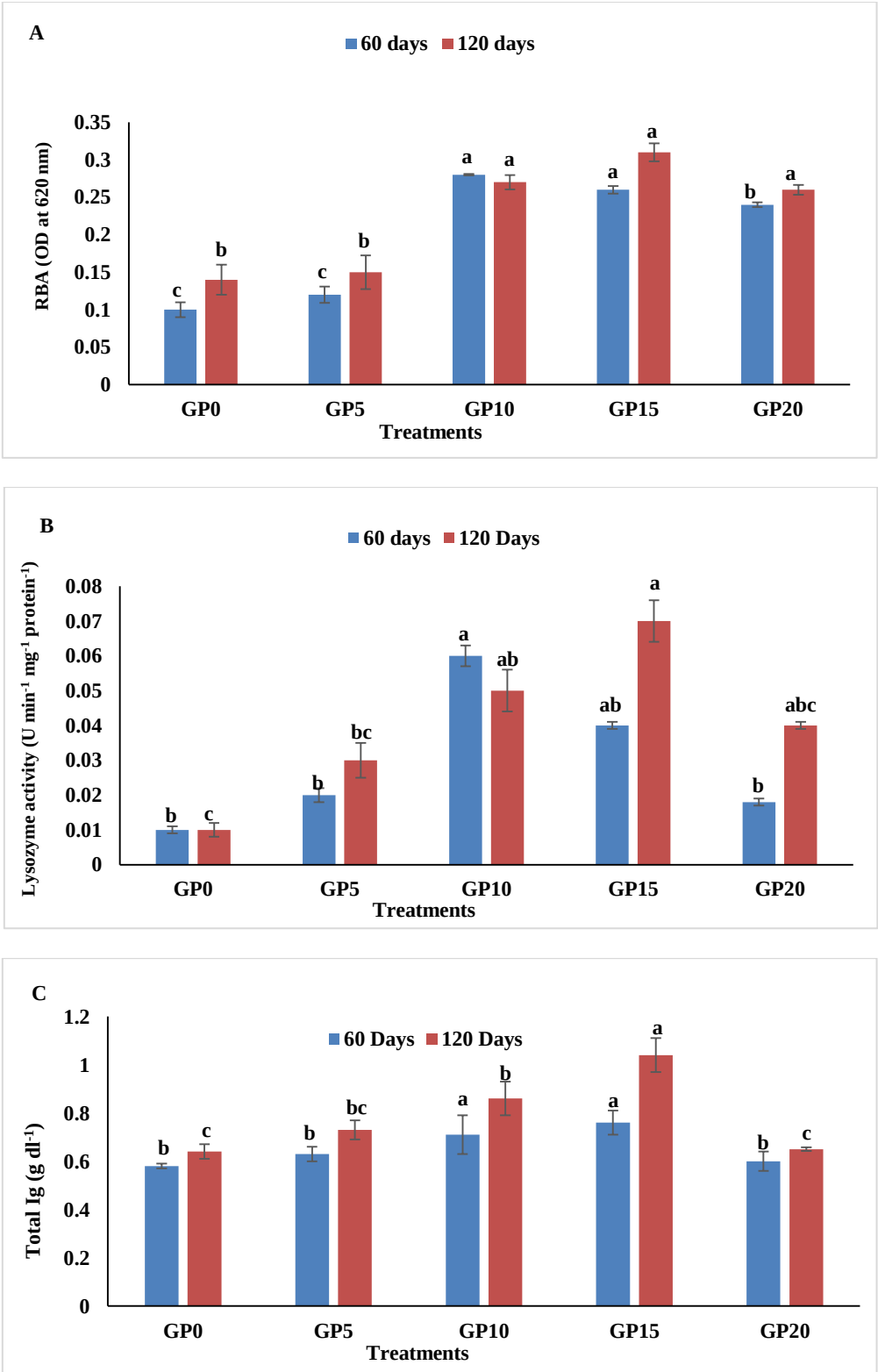


Figure 18. Comparative A) Respiratory burst activity (OD at 620 nm), B) Lysozyme activity (U min^{-1} mg^{-1} $protein^{-1}$) and C) Total Ig (g dl^{-1}) of *L. rohita* in different treatments after 60 and 120 days of the experiment

After 60 and 120 days of feeding trial, the increased total Ig content in all the treatments as compared to GP0 with significant improvement in GP10 and GP15 suggested enhanced immune system. Further, all the non specific parameters follows the same trend, i.e., significant enhancement in GP10 and GP15, after 60 days feeding and in GP15 after 120 days feeding. This clearly indicated that ginger @ 1.5% showed its positive effect in fish after prolonged feeding (120 days). Natural Igs are considered as components of the innate immune system, since they are produced without any apparent antigenic stimulation, found in the serum of healthy vertebrates and are polyreactive showing reactivity for non-self associated molecular patterns like LPS, viral and parasitic products. Fish fed with different level of ginger exhibited significantly enhanced level of total Ig levels in the present study revealed better immunocompetence (Apines-Amar et al., 2012). These results coincide with the investigations of Akrami et al. (2015) and Binaii et al. (2014), who reported enhanced levels of serum Ig level in beluga after feeding with nettle and onion powder respectively. Similarly, Sukumaran et al. (2016) demonstrated that total Ig level was significantly higher in rohu, when fed with 0.8% ginger powder. Hassanin et al. (2014) reported that IgM level of *O. nilioticus* significantly improved with supplementation of the ginger @ 1%. However, Vahedi et al. (2017) reported maximum value of serum total Ig in beluga juveniles, when fed with dietary ginger extract @ 1.5%, but there were no significant differences among the treatments. Ginger is reported to have broad-spectrum activities including activation of phagocytic cells, which is an important component of non specific immune system of fish (MacArthur & Fletcher, 1985). Further, the possible mode of action of ginger as immunostimulant is due to the bioactive component i.e gingerol, which is reported to induce activity of interleukin-6 (IL-6) (Benny et al., 2004).

4.1.5 Blood metabolic profile

4.1.5.1 Lipid profile

Lipid profile was estimated in terms of High density lipids (HDL), Cholesterol and Triglycerides (mg dl^{-1}) in the blood serum of fish fed with/without ginger after completion of experiment (120 days).

4.1.5.1.1 High density lipids (HDL)

Among different treatments, mean HDL (mg dl^{-1}) in blood serum was 30.60, 34.86, 42.81, 54.92 and 39.85 in GP0, GP5, GP10, GP15 and GP20,

respectively with significantly higher ($p \leq 0.05$) value in GP15 (GP15 > GP10 $\geq$ GP20 $\geq$ GP5 $\geq$ GP0) as compared to control and other treatments (Table 16, Fig. 19-A).

4.1.5.1.2 Triglycerides (TG)

Among different treatments, mean triglycerides (mg dl^{-1}) in blood serum was 53.77, 46.58, 48.04, 37.11 and 42.58 in GP0, GP5, GP10, GP15 and GP20, respectively with significantly lowest ($p \leq 0.05$) values in GP15 (GP0 $\geq$ GP10 = GP5 = GP20 > GP15) as compared to control and other treatments (Table 16, Fig. 19-B).

4.1.5.1.3 Cholesterol

Among different treatments, mean Cholesterol (mg dl^{-1}) in blood serum was 65.93, 62.82, 66.07, 61.94 and 67.41 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 16, Fig. 19-C) with insignificant difference among treatments. However, among the treatments and control, lowest value of cholesterol was observed in GP15 (61.94) and highest in GP20 (67.41).

In the present study, maximum value of triglycerides and cholesterol was reported in GP0 (control) and minimum in GP15 as compared to all the treatments, however the differences were insignificant for cholesterol and significant for triglycerides (minimum value in GP15) among control and treatments. The results can be explained, due to the fact that ginger possesses blood thinning properties (Verma et al., 1993) and moreover due to presence of one of the bioactive compound saponin, ginger is capable of improving hyperlipidemia (Talpur et al., 2013). The results of this study are in accordance with of Vahedi et al. (2017), who reported significantly ($p \leq 0.05$) lowest triglycerides in beluga when fed with 1.5% ginger supplemented diets.

In the present study, HDL content was also found to be significantly high in all the treatments (ginger powder supplemented diets) compared to control (GP0). Improved HDL indicated fish to be in good health, as HDL cholesterol is known as the good cholesterol, because it helps in removal of other forms of cholesterol from bloodstream, hence the higher levels of HDL cholesterol are associated with a lower risk of heart disease by lowering triglyceride levels.

Table 16. Blood metabolic profile of *L. rohita* in different treatments at the completion of the experimental period

Parameter	Treatments*				
	GP0	**GP5**	**GP10**	**GP15**	**GP20**
Lipid Profile					
HDL (mg dl^{-1})	30.60 ± 1.77^{d}	34.86 ± 1.40^{cd}	42.81 ± 2.58^{b}	54.92 ± 2.06^{a}	39.85 ± 2.74^{bc}
Triglycerides (mg dl^{-1})	53.77 ± 1.23^{a}	46.58 ± 3.84^{ab}	48.04 ± 3.00^{ab}	37.11 ± 3.54^{c}	42.58 ± 3.60^{ab}
Cholesterol (mg dl^{-1})	65.93±0.71	62.82±2.92	66.07±5.62	61.94±3.28	67.41±8.85
Antioxidant Parameters					
SOD (U mg^{-1}Hb)	0.36 ± 0.01^{c}	0.48 ± 0.12^{bc}	0.79 ± 0.10^{ab}	1.04 ± 0.10^{a}	0.66 ± 0.14^{bc}
LPO (nmol MDA G Hb^{-1})	3.38 ± 0.34^{a}	2.20 ± 0.09^{b}	1.76 ± 0.08^{b}	0.57 ± 0.15^{c}	2.12 ± 0.34^{b}
Liver Profile					
ALT /SGPT (IU l^{-1})	17.72 ± 1.02^{a}	15.91 ± 0.01^{ab}	16.50 ± 0.59^{ab}	14.14 ± 1.01^{b}	15.32 ± 0.59^{ab}
AST/SGOT (IU l^{-1})	3.53 ± 0.01^{ab}	4.71 ± 0.58^{a}	3.53 ± 0.02^{ab}	2.36 ± 0.59^{b}	3.54 ± 0.02^{ab}

*See Table 7 for legends
Values are Mean ± S.E., ($p\leq0.05$), n=3
Values with same superscript (a, b,…..d in a row do not differ significantly ($p\leq0.05$)
SOD = Superoxide dismutase, LPO = Lipid peroxidase, AST = Aspartate transaminase, ALT = Alanine transaminase, HDL = High denity lipid

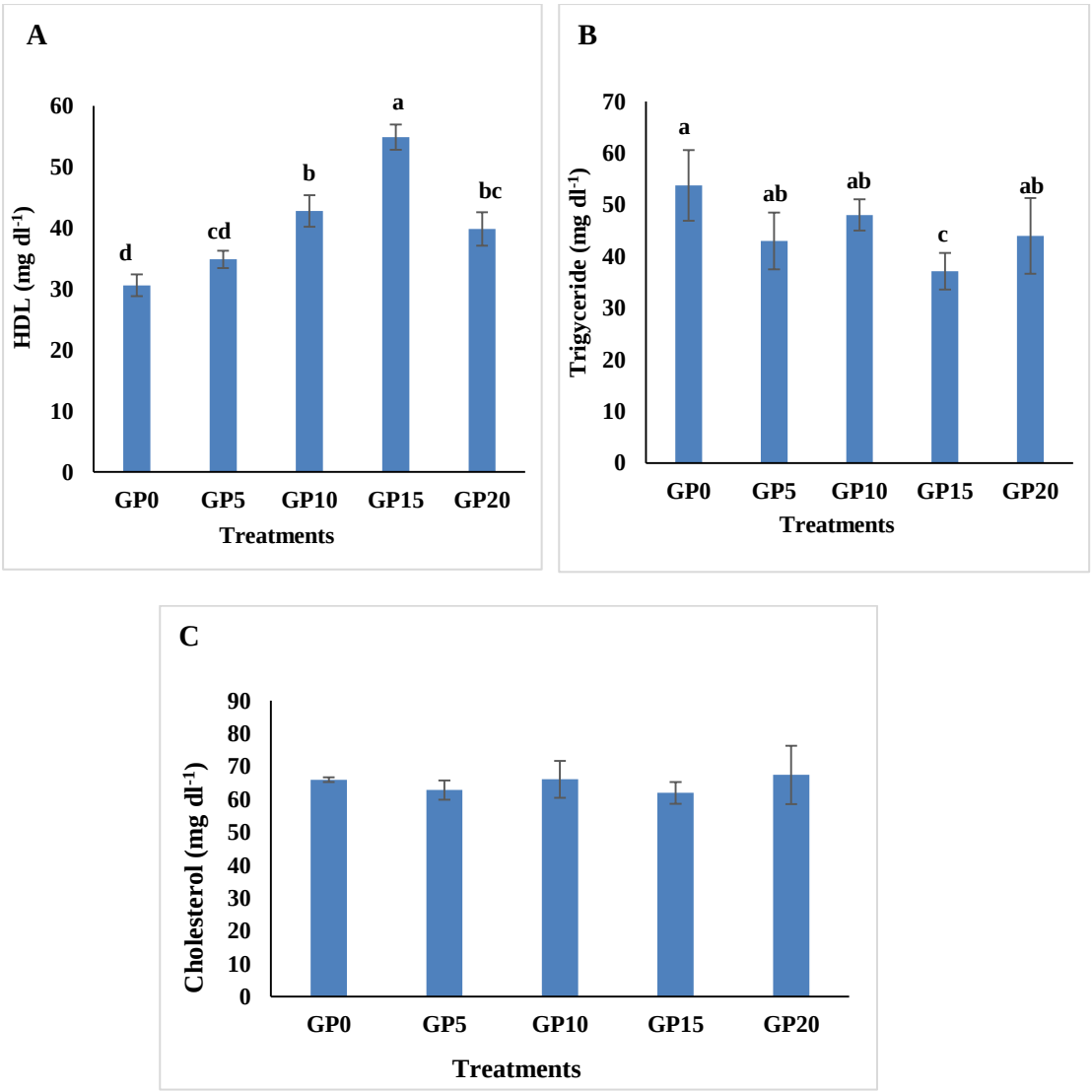


Figure 19. Comparative A) HDL (mg dl^{-1}), B) Triglycerides (mg dl^{-1}) and C) Cholesterol (mg dl^{-1}) in blood serum of *L. rohita* in different treatments at the completion of the experiment

4.1.5.2 Antioxidant Parameters

Antioxidant parameters in haemolysate of *L. rohita* were analyzed at the completion of the experiment (120 days) in terms of superoxide dismutase (SOD) and lipid peroxidation (LPO).

4.1.5.2.1 Superoxide dismutase (SOD)

Among different treatments, mean SOD (Umg Hb^{-1}) activity in haemolysate was 0.36, 0.48, 0.79, 1.04 and 0.66 in GP0, GP5, GP10, GP15 and GP20, respectively with significant difference among treatments (GP15 $\geq$ GP10 $\geq$

GP20 = GP5 ≥ GP0). As compared to control (GP0), significantly highest value ($p \leq 0.05$) of SOD (1.04) was observed in GP15 (Table 16, Fig. 20-A).

4.1.5.2.2 Lipid peroxidation (LPO)

Among different treatments, mean LPO (nmol MDA G Hb^{-1}) activity in haemolysate was 3.38, 2.20, 1.76, 0.57 and 2.12 in GP0, GP5, GP10, GP15 and GP20, repectively with significant difference among treatments (GP0 > GP5 = GP20 = GP10 > GP15). As compared to control (GP0), significantly lowest ($p \leq 0.05$) LPO (0.57) activity was observed in GP15 (Table 16, Fig. 20-B).

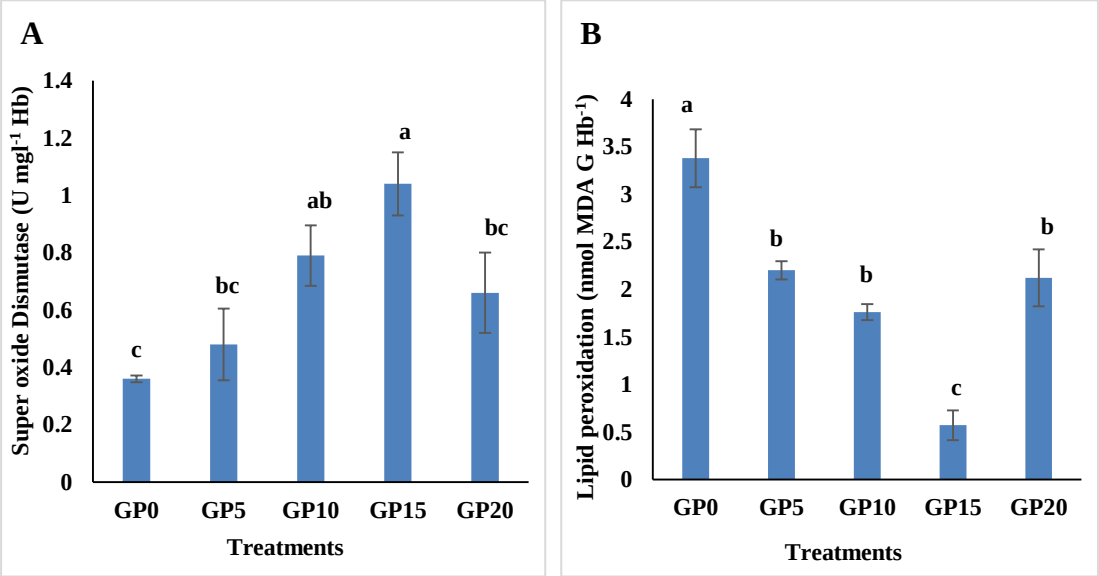


Figure 20. Comparative A) Superoxide Dismutase (U mg Hb^{-1}) and B) Lipid Peroxidation (nmol MDA G Hb^{-1}) in blood serum of *L. rohita* in different treatments at the completion of the experiment

SOD is metalloenzymes that play major role in protection of cells against oxidative damage (Metaxa et al., 2006). In the present study, significant differences in SOD level were observed among treatments and control with significant ($P \leq 0.05$) highest value of SOD in treatment, in which fish was fed with 1.5% ginger powder (GP15) compare to control (GP0). This can be explained as, in general, stress causes oxidative damage to cells, which overcomes that effect through the increased activity of antioxidative enzymes (SOD). It possibly indicates a role in O_2 scavenging (Shivendra et al., 2011; Parihar et al., 1997), indicating the defence response of ginger through the increased activity of SOD. Similarly, results of Jahanjoo et al. (2018) showed high activity of SOD in tilapia, when fed with diets supplemented with 1%

ginger. It has been reported by Mohammadi et al. (2020) that 0.2% ginger extract can effectively improved the SOD activity in common carp. However, unlike this study, Yuan et al. (2007) noticed that there was no significant difference in SOD activity between the 0.5% and 1% herbal immune regulation mixture groups and the control group.

Malondialdehyde (MDA), which is one of the end products of lipid peroxidation caused by oxygen free radicals, is an important oxidative stress indicator, which shows the degree of lipid peroxidation. In the present study, there was a significant decrease in lipid peroxidation level in ginger fed fish as compared to control (GP0). This might be due to the fact that ginger is considered as potent antioxidant agent, which prevents free radicals generation (Kim et al., 2007). On the other hand, increase in MDA level is one of the important indicators of the damage occurred in the body at the cellular level (Yagi, 1984). Phenolic compounds present in ginger (gingerols, shogaols, volatile oils, flavonoids, and phenolic ketone derivatives) encourage antioxidant activity against free radicals and prevent lipid peroxidation (Lebda et al., 2012). The results are in accordance with the studies conducted by Mahmoud et al. (2019) and Sahan et al. (2016) in nile tilapia, where ginger powder resulted in significantly decreased level of LPO @ 1.5% and 1 % respectively.

4.1.5.3 Liver Profile

Liver profile was studied in blood serum of rohu in terms of ALT and AST enzymes activities at the completion (120 days) of experiment.

4.1.5.3.1 Alanine aminotransferase (ALT)

Among different treatments, mean ALT (IU l^{-1}) of fish was 17.72, 15.91, 16.50, 14.14 and 15.32 in GP0, GP5, GP10, GP15 and GP20, respectively with significant ($p \leq 0.05$) decrease value of ALT in GP15 as compared to control and insignificant differences with other treatments (Table 16, Fig. 21-A).

4.1.5.3.2 Aspartate aminotransferase (AST)

Among different treatments, mean AST (IU l^{-1}) of fish was 3.53, 4.71, 3.53, 2.36 and 3.54 in GP0, GP5, GP10, GP15 and GP20, respectively with significant ($p \leq 0.05$) decrease in GP15 as compared to GP5 and insignificant differences among other treatments and control (Table 16, Fig. 21-B).

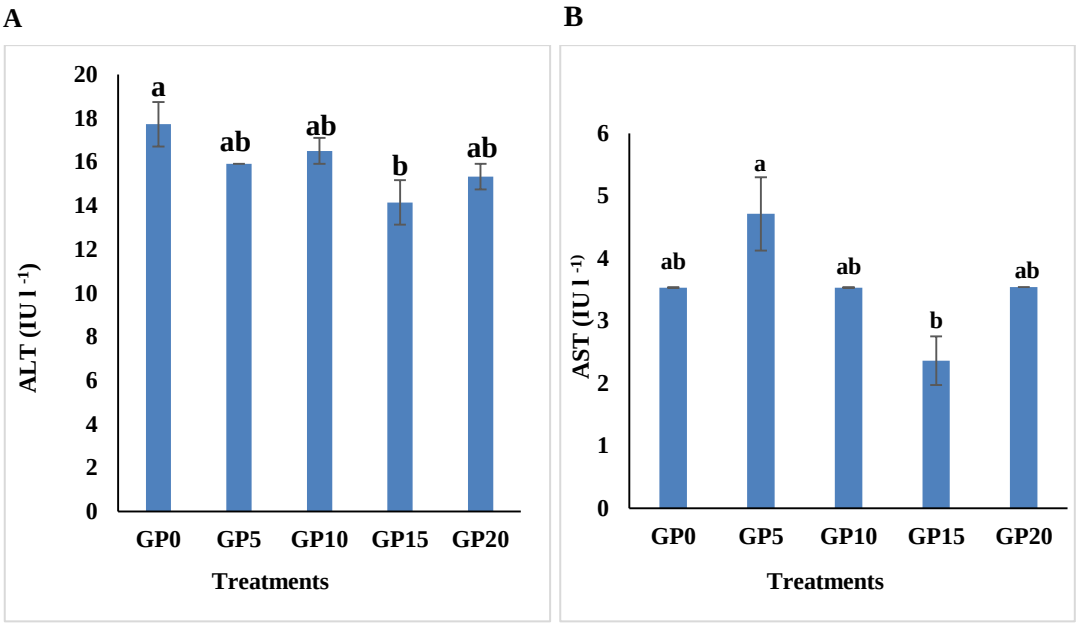


Figure 21. Comparative A) Alanine aminotransferase (IU l^{-1}) and B) Aspartate aminotransferase (IU l^{-1}) in blood serum of *L. rohita* in different treatment at the completion of experiment

Liver enzymes viz. ALT and AST are vital indicators of immune system and hence overall fish health. Elevated levels of these enzymes may indicate degeneration, necrosis, and destruction of the liver due to cellular damage (Bhardwaj et al., 2010). In the present study, significantly lower activity of ALT and AST were found in GP15 (1.5% GP supplementation), which might be due to the presence of bioactive compounds (polyphenols, flavonoids, tannins and saponins) of ginger, and they must have protected the fish from infection by triggering immune system along with prevention of lipid peroxidation of cell membranes and inhibited the release of foresaid enzymes into the plasma. The results of this study are in accordance with the findings of Vahedi et al. (2017), who reported lowering of liver enzymes in juvenile beluga, when fed with ginger supplemented diets. In contrary to this, Gholipour kanani et al. (2014) reported that there were no significant differences in ALT and AST in beluga fed with ginger diets compared with the control.

4.1.5.4 Immunological parameters

4.1.5.4.1 Total Proteins

Among different treatments, total proteins (g dl^{-1}) in blood serum of rohu was 2.10, 2.35, 2.50, 2.14 and 1.97 in GP0, GP5, GP10, GP15 and GP20, respectively. Although, there was no significant differences among treatments and control (Table

17, Fig. 22-A), however, maximum value of total proteins was observed in GP10 (2.50).

4.1.5.4.2 Albumins

Among different treatments, mean albumins (g dl^{-1}) in blood serum of rohu was 0.39, 0.43, 0.52, 0.73 and 0.32 in GP0, GP5, GP10, GP15 and GP20, respectively and difference were significant among treatments and control (GP15 $\geq$ GP10 = GP5 = GP0 $\geq$ GP20) with significantly highest ($p \leq 0.05$) value of albumin in GP15 (Table 17, Fig. 22-B).

Table 17. Immunological parameters of *L. rohita* in different treatments at the completion of the experimental period

Parameter	GP0	GP5	GP10	GP15	GP20
Total Proteins (g dl^{-1})	2.10±0.16	2.35±0.09	2.50±0.17	2.14±0.36	1.97±0.12
Albumins (g dl^{-1})	0.39 ± 0.00^{ab}	0.43 ± 0.01^{ab}	0.52 ± 0.24^{ab}	0.73 ± 0.04^{a}	0.32 ± 0.04^{b}
Globulins (g dl^{-1})	1.71±0.16	1.92±0.11	1.98±0.39	1.41±0.40	1.65±0.08
Alb/Glb ratio	0.23±0.02	0.22±0.02	0.35±0.23	0.62±0.18	0.19±0.02
Glucose (mg dl^{-1})	129.1 ± 15.10^{a}	56.83 ± 1.48^{b}	65.32 ± 13.03^{b}	74.37 ± 6.20^{b}	80.13 ± 6.65^{b}

*See Table 7 for legends
Values are Mean ± S.E., ($p \leq 0.05$), n=3
Values with same superscript (a, b,…..d in a row do not differ significantly ($p \leq 0.05$)

4.1.5.4.3 Globulins

Among different treatments, mean globulins (g dl^{-1}) in blood serum of rohu was 1.71, 1.92, 1.98, 1.41 and 1.65 in GP0, GP5, GP10, GP15 and GP20, respectively. Although, there was no significant differences among treatments and control (Table 17, Fig. 22-C), however, highest value of globulins was observed in GP10 (1.98).

4.1.5.4.4 Albumin/Globulin (Alb/Glb) ratio

Among different treatments, mean Alb/Glb ratio of fish was 0.23, 0.22, 0.35, 0.62 and 0.19 in GP0, GP5, GP10, GP15 and GP20, respectively. Although, there was no significant difference among treatments and control (Table 17) however, highest value of Alb/Glb was observed in GP15 (0.62).

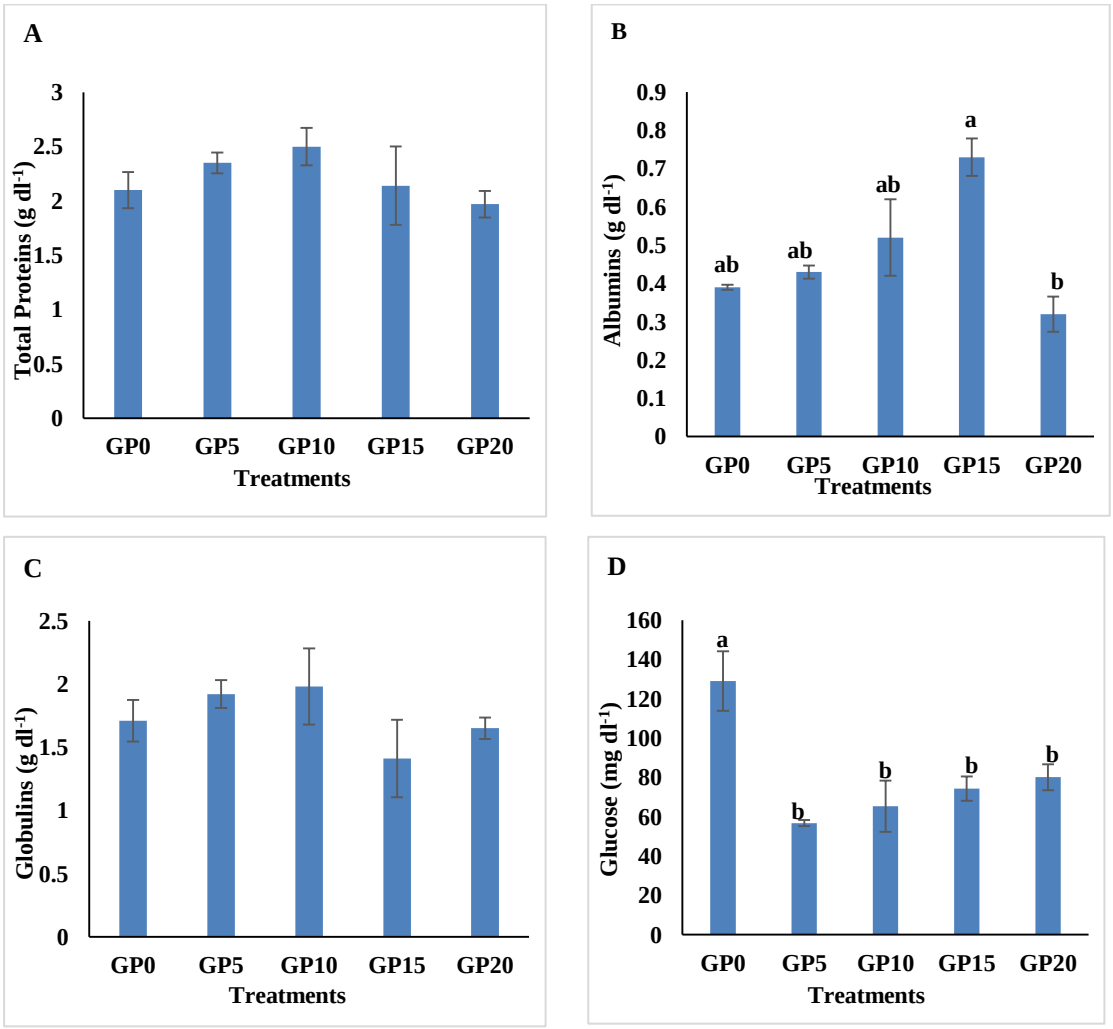


Figure 22. Comparative A) Total Proteins (g dl^{-1}), B) Albumins (g dl^{-1}), C) Globulins (g dl^{-1}) and D) Glucose (mg dl^{-1}) in blood serum of *L. rohita* in different treatment at the completion of experiment

4.1.5.4.5 Glucose

Among different treatments, mean glucose (mg dl^{-1}) level in blood serum of rohu was 129.10, 56.83, 65.32, 74.37 and 80.13 in GP0, GP5, GP10, GP15 and GP20, respectively. As compared to control, glucose level was significantly ($p \leq 0.05$)

reduced in all the treatments with minimum value (56.83) in GP5 (Table 17, Fig. 22-D).

Serum total proteins and globulins are the important indicators of the biochemical, nutritional and health status of the fish (Patriche et al., 2009; Jha et al., 2007). In the present study, total proteins and globulins did not differ significantly among the treatments and control. These results coincide with the investigation of Vahedi et al. (2017), who reported that total protein and globulin content of beluga had no significant difference between ginger fed and non ginger fed (control) diet. In contrast, ginger powder supplementation resulted in increased level of total protein in *L. calcrifer* (Talpur et al., 2013) and juvenile *H. huso* (Gholipour kanani et al., 2014). In the current study, albumin content in blood serum of fish was significantly highest ($p \leq 0.05$) in treatment, where fish was fed with 1.5% (GP15) of ginger powder, which in turn revealed healthy immune system of fish, indicated in terms of improved albumin level (Jha et al., 2007). Previous studies also reported increase in serum albumin content, when tilapia and sea bass was fed with ginger diet (Talpur et al., 2013; Immanuel et al., 2009). On the contrary, Vahedi et al. (2017) indicated that albumin content had no significant difference in beluga fed with diet containing ginger extract, when compared with the control.

The use of ginger has clearly showed its hypoglycaemic potential by decreasing the glucose (Ahmed & Sharma, 1997) level of experimental rohu. The immunostimulants like ginger, due to presence of bioactive compounds (polyphenols, flavonoids and saponin) have the ability to decrease the glucose levels by increasing the level of insulin (Farahi et al., 2010; Sahu et al., 2007a). This might be one of the probable reasons of reduced glucose concentration in the treatments, where fish was fed with ginger supplemented diet as compared to control (GP0). The findings of present study in terms of decreased glucose level are supported by the results of previous studies conducted by Talpur et al. (2013) and Sahu et al. (2007a) after feeding rohu and Asian sea bass with garlic supplemented diets.

4.1.6 Growth hormones

Growth hormones (Triiodothyronine and Thyroxine) were analyzed in blood serum of rohu at 60 and 120 day of experiment.

4.1.6.1 Triiodothyronine (T3)

Among different treatments mean Triiodothyronine (T3) was 2.42, 2.44, 2.48, 2.50 and 2.48 in GP0, GP5, GP10, GP15 and GP20, respectively, after 60 days of culture period with insignificant differences among treatments and control, however, maximum value was observed in GP15. At the completion of the experiment (120 day), mean T3 was 2.44, 2.51, 2.50, 2.57 and 2.52 in GP0, GP5, GP10, GP15 and GP20, respectively (Table 18, Fig. 23-A) with significant ($p \leq 0.05$) enhanced T3 level in GP15 and GP20. Among all the treatments, maximum value of T3 was recorded in GP15 (2.50, 2.57) and minimum in GP0 (2.42, 2.44) at day 60 and 120 respectively.

4.1.6.2 Thyroxine (T4)

Among different treatments mean Thyroxine (T4) was 1.15, 1.24, 1.30, 1.41 and 1.31 in GP0, GP5, GP10, GP15 and GP20, respectively after 60 days of culture period with significant ($p \leq 0.05$) enhanced T4 values in GP15 as compared to control (GP15> GP20 = GP10 = GP5 > GP0). At the completion of the experiment (120 day), mean T4 was 1.26, 1.30, 1.35, 1.37 and 1.36 in GP0, GP5, GP10, GP15 and GP20, respectively after 120 days of culture period (Table 18, Fig. 23-B) with significanly improved T4 levels in all the treatments, except GP5. As compared to control (GP0), significant increase ($p \leq 0.05$) in T4 was observed in GP15 at day 60 and in GP10, GP15, GP20 at day 120.

Table 18. Growth hormone of *L. rohita* in different treatments after 60 and 120 days of the experiment

Parameters	Days	Treatments*				
		GP0	GP5	GP10	GP15	GP20
Triiodothyronine (T3)	60	2.42±0.06	2.44±0.05	2.48±0.02	2.50±0.01	2.48±0.08
	120	2.44±0.02^{b}	2.51±0.03^{ab}	2.50±0.01^{ab}	2.57±0.06^{a}	2.52±0.02^{a}
Thyroxine (T4)	60	1.15±0.04^{b}	1.24±0.06^{ab}	1.30±0.08^{ab}	1.41±0.03^{a}	1.31±0.03^{ab}
	120	1.26±0.04^{b}	1.30±0.01^{ab}	1.35±0.02^{a}	1.37±0.02^{a}	1.36±0.01^{a}

*See Table 7 for legends

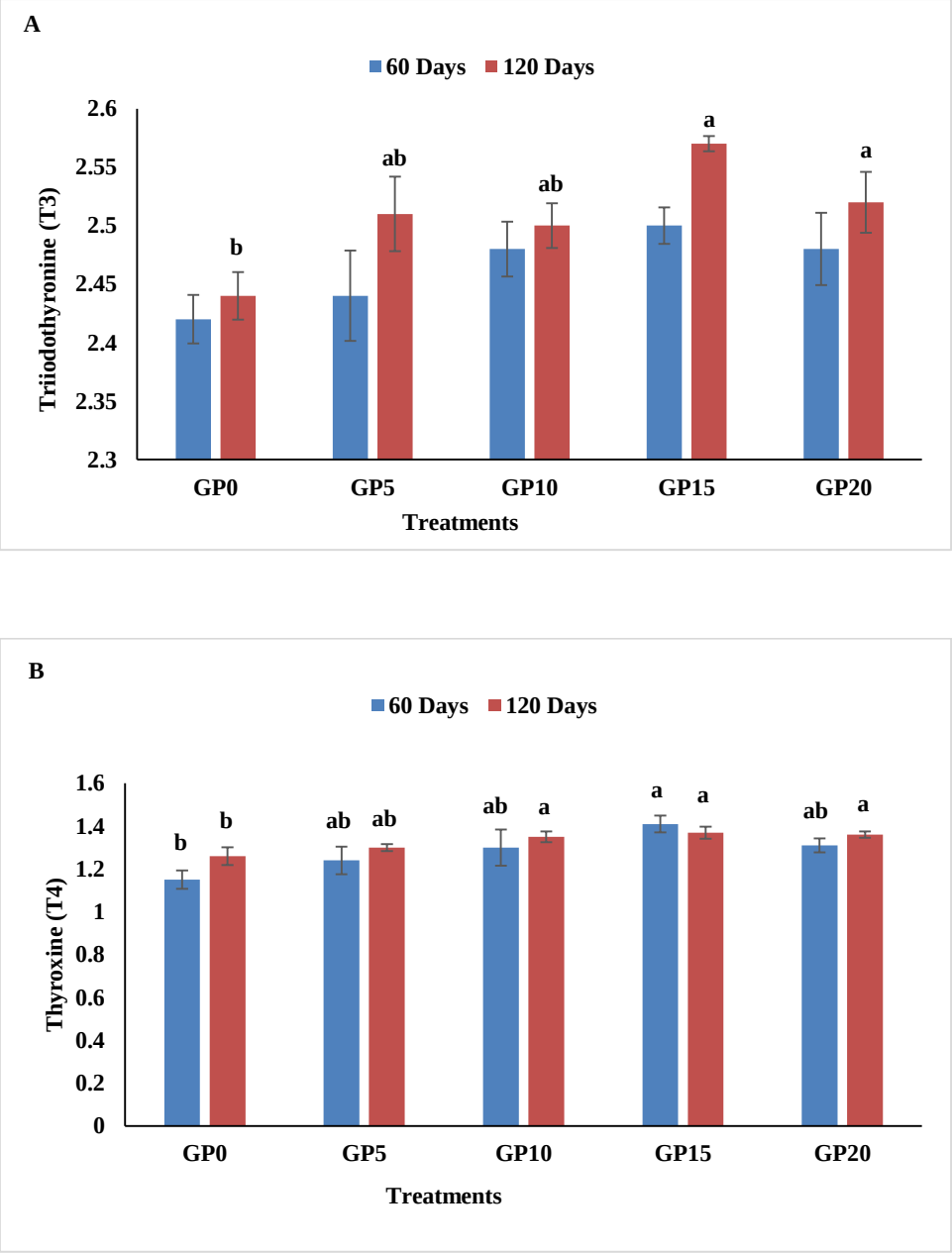


Figure 23. Comparative A) Triiodothyronine (T3) and B) Thyroxine (T4) in blood serum of *L. rohita* in different treatments after 60 and 120 days of the experiment

At the end of feeding trial, the concentrations of T3 and T4 hormones in the serum were significantly ($p \leq 0.05$) elevated in ginger fed fish as compared to the control. Thyroid hormones play an important role in fish growth, development, metabolism and reproduction (Blanton & Specker, 2007; Power et al., 2001). The positive correlation between the tested thyroid hormones and the growth performances in the ginger fed group indicated that these hormones were likely to be involved in improving the growth and development of fish.

4.1.7 Challenge study against *A. hydrophilla*

4.1.7.1 Fish mortality

After 120 days of feeding with control (GP0) and ginger supplemented diets (GP5, GP10, GP15, GP20), fish was challenged with pathogenic *A. hydrophilla* for 15 days (in triplicate) and fish mortality (%) and level of protection (%) was calculated (Table 19). In addition to control and experimental treatments, one negative control (GN) was also kept as reference, in which neither fish was fed with ginger powder, nor it was infected with bacteria.

After 15 days of challenge study, significantly lower fish mortality (13.33%) was observed in GP15 as compared to control and all the ginger powder supplemented diets. Likewise, the level of protection (%) was also highest in GP15 (82%), followed by GP20 & GP10 (64%) and GP5 (46%) respectively.

The disease challenge is an *in-vitro* technique, which helps to determine the performance and immunity of fish, when exposed to pathogenic bacteria present in their natural environment (Arakoosh et al., 2005). The challenge study using *A. hydrophilla* in the present study was undertaken to know the immunity status of rohu after feeding with ginger powder for 120 days and the results revealed that level of protection was maximum, when ginger was fed @ 1.5% (GP15). The results clearly attributed to antimicrobial properties of ginger, which must have provided higher protection to fish as compared to GP0, when challanged with *A. hydrophilla*.

Table 19. Fish mortality (%) and level of protection (%) after 15 days of challenge trial against *A. hydrophilla* in *L. rohita* in different treatments

Parameters	Treatments*					
	GN**	GP0	GP5	GP10	GP15	GP20
Fish Mortality (%)	0	73.33 ± 6.66^{a}	40.0 ±11.54^{b}	26.66 ±6.66^{bc}	13.33 ±6.76^{c}	26.66 ±6.66^{bc}
Level of protection (%)	100	---	46	64	82	64

** GN = Negative control (without ginger powder and without bacterial infection)
* See Table 7 for legends, Values are Mean ± S.E., ($p \leq 0.05$), n=5, Values with same superscript (a, b,…..d) in a row do not differ significantly ($p \leq 0.05$)

Further, the maximum level of protection in GP15, may be due to the prolonged administration of dietary ginger powder (120 days) at an appropriate dose resulted in improvement of immune response of fish against the infection with *A. hydrophila* as compared to GP0. Similarly, Talpur et al. (2013) reported that ginger induce beneficial effects such as disease protection due to improved immune response, which is supported by the higher survival of *L. calcarifer* after infection with *V. harveyi*. Further, Sukumaran et al. (2016) reported that after challenging with *A. hydrophila* all ginger-fed fishes (rohu) exhibited higher survival than control, and significantly highest ($p \leq 0.05$) survival was observed in 0.8 % ginger supplementation.

In common with other plant products, such as *Achyranthes aspera* (Ai et al., 2007) and *Ocimum sanctum* leaf extracts (Logambal et al., 2000), ginger conferred health benefits in terms of reduction in mortalities after bacterial challenge and a heightened effect on non-specific immune mechanisms of fish (Nya & Austin, 2009). Furthermore, bathing *Gyrodactylus turnbulli*-infected fish (*Poecilia reticulata*) in ethanolic ginger extract significantly reduced infection prevalence and intensity, when compared to control (Levy et al., 2015).

4.1.7.2 Fish disease symptoms

During 15 days of challenge study, fish was observed for disease symptoms, whereas post-mortem studies were performed after the completion of challenge trial. The disease symptoms in *L. rohita* challenged with *A. hydrophila* appeared after 72 hrs post injection. In all the treatments, fish showed number of symptoms including loss of balance, excessive mucus secretions on skin, haemorrhages at certain parts of the body surface, torn and black color appearance on fins, skin ulcers penetrated into subcutaneous muscle with pronounced disease symptoms in positive control (GP0) except in negative (GN) control (Table 20, Fig. 24).

Table 20. Clinical symptoms observed during the 15 days challenge trial against *A. hydrophila in L. rohita*

Days	Clinical Disease Symptoms	GN	GP0	GP5	GP10	GP15	GP20
	Dark fins (reddish in colour)	-	+++	++	++	+	++
	Sluggish movement and reddish colour around mouth	-	++	++	++	+	+
	Rejection for feed	-	+++	+++	++	+	+
1-7	Reduced body movements and reddish abdomen	-	++	++	+	+	+
	Increased mucus on body surfaces	-	+++	+++	++	-	-
	Reddish on ventral side, lips and eyes	-	+++	++	++	-	+
	Mortality	-	+++	++	++	+	++
8-15	Survived fish were in good conditions, but reduced feed intake	-	+++	+++	++	+	++

Note: - No effect; + Less effect; ++ Moderate effect; +++ Severe effect

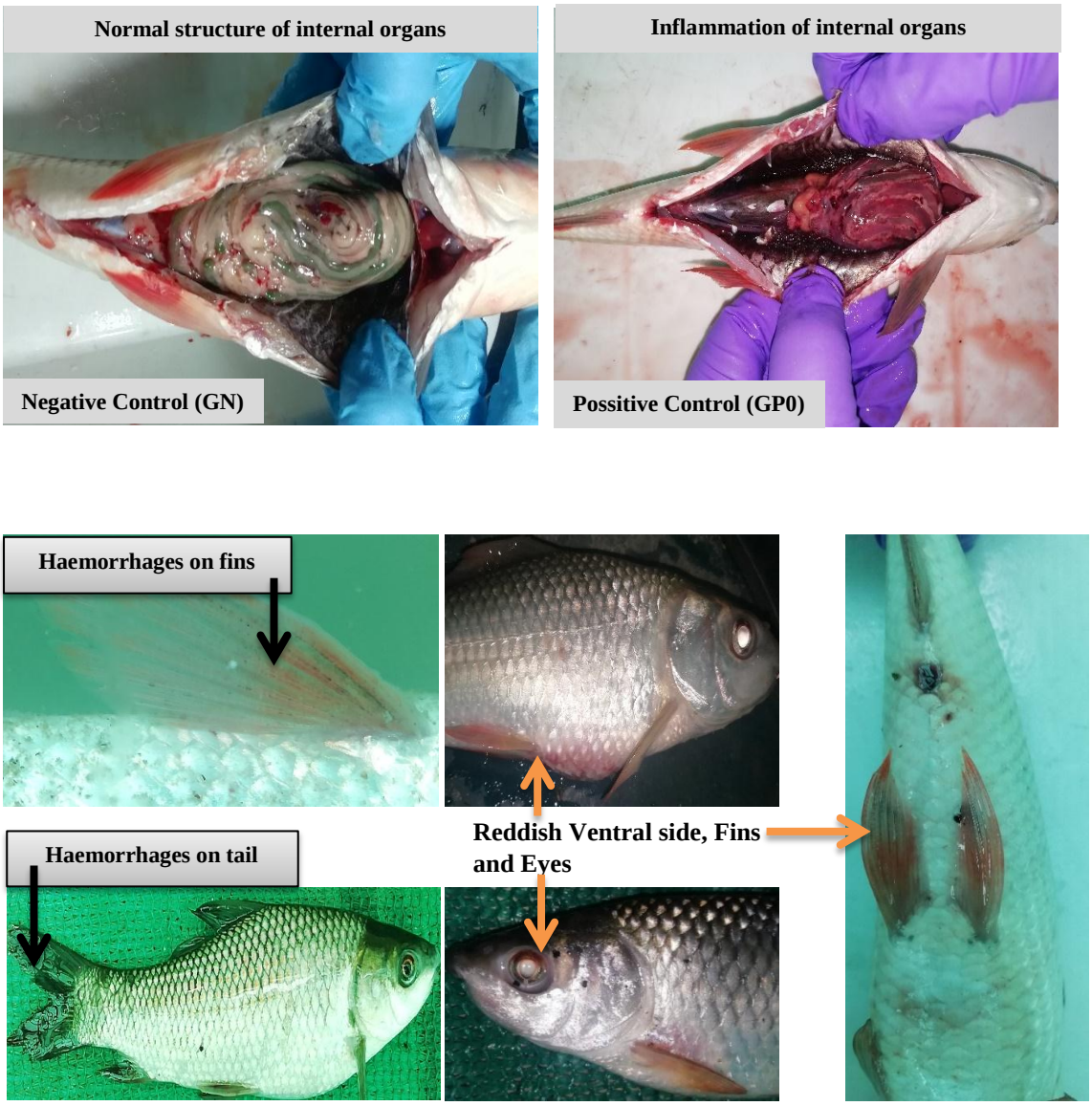


Figure 24. *L. rohita* showing disease symptoms during and after 15 days challenge with *A. hydrophila*

The post mortem studies revealed haemorrhages, inflammation, congestion and enlargement of internal organs in positive control (GP0) and all the treatments (ginger feeding) challenged with *A. hydrophilla*, however the intensity of infection in terms of internal organ injury was severe in positive control (GP0) and minimum in GP15 (Table 19, Fig. 24). The results of the challenge study in terms of fish mortality and post mortem disease symptoms were in accordance to each other. Decreased mortality along with lesser disease incidence in ginger fed fish can be explained with the fact that, bioactive components of ginger resulted in trypsin inhibition, which may

regulate the hydrolysis of protein in vivo and thus balance the mechanism of immune defense against pathogens (Tremacoldi & Pascholati, 2002).

From preent study, (Experiment I), it can be concluded that, among ginger powder (GP) supplemented diets (@ 0.5, 1.0, 1.5 and 2.0%), 1.5% GP supplementation resulted in improvement in survival, growth and health status including hematological, non-specific immune responses and blood metabolic profile of rohu *L. rohita* after 120 days of feeding under semi-intensive culture system. Further, ginger powder also resulted in enhanced immunity of rohu revealed in terms of decreased mortality and increased protection against pathogenic *A. hydrophila*. From overall findings of the present study, ginger proved as one of the potential natural nutraceutical product and can be incorporated at an appropriate level i.e. 1.5% (15 g kg^{-1} diet) for improved growth and health status of fish.

4.2 Experiment II: To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia*

4.2.1 *Artemia* toxicity assay (Pre-treatment study)

4.2.2 *Artemia* challenge assay against *V. parahaemolyticus*

4.2.3 Immunological assay (Expression of defense-related genes in *Artemia*)

4.2.1 *Artemia* toxicity assay (Pre-treatment study)

Artemia larvae were exposed to increasing concentration of ginger extract (GE) under gnotobiotic conditions @ 83.3, 125, 166.6, 208.3, 250, 333.3 ug ml^{-1} for 2 h without pathogen challenge and were counted to calculate the % survival after 48 hours. After 48 hours, survival of *Artemia* in all the groups including negative (C1) and positive controls (C2) was found to be more than 80% (Fig. 25). These results suggest that exposure of GE at the indicated doses (83.3 - 333.3 ug ml^{-1}) appeared to be non-toxic to *Artemia* larvae under the present experimental conditions (Fig. 25).

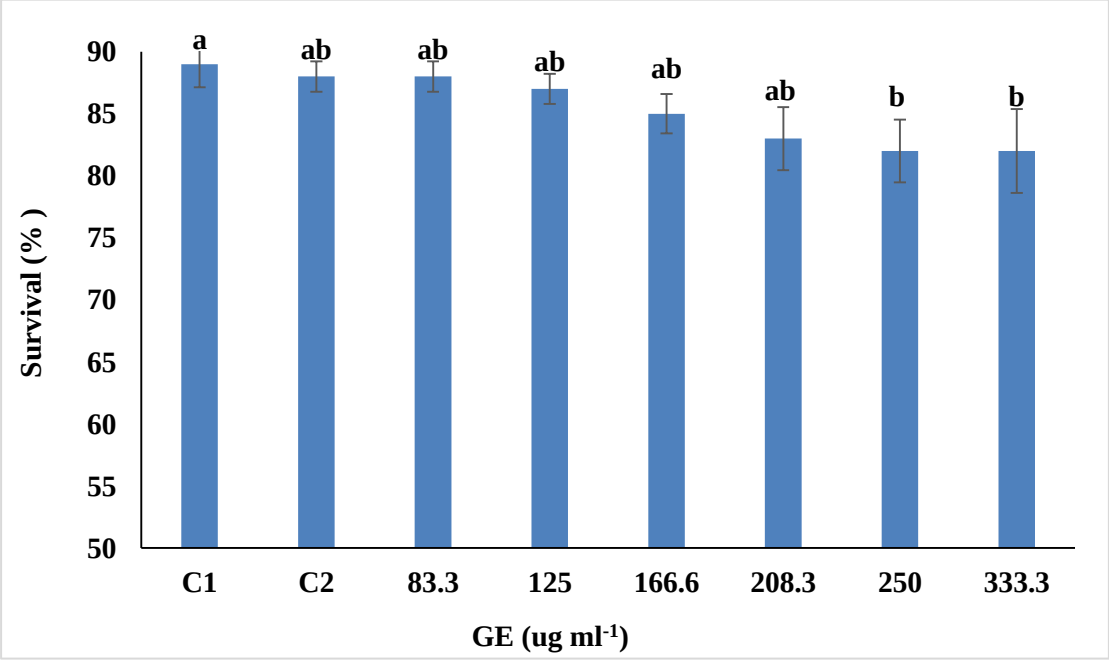


Figure 25. Survival of *Artemia* larvae after 48 hours of toxicity test

4.2.2 *Artemia* challenge assay against *V. parahaemolyticus*

For challenge assay, to study the prophylactic action of GE, *Artemia* larvae were exposed/pre-treated with different doses (83.3, 125, 166.6, 208.3, 250, 333.3 ug ml^{-1}) along with controls (C1 and C2) for two hours and subsequently challenged

against *V. parahaemolyticus* strain 20130629002S01. Results revealed that there was no significant difference found in the *Artemia* survival between the negative and positive controls, suggesting that acetone *per se* had no effect on the survival. However, *Artemia* larvae that received ginger pre-treatment in the range of 166.6 to 333.3 ug ml^{-1} exhibited a significant ($p \leq 0.05$) increase in the survival compared to the controls. Survival at 48 h post challenge was maximum and statistically same at the doses - 166.6, 208.3 and 250 ug ml^{-1} (Fig. 26-A). However, at 54 h post challenge, the best protective GE doses were 250 and 333.3 ug ml^{-1} as compared to the controls (Fig. 26-B). At 67 h post challenge, complete mortality was recorded in the control groups (C1 and C2). However, in the groups that received ginger extract pre-treatment in the range of 83.3 and 333.3 ug ml^{-1}, GE showed its protective effect and maximum survival ($p \leq 0.05$) was observed at concentration of 250 ug ml^{-1} (Fig. 26-C).

Ginger (*Z. officinale*), one of the promising nutraceuticals, has a wide variety of prophylactic and therapeutic properties mainly due to its active polyphenolic components, such as gingerols, shogaols, paradols (Prasad & Tyagi, 2015). The ability of plant-derived phenolic compounds to induce resistance in a host against microbial infection is due to their multi-functional properties, such as bacteriostatic and/or bacteriocidal abilities as well as antioxidant and prooxidant activities, which subsequently are responsible for inducing the host immune system (Baruah et al., 2017; Ozcan et al., 2014; Burt, 2004). In the present study, using the axenic brine shrimp (*Artemia* larvae) and pathogenic strain *V. parahaemolyticus* as the host-pathogen model, it can be demonstrated that pre-treatment of *Artemia* with ginger extract led to the protection of *Artemia* larvae against challenge with *V. parahaemolyticus*. The protective effect of the extract was dose-dependent with the dose of 250 ug ml^{-1} conferring maximum protection to the challenged larvae. Ginger was shown to be endowed with potent antioxidant properties and is an effective scavenger of superoxide radicals, which has been proposed as one of the possible mechanisms of its protective action against pathogenic stressors (Kim et al., 2007; Jagetia et al., 2003; Borex, 2001; Hirahara, 1974).

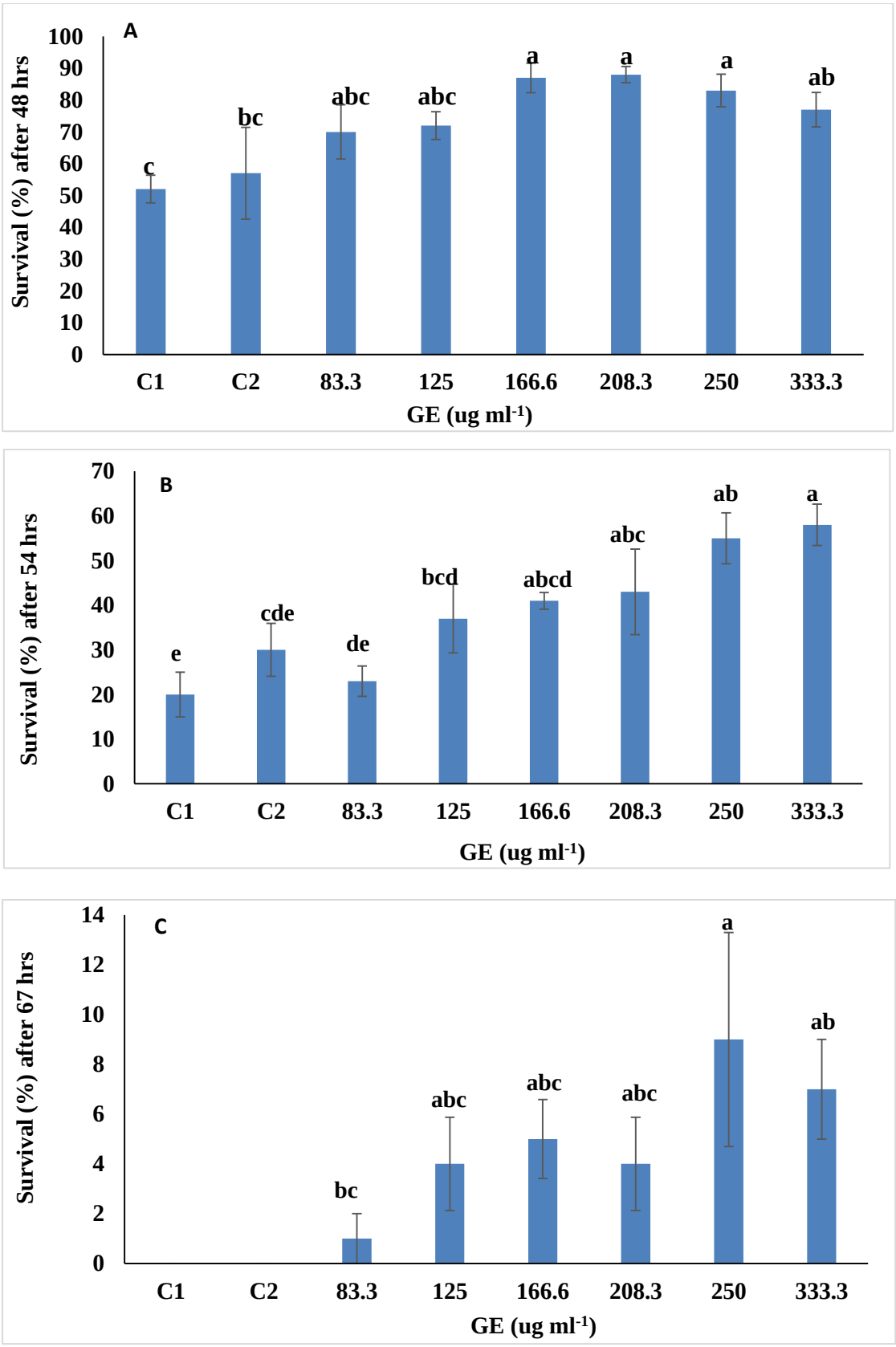


Figure 26. Effect of GE pre-treatment on the survival of *Artemia* larvae at (A) 48 h, (B) 54h and (C) 67h of challenge with *V. parahaemolyticus*

4.2.3 Immunological assay (Expression of defense-related genes in *Artemia*)

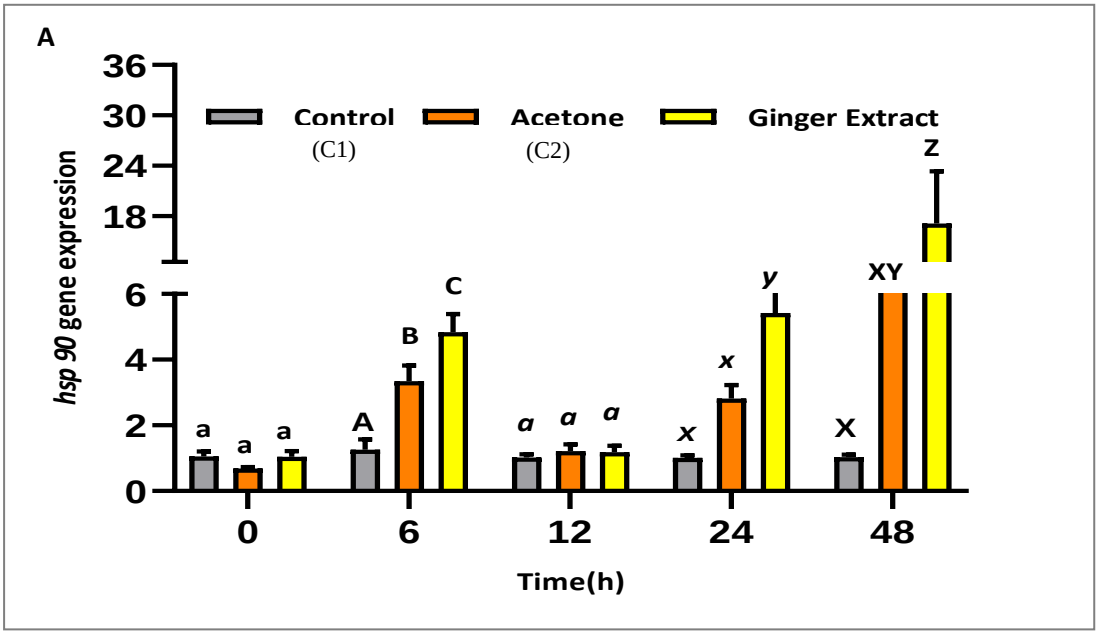
The response of the defense system of *Artemia* due to pre-treatment with GE was studied by measuring the relative mRNA level of the heat shock protein 70 (*hsp70*), heat shock protein 90 (*hsp90*), down syndrome cell adhesion molecule (*dscam*), lipopolysaccharide and β-1,3-glucan-binding protein (*lgbp*), prophenoloxidase (*proPO*), high mobility group box (*hmgb*), peroxinectin (*pxn*), superoxide dismutase (*sod*), transglutaminase 1 (*tgase1*) and transglutaminase 2 (*tgase2*) genes (Fig. 27-33) at different time points (0, 6, 12, 24 and 48h) after challenge with *V. parahaemolyticus*. The dose of GE (250 ug ml^{-1}) that showed the best protective effect was chosen to carry out immunological assay.

The results showed that the expression of *hsp70*, *dscam*, *proPO* and *tgase2* showed no significant differences (Fig. 27B, 29B, 32, 33B) in challenged larvae pretreated with GE as compared to the control at any of the tested time points. However, *hsp90* expression levels became significantly high at 6 h (4.8-fold), 24 h (5.4-fold) and 48 h (17-fold) post challenge in GE pre-treated group as compared to corresponding control.

At 24 and 48 h post challenge, the *hmgb* mRNA level in the larvae was significantly higher in GE pre-treated group by 2.6-fold and 4-fold respectively (Fig. 28), relative to the corresponding controls. Similarly, *lgbp* gene (Fig. 29A) tend to increase at 48 h (11-fold) post challenge ($P \leq 0.05$). Moreover, transcript levels for the *pxn* and *sod* (Fig. 30, 31) were significantly up-regulated at 0 h i.e. prior to infection (3.9-fold; 2.2-fold) and 48 h post infection (4-fold; 4 fold rise) in GE pre-treated group as compared to the corresponding controls. At 48 h post challenge, the *tgase1* mRNA level (Fig. 33A) in the larvae was significantly higher (by 2.9-fold) in the GE pre-treated groups as compared to the respective controls.

Immunological assays help in understanding the mechanism behind the protective effect of GE in challenged *Artemia* larvae. There is a possibility that the protective effect of ginger might be mediated by the induction of stress proteins, like heat shock protein *hsp70, hsp90* which might stimulate the immune response instigating resistance against *V. parahaemolyticus* in the *Artemia* larvae. There is evidence that the heat shock protein family, which acts as 'danger'-signaling molecules, can activate the innate immune system directly by shielding the cells against injury due to pathogens, activating Toll-like receptors, delivering

inflammatory signals, and stabilizing key proteins involved in pathogen destruction (Roberts et al., 2010; Asea et al., 2002; Wallin et al., 2002).



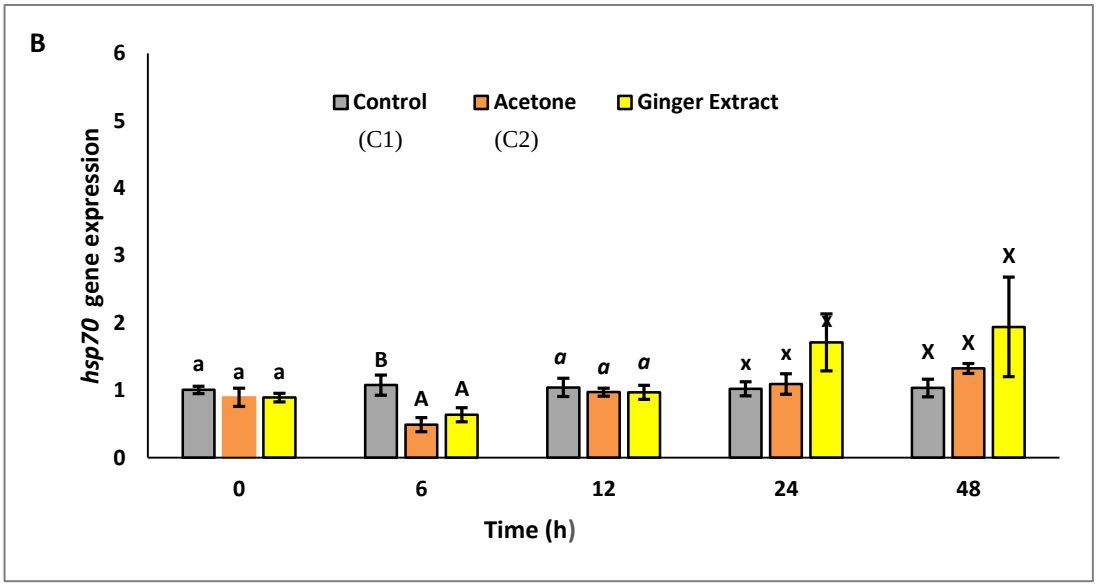


Figure 27. Expression of defense related genes – (A) *hsp90* and (B) *hsp70* in the *Artemia* larvae. Results, which are the mean of 3 replicates, are presented relative to *Artemia ef-1* and *gapdh* gene expression, using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Bars indicate standard error from the mean. Significant differences between the treatment, acetone and control at corresponding time points are indicated by different letters ($p \leq 0.05$)

The *Artemia*, an invertebrate lacks an acquired immune system, unlike the vertebrates, and depends on innate immune factors to build up resistance against pathogens. In this study, heat shock protein 90 (*hsp90*) expression levels became

significantly high at 6 h (4.8-fold), 24 h (5.4-fold), and 48 h (17-fold) post-challenge in GE pre-treated groups, as compared to the corresponding control. While the expression level of *hsp70* remained high at 24 and 48 h post-challenge in the GE pretreated groups, however, the difference was not significant, when compared to the control. The increase in the *hsp90* expression level in the treated group was positively associated with an increase in the survival of *Artemia* in the present study, suggesting the possible role of the stress protein in the induction of resistance against *V. parahemolyticus*. This explanation is consistent with the well known important roles of *hsp90* signaling proteins in defining the resistance of organisms against stressor by performing multifaceted functions, such as acting as a molecular chaperone for proteins, functioning as a danger-associated molecular pattern (DAMP) during inflammation, and various cellular processes (Roh & Sohn, 2018; Chen & Nunez, 2011) and their participation in the activation of cell surface innate immune receptors, thereby modulating many aspects of host's immune responses (Junprung et al., 2017).

The protein high mobility group box-1 (*hmgb*) functions as a nucleosome stabilizer and a regulator of transcription, it was discovered to be a crucial cytokine, that mediates the response to infection, activating Toll-like receptors and delivering inflammatory signals (Tang et al., 2011). With a lot of common characteristics shared, the heat shock protein family, acts as 'danger'-signalling molecules which could activate the innate immune system (Wallin et al., 2002). qRT-PCR results of the present study demonstrated that *hmgb* was significantly upregulated at both time points (24 h and 48 h) in GE pretreated group by 2.6-fold and 4-fold respectively relative to the corresponding controls.

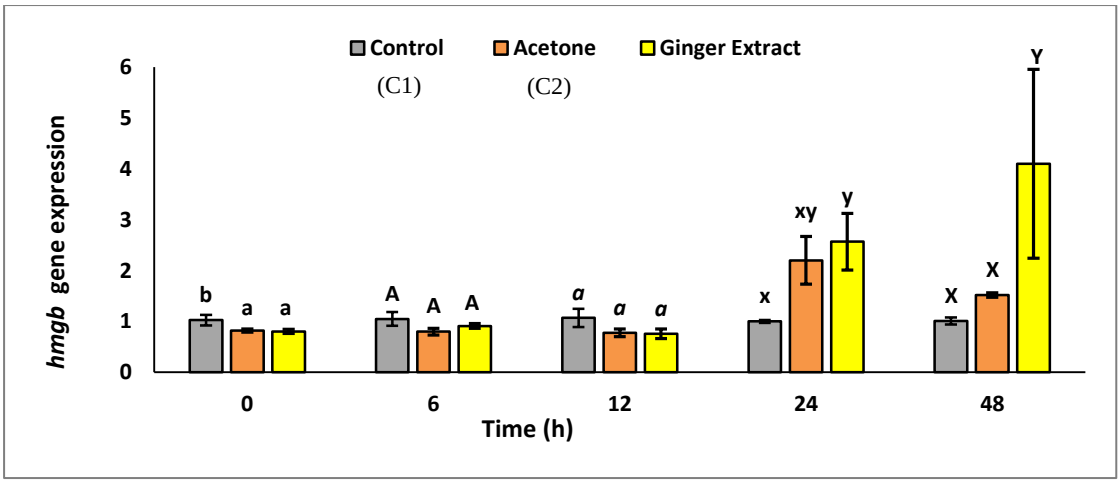


Figure 28. Expression of *hmgb* in the *Artemia* larvae
See Fig. 27 for Legend

lgbp and *dscam* are important pathogen recognition receptor (PRR) and are germline-encoded host sensor of the innate immune system, which detects pathogen-associated molecular patterns (PAMPs) (Mahla et al., 2013). Previous studies had reported that *lgbp* could regulate innate immune defense in invertebrates (including shrimps) against gram-negative bacteria (Amparyup et al., 2012; Kim et al., 2000), thereby conferring protection to the host against infection (Chen et al., 2014). A significant upregulation of the *lgbp* gene at 48 h (11-fold) post-challenge was recorded in the GE pretreated *Artemia* in the present study. These results suggests that *lgpb* in association with the *hsp90* molecule may mediate their effects on the generation of protective immune responses in the host *Artemia* against *V. parahem olyticus*, however, the role of immune effectors could not be ruled out.

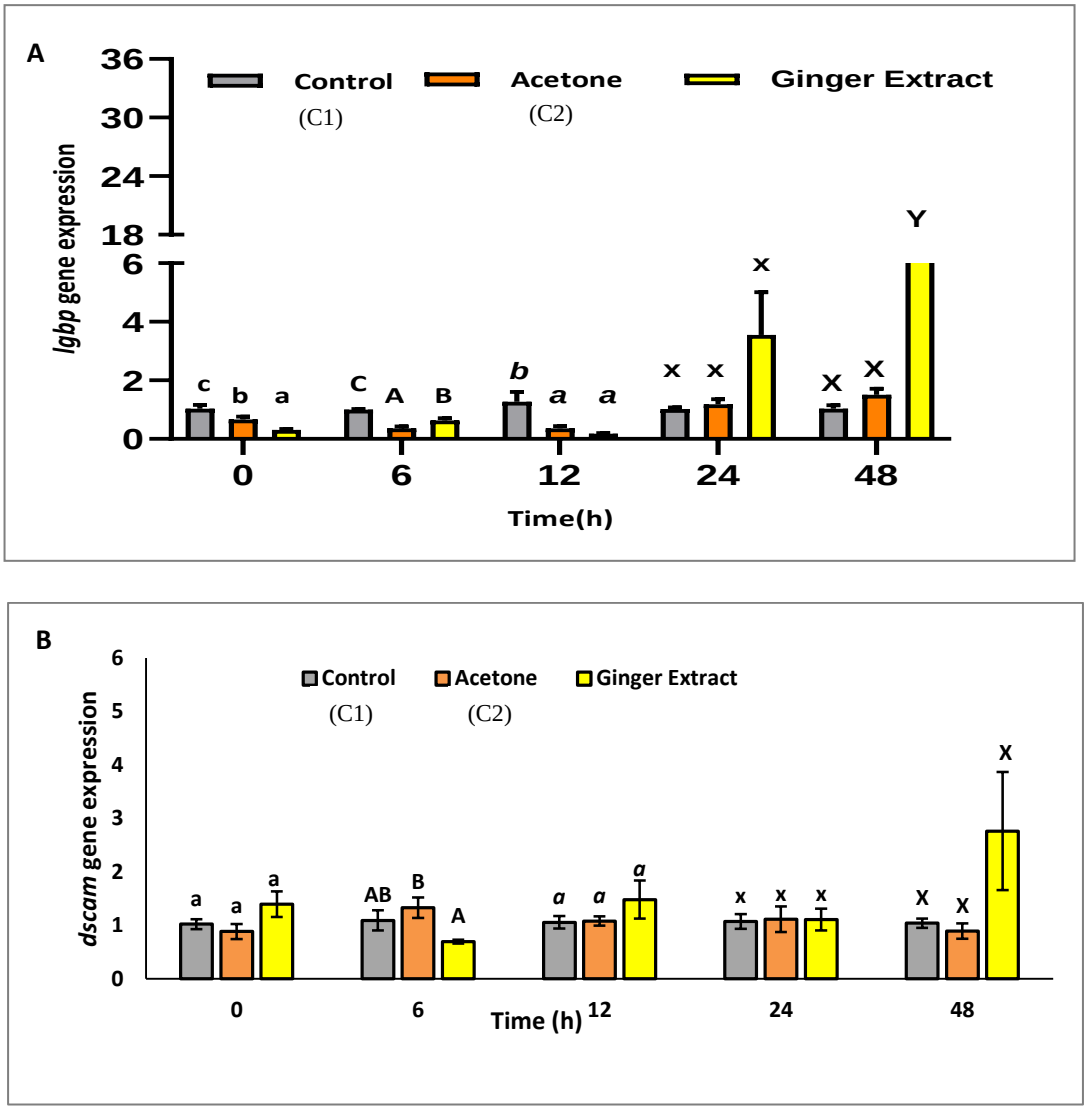


Figure 29. Expression of (A) *lgbp* and (B) *dscam* in the *Artemia* larvae
See Fig. 27 for Legend

dscam is a complex organized hypervariable protein, that detects pathogen PAMPs and the molecular diversity of *dscam* plays an important role in the specificity derived through alternative splicing mechanisms (Wang et al., 2017; Watson, 2005). A recent study in crab showed *dscam* to get upregulated following bacterial challenge, and subsequently, bacterial-induced specific soluble *dscam* isoforms could enhance phagocytosis (Li et al., 2018). However, in the present study, the expression of *dscam* increased by 2.8- folds after 48 h of infection in the group, which was treated with GE but the differences were insignificant.

In shrimp, *sod* plays an important role in protection against *V. parahaemolyticus* and WSSV infection (Kumar et al., 2018; Ji et al., 2011). In the present study, a significant increase in levels of *sod* mRNA was recorded at 0 h i.e. before challenge (2.2-fold) and at 48 h post-challenge (4-fold rise) in GE pre-treated group as compared to the respective controls. *sod* is an intracellular enzyme present in all oxygen-metabolizing cells, which dismutate superoxide radicals into hydrogen peroxide (Rahal et al., 2014). Ginger has been shown to be endowed with potent antioxidant properties and is an effective scavenger of superoxide radicals, which could be regarded as one of the possible mechanisms of its protective action against *V. parahaemolyticus* (Kim et al., 2007; Jagetia et al., 2003) in *Artemia* larvae.

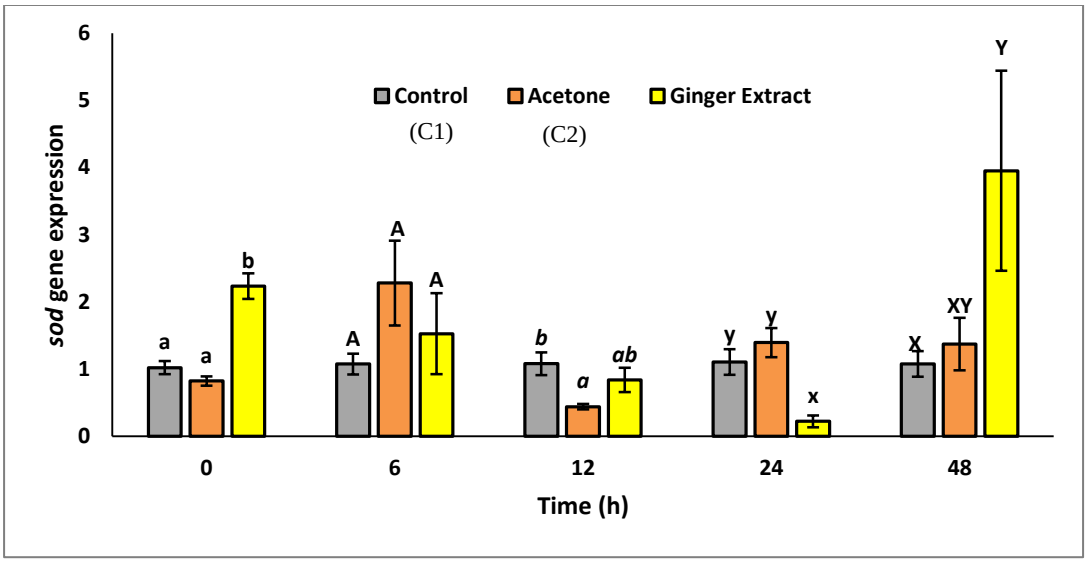


Figure 30. Expression of *sod* in the *Artemia* larvae
See Fig. 27 for Legend

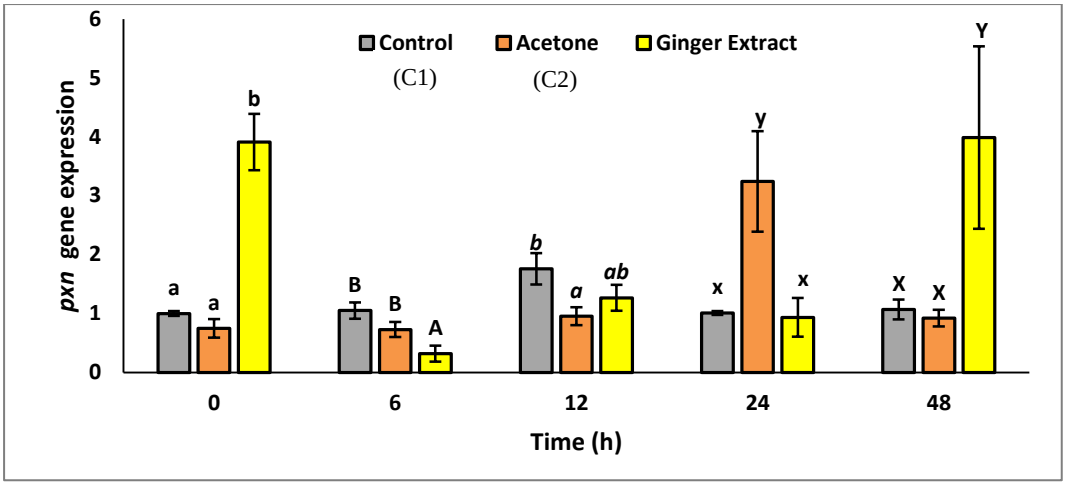


Figure 31. Expression of *pxn* in the *Artemia* larvae
See Fig. 27 for Legend

Similarly, *peroxinectin* (*pxn*) is also one of the important defence-related gene and is a cell-adhesive protein known to be strongly associated with the proPO system (Shanthi et al., 2014). It regulates the expression of antimicrobial peptides (AMPs) in invertebrates (Dong et al., 2011; Sritunyalucksana et al., 2001). It was suggested that *pxn* might produce hypohalic acid from hydrogen peroxide produced by *sod* and function as an efficient microbicidal attack system to the invading microorganisms (Holmblad & Soderhall, 1999). This might be the reason for significant up-regulation of *pxn* at 0 h (3.9-fold) before the challenge, and at 48h post-challenge (4-fold) in the GE pre-treated group.

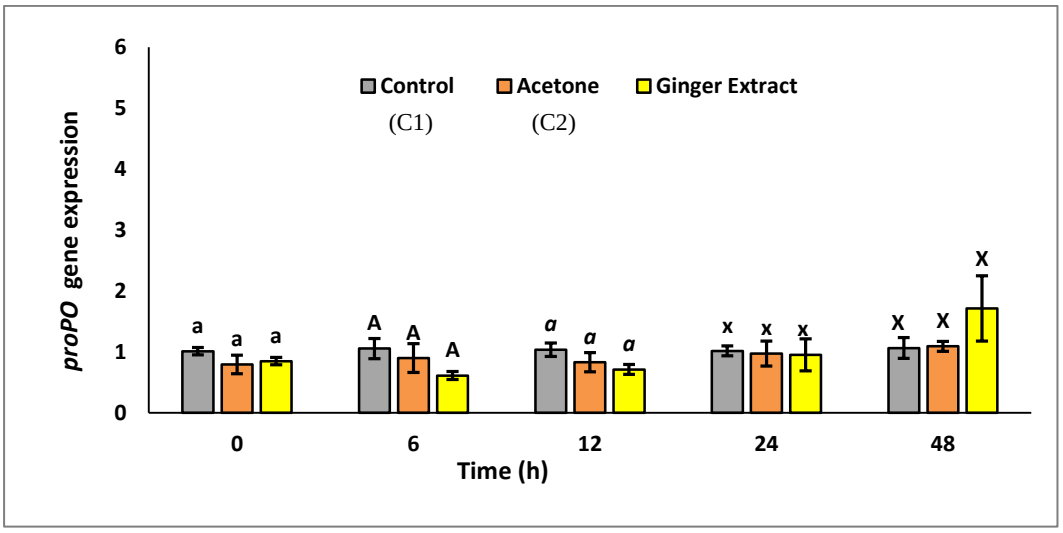


Figure 32. Expression of *proPO* in the *Artemia* larvae
See Fig. 27 for Legend

Moreover, *proPO* activity is one of the primary defences functioning as a non-self recognition system. One of the serine proteases in the cascades named as the ProPO-activating enzyme (ppA) cleaves proPO to generate phenoloxidase (PO) (Cerenius et al., 2008). This active enzyme can produce toxic compounds for microorganisms by oxidizing phenols to melanin. While the expression level of *proPO* remained high at 48 h post-challenge in the GE pretreated groups, however, the difference was not significant, when compared to the control.

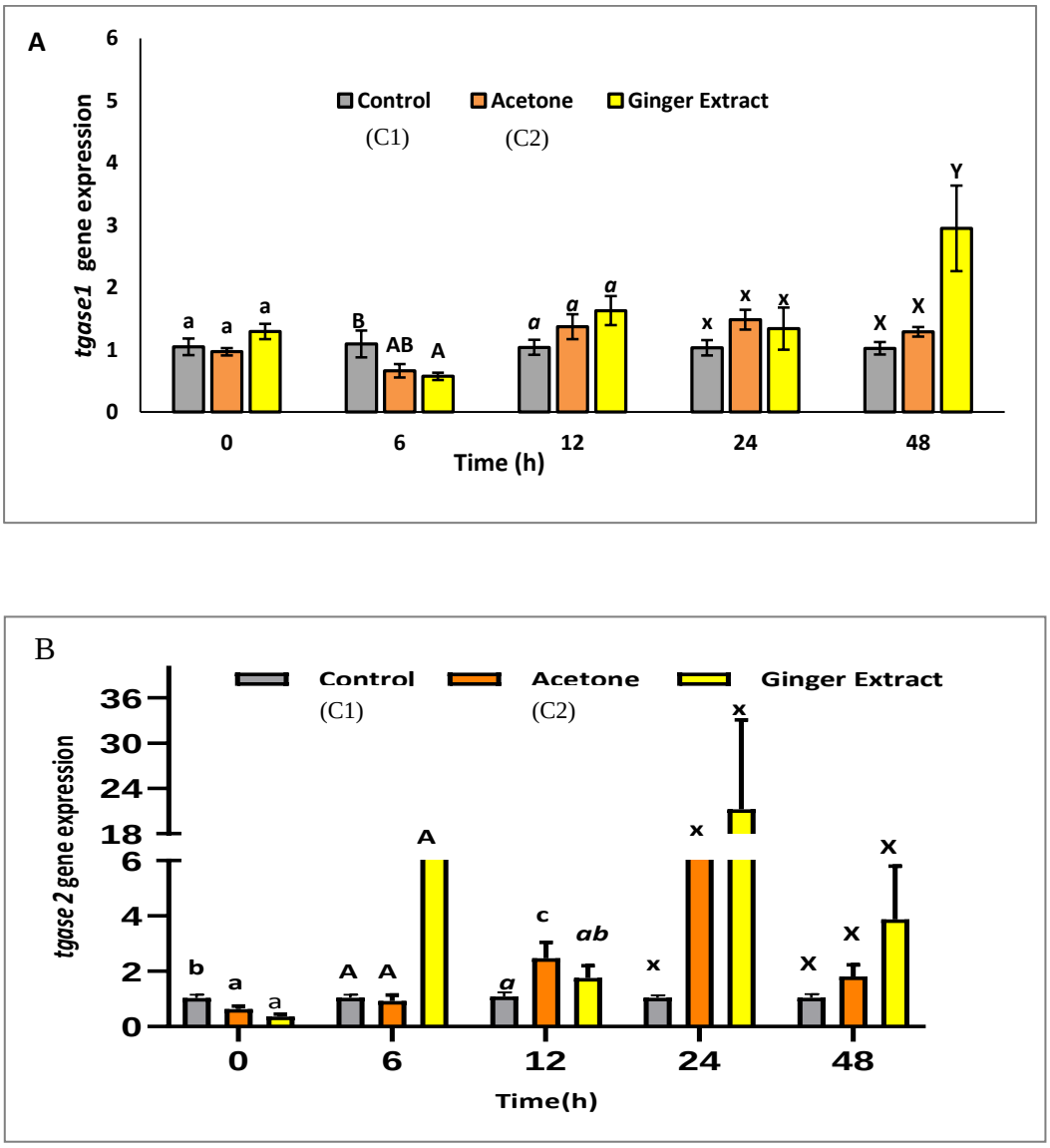


Figure 33. Expression of (A) *tgase1* and (B) *tgase2* in the *Artemia* larvae
See Fig. 27 for Legend

Transglutaminase (*tgase*) is one of the most important innate immune genes in invertebrates, and is known for its protective response against pathogenic bacteria

(Cerenius et al., 2008). It is specifically responsible for catalysing the clotting reaction to prevent the loss of hemolymph through breaks in the exoskeleton (Lin et al., 2008; Chen et al., 2005). The results of the present study revealed that GE significantly increased the expression of the *tgase1* gene by 2.9-fold at 48 h post-challenge. It is possible that the functional protein *tgase* encoded by the GE mediated *tgase* gene assists in the host defense by preventing tissue damage and simultaneously by blocking the progression of *Vibrio* infection in *Artemia*. This is consistent with the findings of Babu et al. (2013), where shrimp fed with a diet containing immunostimulants showed significantly higher expression level of *tgase* and had higher protection against white spot syndrome virus (shrimp pathogen).

In conclusion, the present study (Experiment II) demonstrated that ginger extract could strongly enhance the resistance of the host *Artemia* against *V. parahaemolyticus*. The observed protective effect of the ginger extract is associated with the generation of the innate immune responses. The ability of the ginger extract to boost immunity makes it a potential natural prophylactic agent, that could be used in controlling disease like acute hepatopancreatic necrosis disease (AHPND) caused by *V. parahaemolyticus* in farmed penaeid shrimps. The results of the experimental study add new information about the nutraceutical properties of ginger extract, with special reference to its anti-AHPND effects. Further, the study has also advance our knowledge of this natural extract as a potential antimicrobial agent for application in aquaculture.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study was carried out **"To investigate the nutraceutical properties of ginger in aquaculture feed formulation"** in rohu, *Labeo rohita* under traditional semi-intensive culture system and in *Artemia* under germ free gnotobiotic conditions by undertaking following two experiments

- To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *L. rohita* (Ham.) – **Experiment - I**
- To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia* - **Experiment - II**

5.1 To study the effect of dietary supplementation of ginger (*Zingiber officinale*) powder on survival, growth, immune status and disease resistance of rohu, *L. rohita* (Ham.)

Experiment - I

The study was conducted by undertaking two experiments (Experiment I in India and Experiment II in Sweden). In Experiment I, feeding trial (120 days) was conducted to evaluate the efficacy of ginger powder (GP) as nutraceutical in rohu (*L. rohita*) in terms of growth, health status and resistance against *Aeromonas hydrophila* infection. Study was carried out in cemented outdoor tanks ($20m^2$) in triplicates with a stocking density of 30 fish $tank^{-1}$. Ginger rhizomes were procured from local market of Ludhiana and ginger powder (GP) was prepared (rhizomes were grated, dried and powdered) to incorporate it in control diet (GP0 - without GP) by incorporating GP at different levels (GP5 - 5g kg^{-1} GP0; GP10 -10g kg^{-1} GP0; GP15 - 15g kg^{-1} GP0 and GP20 - 20g kg^{-1} GP0). Fingerlings (Av. Weight 20.55 g) of experimental fish, rohu (*L. rohita)* were procured from fish farm of College of Fisheries, GADVASU and were acclimatized for 15 days in FRP pools under indoor conditions. Acclimatized fish was fed with four experimental (GP5-GP20) and one control (GP0) diet [Rice bran (49%) + Mustard meal (49%) + Vit-Min. mixture (1.5 %) + Salt (0.5%)] for 120 days (May-August, 2019). Fish was fed @

5% body weight with the respective diets for first two months (May and June) and @ 3% body weight for rest of the experimental period (July-August). Observations were recorded w.r.t. physico-chemical parameters [temperature, pH, dissolve oxygen (DO), total alkalinity (TA), total hardness (TH), ammonical nitrogen (NH_3-N)] at 15 day interval; fish growth [total body length (TBL) and body weight (BW)] at 30 day interval and fish survival (%) and growth parameters [TBLG, NWG, SGR, PER, FCR and condition factor (K-value)] at the end of the experiment; haematological parameters (RBC, WBC, Hb, Ht, MCV, MCH and MCHC); non-specific immune responses (RBA, Lysozyme activity and Total Ig); and thyroid hormones at 60 and 120 day interval; blood metabolic profile [lipid profile (HDL, cholesterol and triglycerides), liver profile (AST and ALT), antioxidant parameters (SOD and LPO), immunological parameters (total proteins, albumin, globulins, Alb./Glb ratio and glucose)] at completion of the experiment (120 day). After feeding for 120 days with experimental and control diets, fish were challenged with pathogenic bacteria *Aeromonas hydrophila* for 15 days to know the diseases resistance [(mortality and level of protection (%)] along with disease symptoms) in experimental fish as compared to control.

5.1.1 Physico-chemical parameters of water

The water quality parameters viz. temperature, pH, DO, TA, TH and NH_3-N, varied from 25.63-29.1°C, 7.22 to 8.55, 6.13 to 10.13 mg l^{-1}, 158.66 to 249.33 $CaCO_3$ mg l^{-1}, 180 to 250.66 $CaCO_3$ mg l^{-1} and 0.003 to 0.21 mg l^{-1} respectively, in different treatments. All the water quality parameters remained in optimal range for carp culture throughout the experimental period in all the treatments and control.

5.1.2 Survival and growth parameters

Survival of fish in GP0, GP5, GP10, GP15 and GP20 was 89.39, 92.42, 93.93, 96.96 and 92.42% respectively, with no significant differences in mean values. The results revealed that incorporation of GP enhanced fish survival. Moreover, experimental fishes were healthy with shining body and without any sign of disease or parasitic infection.

At the end of the experimental period (120 days), average final total body length (cm) and TBLG (cm) was highest in GP15 (23.78 and 11.64 cm) and lowest in GP20 (20.39 and 8.58 cm) with significant ($p \leq 0.05$) differences. Average total body

weight (g) and NWG (g) was also maximum in GP15 (157.04, 136.40 g) and minimum in GP20 (110.43 and 88.79g) and the differences for TBW and NWG among different treatments were significant ($p \leq 0.05$). Likewise, SGR and feeding efficiency in terms of FCR and PER significantly improved in GP15 as compared to rest of the treatments and control, however, condition factor (K-value) did not vary significantly among treatments and control.

5.1.3 Haematological parameters

Among different treatments, mean RBC ($x10^6$ mm^{3-1}) was 1.44, 1.37, 1.59, 1.83 and 1.21; 1.69, 1.51, 1.64, 2.31 and 1.69 in GP0, GP5, GP10, GP15 and GP20, respectively after 60 and 120 days of culture period. As compared to control (GP0), significant increase ($p \leq 0.05$) in RBC was observed in GP15 at both the time intervals.

Mean WBC ($x\ 10^4$ mm^{3-1}) was 5.21, 6.43, 5.65, 6.95 and 5.05; 5.70, 6.79, 5.86, 8.51 and 5.64 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days of culture period. As compared to control (GP0), significant increase ($p \leq 0.05$) in WBC was observed in GP5 and GP15 at day 60 and in GP15 at day 120.

Mean Hb (g%) was 7.87, 8.03, 7.94, 8.33 and 7.46; 8.11, 8.28, 8.44, 10.30 and 7.88 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days of culture period and the differences for Hb were significant among different treatments with highest value in GP15 at both the time intervals.

Mean Ht (%) was 19.20, 33.83, 27.40, 28.06 and 19.96; 23.30, 32.76, 33.30, 41.06 and 21.63 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days culture period and the differences were significant among different treatments. As compared to control (GP0), significant increase ($p \leq 0.05$) in Ht was observed in GP5, GP10 and GP15 at day 60 and in GP15 at day 120.

Variations in haematological indices (MCV, MCH and MCHC) were in line with the haematological parameters i.e. RBC, Hb and Ht. In accordance to growth parameters, haematological parameters, too revealed that supplementation of ginger powder @ 1.5% (GP15) helped in improving health status of rohu.

5.1.4 Non-specific immune responses

Among different treatments mean RBA (OD at 620nm) was 0.10, 0.12, 0.28, 0.26 and 0.24; 0.14, 0.15, 0.27, 0.31 and 0.26 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days of culture period. As compared to control (GP0), significantly highest ($p \leq 0.05$) RBA was observed in GP10 and GP15 at day 60 and 120 respectively.

Mean lysozyme (U min^{-1} mg^{-1} $protein^{-1}$) activity was 0.01, 0.02, 0.06, 0.04 and 0.02; 0.01, 0.03, 0.05, 0.07 and 0.04 in GP0, GP5, GP10, GP15 and GP20 respectively after 60 and 120 days of culture period. As compared to control (GP0), significantly highest ($p \leq 0.05$) lysosyme activity was observed in GP10 at day 60 and in GP15 at day 120.

Mean total immunoglobulins (gdl^{-1}) was 0.58, 0.63, 0.71, 0.76 and 0.60; 0.64, 0.73, 0.86, 1.04 and 0.65 in GP0, GP5, GP10, GP15 and GP20 respectively after 60 and 120 days of culture period. Although at day 60, significantly highest ($p \leq 0.05$) value of total Ig was observed in GP10 and GP15, however, the maximum value was reported in GP15. At day 120, GP15 revealed significantly higher total Ig as compared to all the treatments and control (GP0).

5.1.5 Blood metabolic profile

5.1.5.1 Lipid profile

Lipid profile was estimated in terms of HDL, Cholesterol and Triglycerides (mg dl^{-1}) in the blood serum of fish fed with different diets. Among different treatments, mean HDL, cholesterol and triglycerides in fish serum was 30.60, 34.86, 42.81, 54.92 and 39.85; 65.93, 62.82, 66.07, 61.94 and 67.41; 53.77, 46.58, 48.04, 37.11 and 42.58 in GP0, GP5, GP10, GP15 and GP20 respectively, after 120 days of culture period. In accordance to hematological parameters, lipid profile including HDL, Cholesterol and Triglycerides, too revealed that supplementation of dietary ginger improved health status of rohu in terms of lipid profile.

5.1.5.2 Antioxidant Parameters

Antioxidant parameters in haemolysate of *L. rohita* fed with ginger and control diets were analyzed at the completion of the experiment in terms of superoxide dismutase (SOD) and lipid peroxidation (LPO). Mean SOD (U mg^{-1} Hb^{-}

^{1}) and LPO (nmol MDA G Hb^{-1}) activity in haemolysate was 0.36, 0.48, 0.79, 1.04 and 0.66; 3.38, 2.20, 1.76, 0.57 and 2.12 in GP0, GP5, GP10, GP15 and GP20 respectively, with significant difference ($p \leq 0.05$) among treatments.

5.1.5.3 Liver Profile

Liver profile was studied in terms of ALT and AST enzymes activities at the completion of experiment. Among different treatments, mean ALT (IU l^{-1}) and AST (IU l^{-1}) was 17.72, 15.91, 16.50, 14.14 and 15.32; 3.53, 4.71, 3.53, 2.36 and 3.54 in GP0, GP5, GP10, GP15 and GP20 respectively, with significant difference among treatments with lowest value in GP15.

5.1.5.4 Immunological parameters

Immunological parameters were estimated at the completion of experiment in terms of total proteins, albumin, globulins (g dl^{-1}), Alb/Glb ratio and glucose in the serum of fish fed with/without ginger. Among different treatments, mean total proteins, albumin, globulins and Alb/Glb ratio in fish serum was 2.10, 2.35, 2.50, 2.14 and 1.97; 0.39, 0.43, 0.52, 0.73 and 0.32; 1.71, 1.92, 1.98, 1.41 and 1.65 and 0.23, 0.22, 0.35, 0.62 and 0.19 in GP0, GP5, GP10, GP15 and GP20 respectively, with insignificant differences for total proteins, globulins and Alb/Glb ratio among treatments and control. However significantly higher value for albumin was observed in GP15 and maximum value of total protein and globulin was observed in GP10, whereas highest Alb/Glb ratio was observed in GP15.

Among different treatments, mean glucose (mg dl^{-1}) level of fish was 129.10, 56.83, 65.32, 74.37 and 80.13 in GP0, GP5, GP10, GP15 and GP20, respectively. As compared to control, glucose level was significantly reduced in all the treatments with minimum value in GP5.

5.1.6 Growth hormones

Among different treatments, mean Triiodothyronine (T3) was 2.42, 2.44, 2.48, 2.50 and 2.48; 2.44, 2.51, 2.50, 2.57 and 2.52 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days of culture period. Among all the treatments, maximum value of T3 was recorded in GP15 and minimum in GP0 at day 60 and 120, respectively. Mean Thyroxine (T4) was 1.15, 1.24, 1.30, 1.41 and 1.31; 1.26, 1.30, 1.35, 1.37 and 1.36 in GP0, GP5, GP10, GP15 and GP20 respectively, after 60 and 120 days of culture period. At the end of feeding trial, T3 and T4 concentrations in

the serum were significantly ($p<0.05$) elevated in all the treatments as compared to control.

5.1.7 Challenge study against *A. hydrophila*

After 120 days of feeding with control (GP0) and ginger supplemented diets (GP5, GP10, GP15 and GP20), experimental fish were challenged with pathogenic bacteria *A. hydrophilla* for 2 weeks to know the diseases resistance in experimental fish as compared to control in terms of fish mortality (%), level of protection (%) and disease symptoms. In addition to positive control (GP0) and experimental treatments, one negative control (GN) was also kept as reference, in which neither fish was fed with ginger powder, nor it was infected with bacteria.

5.1.7.1 Fish Mortality

After 15 days of challenge study, significantly lower fish mortality (13.33%) was observed in GP15, as comnpared to control and all the ginger supplemented diets. Likewise, the level of protection (%) was also highest in GP15 (82%), followed by GP20 and GP10 (64%) and GP5 (46%) respectively.

5.1.7.2 Disease symptoms

The disease symptoms in *L. rohita* challenged with *A. hydrophila* appeared after 72 hrs post injection. In all the treatments, fish showed loss of balance, excessive mucus secretions on skin, haemorrhages at certain parts the body surface, torn and black color appearance on fins, skin ulcers penetrated into subcutaneous muscle, except in negative control (GN). Further, post mortem study revealed, inflammation in internal organs including congestion and enlargement of internal organs, in positive control (GP0) and all the treatments (ginger supplemented) challenged with *A. hydrophilla*, however the intensity of infection in terms of disease symptoms and internal organ injury was severe in positive control (GP0) and minimum in GP15.

The results in terms of mortality, level of protection and disease symptoms clearly attributed to antimicrobial properties of ginger, which provided higher protection to fish in GP15 (ginger supplementation @ 1.5 %), in the presence of pathogenic *A. hydrophilla.*

5.2 To study the effect of dietary supplementation of ginger (*Zingiber officinale*) extract on survival, disease resistance and immune responses in *Artemia*

Experiment - II

Experiment II was conducted at *Artemia* Lab, Swedish University of Agricultural Sciences (SLU), Sweden. Ginger Extract (GE) was prepared from ginger powder (GP) used in Experiment-I from Nutri Biotech Services Ltd., Malta (Europe) and the concentration of GE (in acetone) was 0.5 grams of dry ginger per ml of diluted extract. *Artemia* were pre-treated with six concentrations (83.3, 125, 166.6, 208.3, 250, 333.3 ug ml^{-1}) of GE along with negative (C1) and positive (C2) control for 2h to evaluate the toxicity assay under gnotobiotic condition. The toxicity assay was performed in quintuplicate (all treatments and control). After toxicity assay, *Artemia* were challenged with *V. parahaemolyticus* strain 20130629002S01 to study the prophylactic action of GE and the survival (%) of *Artemia* was recorded at 48, 54 and 67 h post challenge. The dose that gave maximum protection to larvae against *V. parahemolyticus* challenge was used further to carry out the bulk sampling of *Artemia* for performing immunological assays (defence related genes - *hsp90, hsp70, tgase1, tgase2, lgbp, dscam, proPO, pxn, sod and hmgb*) at different time points (0, 6, 12, 24 and 48h) after challenge with *V. parahaemolyticus* using RT-qPCR.

5.2.1 *Artemia* toxicity assay (Pre-treatment study)

Artemia larvae were exposed to increasing concentration of ginger extract (GE) under gnotobiotic conditions @ 83.3, 125, 166.6, 208.3, 250, 333.3 ug ml^{-1} for 2 h without pathogen challenge and were counted to calculate the % survival after 48 hours. Survival of *Artemia* in all the groups was found to be more than 80% suggesting that exposure of *Artemia* to GE at the indicated doses (83.3-333.3 ug ml^{-1}) appeared to be nontoxic to *Artemia* larvae under axenic conditions.

5.2.2 *Artemia* challenge assay against *V. parahaemolyticus*

For challenge assay, to study the prophylactic action of GE, *Artemia* larvae were exposed/pre-treated with different doses (83.3 - 333.3 ug ml^{-1}) for 2 hours and subsequently challenged with *V. parahaemolyticus* strain 20130629002S01. Results revealed that there was no significant difference found in the *Artemia* survival between the negative and positive controls, suggesting that acetone *per se* had no effect on the survival.

The survival (%) of *Artemia* at 48 h post challenge was significantly high at the doses - 166.6, 208.3 and 250 ug ml^{-1}. However, at 54 h post challenge, the best protective doses were 250 and 333.3 ug ml^{-1} as compared to the controls. At 67 h post challenge, complete mortality was recorded in the control groups. However, GE showed its protective effect and maximum survival ($p \leq 0.05$) was observed at concentration of 250 ug ml^{-1}.

5.2.3 Immunological assay (Expression of defense-related genes in *Artemia*)

The response of the defense system of *Artemia* due to pre-treatment with GE was studied by measuring the expression level of the *proPO, tgase1, tgase2, lgbp, hsp70, hsp90, dscam, pxn, sod and hmgb* genes at different time points (0, 6, 12, 24 and 48h) after challenge with *V. parahaemolyticus* using RT-qPCR.

The results revealed that the expression of *hsp70, dscam, proPO* and *tgase2* showed no significant ($p \leq 0.05$) differences in challenged larvae pre-treated with GE compared to the control at any of the tested time points. However, *hsp90* expression levels became significantly high at 6 h (4.8-fold), 24 h (5.4-fold) and 48 h (17-fold) post challenge in GE pre-treated group as compared to corresponding control. At 24 and 48 h post challenge, the *hmgb* mRNA level in the larvae was significantly higher in GE pretreated group by 2.6-fold and 4-fold respectively relative to the corresponding controls. A marked increase was observed in *lgbp* gene at 48 h (11-fold) post challenge ($p \leq 0.05$). Moreover, transcript levels for the *pxn and sod* were significantly up-regulated at 0 h i.e. prior to infection (3.9-fold; 2.2-fold) and 48 h post infection (4-fold; 4 fold rise) in GE pre-treated group compared to the corresponding controls. At 48 h post challenge, the *tgase1* mRNA level in the larvae was significantly higher (2.9-fold) in the GE pre-treated groups compared to the respective controls.

5.3 Conclusions

Under Xenic/Conventional (Semi-intensive) Condition

- Among ginger powder (GP) supplemented diets (@ 0.5, 1.0, 1.5 and 2.0%), 1.5% GP (15g kg^{-1} GP0) supplementation resulted in improvement in survival, growth and health status including hematological, non-specific immune responses and blood metabolic profile of rohu *L. rohita*

- Challenge study with *A. hydrophila* indicated reduced mortality (13.33%) and increased level of protection (82%) @ 1.5% ginger supplementation

Under Axenic/Gnotobiotic Condition

- Exposure of *Artemia* to increasing doses (83.3, 125, 166.6, 208.3, 250, 333.3ug ml^{-1}) of ginger extract (GE) appeared non-toxic even at highest concentration (333.3ug ml^{-1}) of GE
- Significant protection was observed against *V. parahaemolyticus* to *Artemia* larvae pretreated with GE @ 250 ug ml^{-1}
- Exposure of GE @ 250 ug ml^{-1} significantly ($p<0.05$) enhanced the expression of a core set of defense related genes in *Artemia* (*tgase1, hmgb, sod, pxn, lgbp, hsp70 and hsp90*) post infection

Overall, Under Xenic and Axenic Conditions for fish (rohu) and shellfish (*Artemia*) respectively, Ginger (Powder and Extract) acted as: a potent '**NUTRACEUTICAL**'

- Ginger powder and ginger extract - ***Nutritional Benefits*** (in terms of growth and survival)
- Ginger powder and ginger extract - ***health beneficial effects*** in terms of:
  - ✓ acting as a health booster in the form of immunostimulant: ***Prophylactic Potential***
  - ✓ Effective Agent for controlling *A. hydrophilla* and *V. parahaemolyticus* infection in aquaculture: ***Therapeutic Potential***

REFERENCES

Abo-Esa, J. F. K. (2008). Study on Some Ectoparasitic Diseases of Catfish, *Clarias gariepinus* with their control by ginger, *Zingiber officiale*. *Mediterranean Aquaculture Journal*, 1(1), 1-9.

Abolaji, A. O., Ojo, M., Afolabi, T. T., Arowoogun, M. D., Nwawolor, D., & Farombi, E. O. (2017). Protective properties of 6-gingerol-rich fraction from *Zingiber officinale* (ginger) on chlorpyrifos-induced oxidative damage and inflammation in the brain, ovary and uterus of rats. *Chemico Biological Interactions*, 270, 15-23.

Agarwal, M., Walia, S., Dhingra, S., & Khambay, B. P. S. (2001). Insect growth inhibition, antifeedant and antifungal activity of compounds isolated/ derived from *Zingiber officinale* Roscoe (ginger) rhizomes. *Pest Management Science*, 57, 289-300.

Ahilan, B. A., Nithiyapriyatharshini, A., & Ravaneshwaran, K. (2010). Influence of certain herbal additives on the growth, survival and disease resistance of goldfish, *Carassius auratus* (Linnaeus). *Tamilnadu Journal of Veterinary and Animal Science*, 6, 5-11.

Ahmadifar, E., Sheikhzadeh, N., Roshanaei, K., Dargahi, N., & Faggio, C. (2019). Can dietary ginger (*Zingiber officinale*) alter biochemical and immunological parameters and gene expression related to growth, immunity and antioxidant system in zebrafish (*Danio rerio*)? *Aquaculture*, 507, 341-348.

Ahmed, R. S., & Sharma, S. B. (1997). Biochemical studies on combined effects of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) in albino rats. *Indian Journal of Experimental Biology*, 35, 841-843.

Ai, Q., Mai, K., Zhang, L., Tan, B., Zhang, W., Xu, W., & Li, H. (2007). Effects of dietary b-1, 3-glucan on innate immune response of large yellow croaker, *Pseudosciena crocea*. *Fish and Shellfish Immunology*, 22, 394-402.

Ajeel, S. B., & Al-Faragi, J. K. (2013). Effect of ginger (*Zingiber officinale*) and garlic (*Allium sativum*) to enhance health of common carp *Cyprinus carpio*. *The Iraqi Journal of Veterinary Medicine*, 37(1), 59-62.

Ajlan, A. (2016). Medicinal Plants: A Review. *Natural Products: An Indian Journal*, 12(3), 1-6.

Akinyemi, A. J., Thome, G. R., Morsch, V. M., Stefanello, N., Goularte, J. F., Bello-Klein, A., Oboh, G., & Schetinger, M. R. C. (2015). Effect of dietary supplementation of ginger and turmeric rhizomes on angiotensin-1 converting enzyme (ACE) and arginase activities in L-NAME induced hypertensive rats. *Journal of Functional Foods*, 17, 792-801.

Akrami, R., Gharaei, A., Razeghi, M. M., & Galeshi, A. (2015). Effects of dietary onion (*Allium cepa*) powder on growth, innate immune response and hemato-biochemical parameters of beluga (*Huso huso*) juvenile. *Fish and Shellfish Immunology*, 45(2), 828-834.

Alsaiad, M. A., & Al-Zayat, A. M. (2019). Utilization of Ginger Extract in Nile Tilapia (*Oreochromis niloticus*) diets. *Egyptian Journal of Aquatic Biology & Fisheries*, 23(4), 23- 37.

Amend, D. F. (1981). Potency Testing of Fish Vaccines. *Fish Biologics: Serodiagnostics and Vaccines*, 49, 447-454.

Amparyup, P., Sutthangkul, J., Charoensapsri, W., & Tassanakajon, A. (2012). Pattern recognition protein binds to lipopolysaccharide and β-1,3-glucan and activates shrimp prophenoloxidase system. *Journal of Biological Chemistry*, 287, 10060-10069.

Anderson, D. P., & Siwicki, A. K. (1995). Basic haematology and serology for fish health programs. In Shariff M. Arthur J. R. & R. P. Subasinghe (Eds.), Diseases in Asian aquaculture II (pp. 185-202) Fish Health Section, Asian Fisheries Society, Manila, Phillipines.

Antache, A., Cristea, V., Grecu, I., Dediu, L., Cretu, M., & Petrea, S. M. (2014). The influence of some phytobiotics on haematological andsome biochemical indices at *Oreochromis niloticus*. *Animal Science and Biotechnologies*, 47(1), 192-199.

AOAC. (2012). Official methods of analysis of the Association of Official Analytical Chemists (19th ed.). Washington D.C. USA.

APHA. (2012). Standard Methods for the Examination of Water and Wastewater (22nd ed.) Washington D.C. USA.

Apines-Amar, M. J. S., Amar, E. C., Faisan, J. J. P., Pakingking, J. R. V., & Satoh, S. (2012). Dietary onion and ginger enhance growth, hemato-immunological responses, and disease resistance in brown-marbled grouper, *Epinephelus fuscoguttatus*. *Aquaculture, Aquarium, Conservation & Legislation International Journal of the Bioflux Society*, 5(4), 231-239.

Arakoosh, M. R., Boylen, D., Stafford, C. L., Johnson, L. L., & Collier, T. K. (2005). Use of disease challenge assay to assess immunotoxicity of xenobiotics in fish. *National Oceanic and Atmospheric Administration*, 2, 19-22.

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A., & Calderwood, S. K., (2002). Novel signal transduction pathway utilized by extracellular HSP70 role of Toll-like receptor (TLR)-2 and TLR-4. *Journal of Biological Chemistry*, 277, 15028-15034.

Awad, E., & Awaad, A. (2017). Role of medicinal plants on growth performance and immune status in fish. *Fish and Shellfish Immunology*, 67, 40-54.

Babu, D. T., Antony, S. P., Joseph, S. P., Bright, A. R., & Philip, R. (2013). Marine yeast *Candida aquaetextoris* S527 as a potential immunostimulant in black tiger shrimp *Penaeus monodon*. *Journal of Invertebrate Pathology*, 112, 243-252.

Baruah, K., Norouzitallab, P., Linayati, L., Sorgeloos, P., & Bossier, P. (2014). Reactive oxygen species generated by a heat shock protein (Hsp) inducing product contributes to Hsp70 production and Hsp70-mediated protective immunity in *Artemia franciscana* against pathogenic vibrios. *Developmental and Comparative Immunology*, 46, 470-479.

Baruah, K., Norouzitallab, P., Phong, H. P. P. D., Smagghe, G., & Bossier, P. (2017). Enhanced resistance against *Vibrio harveyi* infection by carvacrol and its association with the induction of heat shock protein 72 in gnotobiotic *Artemia franciscana*. *Cell Stress and Chaperone*, 22(3), 377-387.

Baruah, K., Phong, H. P. P. D., Norouzitallab, P., Defoirdt, T., & Bossier, P. (2015). The gnotobiotic brine shrimp (*Artemia franciscana*) model system reveals that the phenolic compound pyrogallol protects against infection through prooxidant activity. *Free Radical Biology and Medicine*, 89, 593-601.

Basha, K. A., Raman, R. P., Prasad, K. P., Kumar, K., Nilavan, E., & Kumar, S. (2013). Effect of dietary supplemented andrographolide on growth, non-specific immune parameters and resistance against *Aeromonas hydrophila* in *Labeo rohita* (Hamilton). *Fish and Shellfish Immunology*, 35, 1433-1441.

Behera, T., Swain, P., Sahoo, S. K., Mohapatra, D., & Das, B. K. (2011). Immunostimulatory effects of curcumin in fish, *Labeo rohita*. *Indian Journal of Natural Products and Resources*, 2, 184-88.

Benny, K. H., Tan, B. K., & Vanitha, J. (2004). Immunomodulatory and antimicrobial effects of some traditional chinese medicinal herbs: a review. *Current Medicinal Chemistry*, 11, 1423-1430.

Bhardwaj, S., Srivastava, M. K., Kapoor, U., & Srivastava, L. P. (2010). A 90 days oral toxicity of imidacloprid in female rats: morphological, biochemical and histopathological evaluations. *Food and Chemistry Toxicology*, 48, 1185-1190.

Bhatnagar, A., Jana, S. N., Garg, S. K., Patra, B. C., Singh, G., & Barman, U. K. (2004). *Water quality management in aquaculture*. Course manual of summer school on Development of sustainable aquaculture technology in fresh and saline waters. pp 203-210. CCS Haryana Agricultural University, Hisar (India).

Bhavan, P. S., Jeyanthi, S., & Rebecca, A. A. (2011). Growth performance of the fresh water prawn *Macrobrachium rosenbergii* post larvae fed with *Ocimum sanctum* (Tulsi) and *Withania somnifera* (Ashwagandha) incorporated feeds. *International Journal of Biological Research and Devolopment*, 1(1), 34-53.

Binaii, M., Ghiasi, M., Farabi, S. M. V., Pourgholam, R., Fazli, H., Safari, R., Alavi, S. E., Taghavi, M. J., & Bankehsaz, Z. (2014). Biochemical and hemato-immunological parameters in juvenile beluga (*Huso huso*) following the diet

supplemented with nettle (*Urtica dioica*). *Fish and Shellfish Immunology*, 36, 46-51.

Birchard, G. F. (1997). Optimal hematocrit: theory, regulation and implications. *American Zoologist*, 37(1), 65-72.

Blanton, M. L., & Specker, J. L. (2007). The hypothalamic-pituitary-thyroid axis in fish and its role in fish development and reproduction. *Critical Reviews in Toxicology*, 37, 97-115.

Borex, C., (2001). Antioxidant health effects of aged garlic extract. *Journal of Nutrition*, 131, 1010-1015.

Boyd, C. E., & Tucker, C. S. (1998). *Pond aquaculture water quality management*. Kluwer Academic Pulishers, Boston, London. 700p.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology*, 94, 223-53.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*, 8, 1137-1144.

Cerenius, L., Lee, B. L., & Soderhall, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, 29(6), 263-271.

Chakraborty, D., Bishayee, K., Ghosh, S., Biswas, R., Mandal, S. K., & Khuda-Bukhsh, A. R. (2012). [6]-Gingerol induces caspase 3 dependent apoptosis and autophagy in cancer cells: drug-DNA interaction and expression of certain signal genes in HeLa cells. *European Journal of Pharmacology*, 694, 20-29.

Chakraborty, S. B., & Hancz, C. (2011). Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. *Reviews in Aquaculture*, 3, 103-19.

Chang, J. S., Wang, K. C., Yeh, C. F., Shieh, D. E., & Chiang, L. C. (2013). Fresh ginger (*Zingiber officinale*) has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines. *Journal of Ethnopharmacology*, 145, 146–151.

Chang, Y. P., Liu, C. H., Wu, C. C., Chiang, C. M., Lian, J. L., & Hsieh, S. L. (2012). Dietary administration of zingerone to enhance growth, non-specific immune response, and resistance to *Vibrio alginolyticus* in Pacific white shrimp (*Litopenaeus vannamei*) juveniles. *Fish and Shellfish Immunology*, 32, 284-290.

Chanu, T. I., Roy, S. D., Sharma, A., Biswas, P., Siddhya, G. M., & Das, A. (2014). Antistress potential of acetone extract of *Zingiber officinale* on biochemical and oxidative stress parameters in *Labeo calbasu* fingerlings subjected to acid stress. *Indian Journal of Fisheries*, 61(4), 69-77.

Chen, C., Kuo, M., Wu, C., & Ho, C. (1997). Pungent compounds of ginger (*Zingiber officinale*) extracted by liquid carbon dioxide. *Journal of Agricultural and Food Chemistry*, 34, 477-480.

Chen, G. Y., & Nunez, G. (2011). Are heat shock proteins DAMPs? *Nature Reviews Immunology*, 11, 565-565.

Chen, M. Y., Hu, K. Y., Huang, C. C., & Song, Y. L. (2005). More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation. *Developmental and Comparitive Immunology*, 29, 1003-1016.

Chen, Y. E., Jin, S., Zhao, Q. S., Zhang, Y., & Wang, C. L. (2014). LGBP, a pattern recognition receptor in Portunus trituberculatus involved in the immune response against different challenges. *Acta Biologica Hungarica*, 65, 294-304.

Chitmanat, C., Tongdonmuan, K., Khanom, P., Pachontis, P., & Nunsong, W. (2003). *Antiparasitic, antibacterial, and antifungal activities derived from a Terminalia catappa solution against some tilapia (Oreochromis niloticus) pathogens*. III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics, 678, 179-82.

Chrubasik, S., Pittler, M. H., & Roufogalis, B. D. (2005). *Zingiberis rhizoma*: A comprehensive review on the ginger effect and efficacy profiles. *Phytomedicine*, 12, 684-701.

Citarasu, T. (2010). Herbal biomedicines: a new opportunity for aquaculture industry. *Aquaculture International*, 18, 403-14.

Citronberg, J., Bostick, R., Ahearn, T., Turgeon, D. K., Ruffin, M. T., Djuric, Z., Sen, A., Brenner, D. E., & Zick, S. M. (2013). Effects of ginger supplementation on cell-cycle biomarkers in the normal-appearing colonic mucosa of patients at increased risk for colorectal cancer: Results from a pilot, randomized, and controlled trial. *Cancer Prevention Research*, 6, 271-281.

Dalmo, R. A., Ingebrigtsen, K., & Gwald, J. (1997). Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *Journal of Fish Disease*, 20, 241-73.

Dey, R. K., & Chandra, S. (1995). Preliminary studies to raise disease resistant seed (fry) of Indian major carp *Catla catla* through herbal treatment of spawn. *Fishing Chimes*, 14, 23-25.

Dong, C., Wei, Z., & Yang, G. (2011). Involvement of peroxinectin in the defence of red swamp crayfish *Procambarus clarkii* against pathogenic *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, 30, 1223-1229.

Dugenci, S. K., Arda, N., & Candan, A. (2003). Some medicinal plants as immunostimulant for fish. *Journal of Ethnopharmacology*, 88, 99-106.

Duncan, D. B. (1955). Multiple range and multiple F tests. Biometrics, 11, 1-41.

El-Desouky, H., El-Asely, A., Shaheen, A. A., & Abbass, A. (2012). Effects of *Zingiber officinalis* and *Cyanodon dactylon* on the growth performance and immune parameters of *Macrobrachium rosenbergii*. *World Journal of Fish and Marine Sciences*, 4, 301-307.

El-Feki, M. A., Tawfek, N. S., & Awad, E. M. (1993). Ulcerative dermal necrosis, a freshwater fish disease and haematological studies. *Journal of the Egyptian Society of Toxicology*, 10, 25-28.

El-Sayed, H. M., Hakim, Y., & El-Sayed, B. M. (2014). Dietary effect of ginger (*Zingiber officinale* Roscoe) on growth performance, immume response of nile tilapia (*Oreochromis niloticus*) and disease resistance against *Aeromonas hydrophila. Abbasa International Journal of Aquaculture*, 7(1), 35-52.

El-Tahir, K. E., Ashour, M. M., & Al- Harbi, M. M. (1993). The respiratory effects of the volatile oil of the black seed (*Nigella sativa*) in guinea pigs: Elucidation of the mechanisms of action. *General Pharmacology*, 24, 1115-1122.

Encarnacao, P. (2016). Functional feed additives in aquaculture feeds. In: S.F. Nates (ed.), *Aquafeed Formulation*, Academic Press, 217-237.

Endo, K., Kanno, E., & Oshima, Y. (1990). Structures of antifungal diarylheptenones, gingerenones A, B, C and isogingerenone B, isolated from the rhizomes of *Zingiber officinale*. *Phytochemistry*, 29, 797-799.

Ernst, E., & Pittler, M. H. (2000). Efficacy of ginger for nausea and vomiting: A systematic review of randomized clinical trials. *British Journal of Anaesthesia*, 84, 367-371.

Fadeifard, F., Raissy, M., Jafarian, M., Rouhi, B. H., Rahimi, M., & Faghani, M. (2018). Effects of black seed (*Nigella sativa*), ginger (*Zingiber officinale*) and cone flower (*Echinacea angustifolia*) on the immune system of rainbow trout, *Oncorhynchus mykiss*. *Brazilian Journal of Veterinary and Animal Sciences*, 70(1), 199-204.

FAO. (2020). *The State of World Fisheries and Aquaculture Production Statistics* 2006-2020. FAO Fisheries and Aquaculture Department, Rome, Italy.

Farahi, A., Kasiri, M., Sudagar, M., Iraei, M. S., & Shahkolaei, M. D. (2010). Effect of garlic (*Allium sativum*) on growth factors, some hematological parameters and body compositions in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture, Aquarium, Conservation and Legislation International Journal of the Bioflux Society*, 3, 317-323.

Fierro-Coronado, J. A., Luna-Gonzalez, A., Caceres-Martinez, C. J., Ruiz-Verdugo, C. A., Escamilla-Montes, R., Diarte-Plata, G., Flores-Miranda, M. D. C., Alvarez-Ruiz, P., & Peraza-Gomez, V. (2019). Effect of medicinal plants on the survival of white shrimp (*Penaeus vannamei*) challenged with WSSV and *Vibrio parahaemolyticus*. *Latin American Journal of Aquatic Research*, 47(2), 377-381.

Fossati, P., Prencipe, L., & Berti, G. (1983). Enzymic creatinine assay: A new colorimetric method based on hydrogen peroxide measurement. *Clinical Chemistry***,** 29, 1494-96.

Ghadikolaei, A. H., Kamali, A., Soltani, M., & Sharifian, M. (2017). Effects of *Zingiber officinale* powder on growth parameters, survival rate and biochemical composition of body in juvenile common carp (*Cyprinus carpio*). *Iranian Journal of Fisheries Sciences*, 16(1), 67-85.

Gholipour, K. H., Nobahar, Z., Kakoolaki, S., & Jafarian, H. (2014). Effect of ginger- and garlic supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiology and Biochemistry*, 40, 481- 90.

Giarma, E., Amanetidou, E., Toufexi, A., & Touraki, M. (2017). Defense systems in developing *Artemia franciscana* nauplii and their modulation by probiotic bacteria offer protection against a *Vibrio anguillarum* challenge. *Fish and Shellfish Immunology*, 66, 163-172.

Gomez-San-Juan, M., Bogdanski, A., & Dubois, O. (2019). *Towards sustainable bioeconomy - Lessons learned from case studies.* Rome, FAO. 132. ISBN: 978-92-5-131424-1

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *The Journal of Biological Chemistry*, 177, 751-66.

Grzanna, R., Lindmark, L., & Frondoza, C. G. (2005). Ginger- a herbal medicinal product with broad anti-inflammatory actions. *Journal of Medicinal Food*, 8, 125-132.

Haghighi, M., & Rohani, M. (2013). The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*). *Journal of Medicinal Plant and Herbal Therapy Research*, 1, 8-12.

Hai, N. V. (2015). The use of medicinal plants as immunostimulants in aquaculture: a review. *Aquaculture*, 446, 88-96.

Hajek, G. J., Klyszejko, B., & Dziaman, R. (2006). The anesthetic effect of clove oil on Common carp, *Cyprinus carpio*. *Acta Ichthyologica Et Piscatoria*, *36*, 93-97.

Halver, J. E. (1976). *The nutritional requirements of cultivated warm water and cold water fish species.* FAO Technical Conference on Aquaculture, Kyoto, Japan. pp. 9.

Han, B., Baruah, K., Nguyen, D. V., Williams, D. L., Devriendt, B., Cox, E., & Bossier, P. (2020). Beta-glucan's varying structure characteristics modulate survival and immune-related genes expression from *Vibrio harveyi*-infected *Artemia franciscana* in gnotobiotic conditions. *Fish and Shellfish Immunology*, 102, 307-315.

Han, B., Kaur, V. I., Baruah, K., Nguyen, V. D., & Bossier, P. (2019). High doses of sodium ascorbate act as a prooxidant and protect gnotobiotic brine shrimp larvae (*Artemia franciscana*) against *Vibrio harveyi* infection coinciding with heat shock protein 70 activation. *Developmental and Comparative Immunology*, 92, 69-76.

Harikrishnan, R., Balasundaram, C., & Heo, M. S. (2011). Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*, 317, 1-15.

Hashim, M. M., Mingsheng, D., Iqbal, M. F., & Xiaohong, C. (2011). Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochemistry*, 570-576.

Hassanin, M. E. S., Hakim, Y., & Badawi, M. E. S. (2014). Dietary effect of ginger (*Zingiber officinal*e Roscoe) on growth performance, immune response of nile tilapia (*Oreochromis niloticus*) and disease resistance against *Aeromonas hydrophila*. *Abbassa International Journal of Aquaculture*, 7, 35-52.

Hatai, K., Kamada, T., Lau, L. M., Kulip, J., Phan, C. S., & Vairappan, C. S. (2018). In vitro Inhibitory effects of two bornean medicinal wild gingers against Pathogenic *Lagenidium thermophilum* infected mud crab *Scylla tranquebarica*. *Biocontrol Science*, 23(1), 35-39.

Hepher, B., Liao, I. C., Cheng, S. H., & Hsieh, C. S. (1983). Food utilization by red tilapia- affects of diet composition, feeding level and temperature on utilization efficiencies for maintenance and growth. *Aquaculture*, 32, 255-75.

Hirahara, F., (1974). Anti oxidative activity of various spices on oils and fats: Anti oxidative activity towards oxidation on storage and heating. *Japanese Journal of Nutrition and Dietetics*, 32, 1-5.

Ho, S., Chang, K., & Lin, C. (2013). Anti-neuroinflammatory capacity of fresh ginger is attributed mainly to 10-gingerol. *Food Chemistry*, 141, 3183-3191.

Holmblad, T., & Soderhall, K. (1999). Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture*, 172(1-2) 111-123.

Hoseini, S. M., Mirghaed, A. T., Iri, Y., & Ghelichpour, M. (2018). Effects of dietary cineole administration on growth performance, hematological and biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 495, 766-772.

Huet, M. (1975). Breeding and cultivation of salmonids, a fish culture in cold water. In *Textbook of Fish Culture, Breeding and Cultivation of Fish*. (pp 59-112). Fishing News Books Ltd, England.

Immanuel, G., Uma, R., Iyapparaj, P., Citarasu, T., Punitha, P. S., & Michael, B. M. (2009). Dietary medicinal plant extracts improve growth, immune activity and survival of tilapia *Oreochromis mossambicus*. *Journal of Fish Biology*, 74, 1462-75.

Jafarinejad, R., Gharaei, A., & Mirdar, H. J. (2020). Dietary ginger improve growth performance, blood parameters, antioxidant capacity and gene expression in *Cyprinus carpio*. *Iranian Journal of Fisheries Sciences*, 19(3), 1237-1252.

Jagetia, G. C., Baliga, M. S., Venkatesh, P., & Ulloor, J. N. (2003). Influence of ginger rhizome (*Zingiber officinale*) on survival, glutathione and lipid peroxidation in mice after whole-body exposure to gamma radiation. *Radiation research*, 160, 584-92.

Jahanjoo, V., Yahyavi, M. I., Akrami, R., & Houshang, A. (2018). Influence of adding garlic (*Allium sativum*), ginger (*Zingiber officinale*), thyme (*Thymus vulgaris*) and their combination on the growth performance, haematoimmunological parameters and disease resistance to *Photobacterium damselae* in sobaity sea bream (*Sparidentex hasta*) fry. *Turkish Journal of Fisheries and Aquatic Sciences*, 18, 633-645.

Jena, J. K., & Das, P. C. (2006). Carp culture. In: S. A., Verma, A. T. Kumar and S. Pradhan (eds.), *Handbook of Fisheries and Aquaculture* (pp. 265-82). ICAR, New Delhi.

Jha, A. K., Pal, A. K., Sahu, N. P., Kumar, S., & Mukherjee, S. C. (2007). Haematoimmunological responses to dietary yeast RNA, w-3fatty acid and b-carotene in *Catla catla* juveniles. *Fish and Shellfish Immunology*, 23, 917-927.

Jhingran, V. G. (1991). *Fish and fisheries of India*. 5th edn. (pp. 727). Hindustan Publishing Corporation, New Delhi.

Ji, K., Fang, L., Zhao, H., Li, Q., Shi, Y., Xu, C., Wang, Y., Du, L., Wang, J., & Liu, Q. (2017). Ginger oleoresin alleviated gamma-ray irradiation-induced reactive oxygen species via the Nrf2 protective response in human mesenchymal stem cells. *Oxidative Medicine and Cellular Longevity*, 1-12.

Ji, P. F., Yao, C. L., & Wang, Z. Y. (2011). Reactive oxygen system plays an important role in shrimp *Litopenaeus vannamei* defense against *Vibrio parahaemolyticus* and WSSV infection. *Disease of Aquatic Organism*, 96, 9-20.

Junprung, W., Supungul, P., & Tassanakajon, A. (2017). HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock. *Fish and Shellfish Immunology*, 60, 237-246.

Kaur, A., Holeyappa, A. S., Bansal, N., Kaur, V. I., & Tyagi, A. (2020). Ameliorative effect of turmeric supplementation in feed of *Labeo rohita* (Linn.) challenged with pathogenic *Aeromonas veronii*. *Aquaculture International*, 28(3), 1-14.

Kaur, Y., Dhawan, A., & Shanthanagouda, A. H. (2019). Effect of neem leaf extract incorporated diet on survival, growth and flesh quality of common carp (*Cyprinus carpio*). *Indian Journal of Ecology*, 44(4), 860-63.

Khan, I., Dhawan, A., & Ansal, M. D. (2018). Effect of Aloe vera supplemented feed on survival and growth of common carp (*Cyprinus carpio* L.). *Indian Journal of Animal Nutrition*, 35(2), 206-13.

Kiczorowska, B., Samolinska, W., Al-Yasiry, A. R. M., Kiczorowski, P., & Meiczan, A. W. (2017). The natural feed additives as immunostimulants in monogastric animal nutrition- a review. *Annals of Animal Science*, 17(3), 605-25.

Kim, J. K., Kim, Y., Na, K. M., Surh, Y. J., & Kim, T. Y. (2007). Gingerol prevents UVB-induced ROS production and COX-2 expression in vitro and in vivo. *Free Radical Research*, 41, 603-614.

Kim, Y., Ryu, J., Han, S., Choi, K., Nam, K., Jang, I., Lemaitre, B., Brey, P. T., & Lee, W. J. (2000). Gram negative bacteria binding protein, a pattern recognition receptor fo lipopolysaccharide and beta-1,3-glucan, which mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. *Journal of Biological Chemistry*, 275(42), 32721-32727.

Korni, F. M. M., & Khalil, F. (2017). Effect of ginger and its nanoparticles on growth performance, cognition capability, immunity and prevention of motile *Aeromonas* septicaemia in *Cyprinus carpio* fingerlings. *Aquaculture Nutrition*, 23, 1492-1499.

Kumar, R., Rao, A. C., Daggula, N., Guguloth, G., Das, B., & Indhuri, A. (2019). Growth promoter effect of ginger, garlic and fenugreek on pacific white leg shrimp (*Litopenaeus vannamei*). *International Journal of Current Microbiology and Applied Sciences*, 8, 2993-3001.

Kumar, R., Sharma, B. K., & Sharma, L. L. (2007). Impact of *Glycyrrhiza glabra* Linn as growth promoter in the supplementary feed of an Indian major carp, *Cirrhinus mrigala*. *Indian Journal of Animal Resource*, 41(1), 35-38.

Kumar, V., Baruah, K., Nguyen, D. V., Smagghe, G., Vossen, E., & Bossier, P. (2018). Phloroglucinol mediated Hsp70 production in crustaceans: Protection

against *Vibrio parahaemolyticus* in *Artemia franciscana* and *Macrobrachium rosenbergii*. *Frontiers in Immunology*, 9, 1091.

Labrador, J. R. P., Guinares, R. C., & Hontiveros, G. J. S. (2016). Effect of garlic powder-supplemented diets on the growth and survival of Pacific white leg shrimp (*Litopenaeus vannamei*). *Cogent Food & Agriculture*, 2(1), 1-8.

Lebda, M. A., Taha, N. M., Korshom, M. A., Mandour, A. E. A., & El- Morshedy, A. M. (2012). Biochemical effect of ginger on some blood and liver parameters in male New Zealand rabbits. *Journal of Animal and Feed Research*, 2, 197-202.

Lee, K., Jeong, H., & Cho, S. (2020). Dietary inclusion effect of yacon, ginger, and blueberry on growth, body composition, and disease resistance of juvenile black rockfish (*Sebastes schlegeli*) against *Vibrio anguillarum*. *Fisheries and Aquatic Sciences*, 23(1), 1-8.

Leger, P., Bengtson, D. A., Simpson, K. L., & Sorgeloos, P. (1986). The use and nutritional value of *Artemia* as a food source. In M. Barnes (Ed.), *Marine Biology and Oceanography: An Annual Review* (pp. 521-623). Aberdeen University Press.

Levy, G., Zilberg, D., Paladini, G., & Fridman, S. (2015). Efficacy of ginger-based treatments against infection with *Gyrodactylus turnbulli* in the guppy (*Poecilia reticulate*). *Veterinary Parasitology*, 209, 235-241.

Li, X. J., Yang, L., Li, D., Zhu, Y. T., Wang, Q., & Li, W. W. (2018). Pathogen-specific binding soluble down syndrome cell adhesion molecule (dscam) regulates phagocytosis via membrane-bound dscam in crab, *Frontiers Immunology*, 9, 801.

Lin, X., Soderhall, K., & Soderhall, I. (2008). Transglutaminase activity in the hematopoietic tissue of a crustacean, *Pacifastacus leniusculus*, importance in hemocyte homeostasis. *BMC Immunology*, 9, 58.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402-408.

Logambal, S. M., Venkatalakshmi, S., & Michael, R. D. (2000). Immunostimulatory effect of leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus*. *Hydrobiologia*, 430, 113-120.

MacArthur, J. I., & Fletcher, T. C. (1985). Phagocytosis in fish. In M.J. Manning & M.F. Tatner (eds.), *Fish Immunology* (pp. 29–46). Academic Press, London.

Magnadottir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology*, 20(2), 137-51.

Mahadavi, M., Hajimoradloo, A., & Ghorbani, R. (2013). Effect of Aloe vera extract on growth parameters of common carp (*Cyprinus carpio*). *World Journal of Medical Sciences*, 9(1), 55-60.

Mahla, R. S., Reddy, M. C., Prasad, V. R. D., Kumar, H., (2013). PAMPs: Role of sugar complexed in innate immunity and vaccine biology. *Frontiers Immunology*, 4, 1-16.

Mahmoud, R. H., & Elnour, W. A. (2013). Comparative evaluation of the efficacy of ginger and orlistat on obesity management, pancreatic lipase and liver peroxisomal catalase enzyme in male albino rats. *European Review for Medical and Pharmacological Sciences*, 17, 75-83.

Mahmoud, R., Aziza, A., Marghani, B., & Eltaysh, R. (2019). Influence of ginger and garlic supplementation on growth performance, whole body composition and oxidative stress in the muscles of nile tilapia (*O. niloticus*). *Advances in Animal and Veterinary Science*, 7(5), 397-404.

Mao, Q. Q., Xu, X. Y., Cao, S. Y., Gan, R. Y., Corke, H., Beta, T., & Li, H. B. (2019). Bioactive Compounds and Bioactivities of Ginger (*Zingiber oficinale*). *Foods*, 8, 1-21.

Maqsood, S., Singh, P., Samoon, M. H., & Munir, K. (2011). Emerging role of immunostimulants in combating the disease outbreak in aquaculture. *International Aquatic Research*, 3, 147-163.

Martins, A. P., Salgueiro, L., Goncalves, M. J., Proenca, C. A., & Vila, R. (2001). Essential oil composition and antimicrobial activity of three Zingiberaceae from *S. tome*. *Planta Medicine*, 67, 580-584.

Metaxa, E., Deviller, G., Pagand, P., Alliaume, C., Casellas, C., & Blancheton, J. P. (2006). High-rate algal pond treatment for water reuse in a marine fish recirculation: water purification and fish health. *Aquaculture*, 252, 92-101.

Misra, C. K., Das, J., Pradhan, P., Pattnaik, S., Sathi, S., & Mukherjee, S. C. (2004). Changes in lysozymal enzyme activity and protection against Vibrio infection in *Macrobrachium rosenbergii* post larvae after bath immunostimulation with b-glucan. *Fish and Shellfish Immunology*, 17, 389-395.

Mohammadi, G., Rashidian, G., Hoseinifar, S. H., Naserabad, S. S., & Van, D. H. (2020). Ginger (*Zingiber officinale*) extract affects growth performance, body composition, haematology, serum and mucosal immune parameters in common carp (*Cyprinus carpio*). *Fish and Shellfish Immunology*, 99, 267-273.

Mohammed, E., Kamel, M., El- Iraqi, K., Tawfik, A. M., Khattab, M. S., & Elsabagh, M. (2020). *Zingiber officinale* and *Glycyrrhiza glabra*, individually or in combination, reduce heavy metal accumulation and improve growth performance and immune status in Nile tilapia, *Oreochromis niloticus*. *Aquaculture Research*, 51, 1933-1941.

Monoppo, H., Kolopita, F. E. M., & Malatunduh, R. (2016). Growth promoting effect of garlic (*Allium sativum*) on carp (*Cyprinus carpio*). *International Journal of Pharmacology Technical Research*, 9(4), 283-88.

Moon, Y., Lee, H., & Lee, S. (2018). Inhibitory effects of three monoterpenes from ginger essential oil on growth and aflatoxin production of *Aspergillus flavus* and their gene regulation in aflatoxin biosynthesis. *Applied Biological Chemistry*, 61, 243-250.

Mukherjee, K. L. (1988). *Medical Laboratory Technology*. Vol 1 pp. 242-43. Tata Mc-Graw hill publishing company Ltd.

Muzaffar, F., Ansal, M. D., & Dhawan, A. (2017). Effect of Garlic (*Allium sativum*) supplemented feed on survival and growth of common carp (*Cyprinus carpio*). *Indian Journal of Animal Nutrition*, 34, 91-93.

Nile, S. H., & Park, S. W. (2015). Chromatographic analysis, antioxidant, anti-inflammatory, and xanthine oxidase inhibitory activities of ginger extracts and its reference compounds. *Industrial Crops and Products*, 70, 238-244.

Nishikimi, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochemical and Biophysical Research Communications*, 46, 849-64.

Nishiyama, T., Mae, T., Kishida, H., Tsukagawa, M., Mimaki, Y., Kuroda, M., Sashida, Y., Takahashi, K., Kawada, T., Nakagawa, K., & Kitahara, M. (2005). Curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa*) suppress an increase in blood glucose level in type 2 diabetic KK-Ay mice. *Journal of Agricultural and Food Chemistry*, 53, 959-963.

Niu, Y., Norouzitallab, P., Baruah, K., Dong, S., & Bossier, P. (2014). A plant-based heat shock protein inducing compound modulates host-pathogen interactions between *Artemia franciscana* and *Vibrio campbellii*. *Aquaculture*, 430, 120-7.

Nya, E. J., & Austin, B. (2009). Use of garlic, *Allium sativum* to control *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, 32, 963-70.

Olaniyi, C. O., Atoyebi, M. O., Obafunmiso, H. T., & Salaam, K. A. (2020). Effect of ginger (*Zingiber officinale*) in the nutrition of african catfish- a cholesterol reducer and fertility enhancer. *International Journal of Aquaculture and Fishery Sciences*, 6, 21-28.

Ozcan, T., Akpinar-Bayizit, A., Yilmaz-Ersan, L., & Delikanli, B. (2014). Phenolics in human health. *International Journal of Chemical Engineering and Applications*, 5, 393-96.

Palmero, F. A., Cocci, P., Angeletti, M., Felici, A., Polzonetti-Magni, A. M., & Mosconi, G. (2012). Dietary aloe vera components effects on cholesterol

lowering and estrogenic responses in juvenile goldfish, *Carassius auratus*. *Fish Physiology and Biochemistry*, 39, 851-61.

Parameswaran, S., Krishan, R. S., Selvovary, C., & Buyan, B. R. (1971). Fish yield from Assam ponds kept under different experimental conditions. *Indian Journal of Fisheries*, 18, 67-83.

Parihar, M. S., Javeri, T., Hemnani, T., Dubey, A. K., & Prakash, P. (1997). Responses of superoxide dismutase, glutathioneperoxidase and reduced glutathione antioxidant defences in gills of the freshwater catfish (*Heteropneustes fossills*) to short-term elevated temperature. *Journal of Thermal Biology*, 22(2), 151-156.

Parma, M. J., Loteste, A., Campana, M., & Bacchetta, C. (2007). Changes of hematological parameters in *Prochilodus lineatus* exposed to sublethal concentration of cypermethrin. *Journal of Environmental Biology*, 28, 147-149.

Parry, R. M., Chandau, R. C., & Shahani, R. M. (1965). A rapid and sensitive assay of muramidase. *Proceedings of the Society for Experimental Biology and Medicine*, 119, 384-86.

Patriche, T., Patriche, N., & Tenciu, M. (2009). Cyprinids total blood proteins determination. *Scientific Papers, Animal Science and Biotechnologies*, 42, 95-101.

Payung, C. N., Tumbol, R. A., & Manoppo, H. (2017). Dietary ginger (*Zingiber officinale*) enhance resistance of Nile tilapia (*Oreochromis niloticus*) against *Aeromonas hydrophila*. *Aquaculture, Aquarium, Conservation & Legislation International Journal of the Bioflux Society*, 10(4), 962-968.

Placer, Z. A., Cushman, L. L., & Johnson, B. C. (1966). Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Annals of Biochemistry*, 16, 359-64.

Platel, K., & Srinivason, K. (2000). Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. *Nahrung*, 44, 42-46.

Poongodi, R., Bhavan, P. S., Muralisankar, T., & Radhakrishnan, S. (2012). Growth promoting potential of garlic, ginger, turmeric and fenugreek on the freshwater prawn *Macrobrachium rosenbergii*. *International Journal of Pharma and Bio Sciences*, 3(4), 914- 926.

Power, D. M., Llewellyn, L., Faustino, M., Nowell, M. A., Bjornsson, B. T., Einarsdottir, I. E., Canario, A. V. M., & Sweeney, G. E. (2001). Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology*, 130, 447-459.

Prasad, S., & Tyagi, A. K. (2015). Ginger and its constituents: role in prevention and treatment of gastrointestinal cancer. *Gastroenterology Research and Practice*, 1-11.

Radhakrishnan, E. K., Bava, S. V., Narayanan, S. S., Nath, L. R., Thulasidasan, A. K. T., Soniya, E. V., & Anto, R. J. (2014). [6]-Gingerol induces caspase-dependent apoptosis and prevents pma-induced proliferation in colon cancer cells by inhibiting Mapk/Ap-1 signaling. *Plos One*, 9(8), 1-13.

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMed Research International*, 761264.

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. *Aquaculture*, 433, 50-61.

Roberts, R. J., Agius, C., Saliba, C., Bossier, P., & Sung, Y. Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *Journal of Fish Disease*, 33, 789-801.

Roeschlau, P., Bernt, E., & Gruber, W. (1974). Enzymatic determination of total cholesterol in serum. *Journal of Clinical Chemistry and Biochemistry*, 12, 226-22.

Roh, J. S., & Sohn, D. H. (2018). Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Network*, 18, 1-14.

Roo, J., Hernandez-Cruz, C. M., Mesa-Rodriguez, A., Fernandez-Palacios, H., & Izquierdo, M. S. (2019). Effect of increasing n-3 HUFA content in enriched Artemia on growth, survival and skeleton anomalies occurrence of greater amberjack Seriola dumerili larvae. *Aquaculture*, 500, 651-659.

Roy, S., Kumar, V., Bossier, P., Norouzitallab, P., & Vanrompay, D. (2019). Phloroglucinol treatment induces transgenerational epigenetic inherited resistance against Vibrio infections and thermal stress in Brine Shrimp (*Artemia franciscana*) model. *Frontiers in Immunology*, 10, 2745.

Sahan, A., Ozutok, S., & Kurutas, B. E. (2016). Determination of some hematological parameters and antioxidant capacity in nile tilapia (*Oreochromis niloticus*) fed ginger (*Zingiber officinale*) to *Aeromonas hydrophila*. *Turkish Journal of Fisheries and Aquatic Sciences*, 16, 197-204.

Sahli, T. (1962). *Textbook of chemical pathology*. In Scward E (Ed.) pp. 35. Williams and Williams C. Baltimore, USA.

Sahu, S., Das, B. K., Mishra, B. K., Pradhan, J., & Sarangi, N. (2007a). Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Journal of Applied Ichthyology*, 23, 80-86.

Sahu, S., Das, B. K., Mishra, B. K., Pradhan, J., Samal, S. K., & Sarangi, N. (2008). Effect of dietary *Curcuma longa* on enzymatic and immunological profiles of rohu, *Labeo rohita* (Ham.), infected with *Aeromonas hydrophila*. *Aquaculture Research*, 39, 1720-30.

Sahu, S., Das, B. K., Pradhan, J., Mohapatra, B. C., Mishra, B. K., & Sarangi, N. (2007b). Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. *Fish and Shellfish Immunology*, 23, 109-18.

Sampath, C., Rashid, M. R., Sang, S., & Ahmedna, M. (2017). Specific bioactive compounds in ginger and apple alleviate hyperglycemia in mice with high fat diet-induced obesity via Nrf2 mediated pathway. *Food Chemistry*, 226, 79-88.

Sankaran, K., & Gurnani, S. (1972). On the variation in the catalytic activity of lysozyme in fishes. *Indian Journal of Biochemistry and Biophysics*, 9, 162-165.

Santra, S., Sihna, A., & Mondal, C. (2016). Effect of Herbal plant (garlic) against common disease in gold fish, *Carassius auratus*. *International Journal of latest Technology in Engineering, Management and Applied Science*, 5(3), 42-44.

Schadich, E., Hlavac, J., Volna, T., Varanasi, L., Hajduch, M., & Dzubak, P. (2016). Effects of ginger phenylpropanoids and quercetin on Nrf2-ARE pathway in human BJ fibroblasts and HaCaT keratinocytes. *Biomedical Research International*, 1-6.

Secombes C. J., & Olivier G., (1997). Host pathogen interaction in samonids. In: E. M., Bernoth, A. E., Ellis, P. J., Midtlying, G., Olivier & P. Smith (Eds.) *Furunculosis: Multidisciplinary Fish Disease Research* (pp. 269-296) Academic Press, New York.

Shakya, S. R., (2015). Medicinal uses of ginger (*Zingiber officinale* Roscoe) improves growth and enhances immunity in aquaculture. *International Journal of Chemical Studies*, 3(2), 83 -7.

Shaluei, F., Nematollahi, A., Naderi-Farasani, H. R., Rahimi, R., & Kaboutari, K. J. (2017). Effect of ethanolic extract of *Zingiber officinale* on growth performance and mucosal immune responses in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, 23, 814-821.

Shanthi, R., Bhavan, P. S., & Radhakrishnan, S. (2012). Influence of medicinal herbs (*Andrographis paniculata*, *Cissus quadrangularis* and *Eclipta alba*) on growth, digestive enzymes, biochemical constituents and protein profile of the freshwater prawn *Macrobrachium rosenbergii*. *Elixir Biotechnology*, 42, 6478-84.

Shanthi, S., Manju, S., Rajakumaran, P., & Vaseeharan, B. (2014). Molecular cloning of peroxinectin gene and its expression in response to peptidoglycan and

Vibrio harveyi in indian white shrimp *Fenneropenaeus indicus*. *Cell Communication and Adhesion*, 21, 281-289.

Sharma, A., Deo, A. D., Riteshkumar, S. T., Chanu, T. I., & Das, A. (2010). Effect of *Withania somnifera* root as a feed additive on immunological parameters and disease resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. *Fish and Shellfish Immunology*, 29, 508-12.

Shivendra, K., Sahu, N. P., Pal, A. K., Subramanian, S., Priyadarshi, H., & Kumar, V. (2011). High dietary protein combats the stress of *Labeo rohita* fingerlings exposed to heat shock. *Fish Physiology and Biochemistry*, 37, 1005-1019.

Singh, N., Tailang, M., & Mehta, S. C. (2016). A Review on Herbal Plants as Immunomodulators. *International Journal of Pharmaceutical Science and Research*, 7(9), 3602-10.

Sivaram, V., Babu, M., Immanuel, G., Murugadass, S., Citarasu, T., & Marian, M. (2004). Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. *Aquaculture*, 237, 9-20.

Soowannayan, C., Boonmee, S., Puckcharoen, S., Anatamsombat, T., Yatip, P., Ng, W. K., Thitamadee, S., Tuchinda, P., Munyoo, B., Nuangsaeng, B., Sonthi, M., & Withyachumnarnkul, B. (2019). Ginger and its component shogaol inhibit Vibrio biofilm formation in vitro and orally protect shrimp against acute hepatopancreatic necrosis disease. *Aquaculture*, 504, 139-147.

Sorgeloos, P., Lavens, P., Leger, P., Tackaert, W., & Versichele, D. (1986). *Manual for the Culture and Use of Brine Shrimp Artemia in Aquaculture.* Artemia Reference Center. Ghent University, Gent, Belgium.

Spranger, J., Kroke, A., Mohlig, M., Hoffmann, K., Bergmann, M. M., Ristow, M., Boeing, H., & Pfeiffer, A. F. (2003). Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes*, 52(3), 812-817.

Sritunyalucksana, K., Wongsuebsantati, K., Johansson, M. W., & Soderhall, K. (2001). Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. *Developmental and Comparative Immunology*, 25, 353-363.

Srivastava, A., Ansal, M. D., & Khairnar, S. O. (2019). Effect of amla (*Phyllanthus emblica*) fruit powder supplemented feed on growth performance and proximate composition of an Indian major carp, *Labeo rohita* fingerlings. *Journal of Entomology and Zoology Studies*, 7(3), 955-959.

Suk, S., Kwon, G. T., Lee, E., Jang, W. J., Yang, H., Kim, J. H., Thimmegowda, N. R., Chung, M., Kwon, J. Y., & Yang, S. (2017). Gingerenone, a polyphenol present in ginger, suppresses obesity and adipose tissue inflammation in high-fat diet-fed mice. *Molecular Nutrition Food Research*, 61(10), 1-15.

Sukumaran, V., Park, S. C., & Giri, S. S. (2016). Role of dietary ginger *Zingiber officinale* in improving growth performance and immune functions of *Labeo rohita* fingerlings. *Fish and Shellfish Immunology*, 57, 362-70.

Swain, S., Pawase, A. S., Pai, R. K., Tibile, R. M., Indulkar, S. T., & Pawar, R. A. (2018). Effect of ginger (*Zingiber officinale* Roscoe) incorporated diet on growth performance of striped catfish, *Pangasianodon hypophthalmus*. *Journal of Entomology and Zoology Studies*, 6(4), 1094-1098.

Swingle, H. S. (1967). Standardization of chemical analysis of waters and pond muds. *FAO Fish Reproduction*, 44(4), 397-21.

Syed-Ali-Fathima, K. M., Annalakshmi, T., Innocent, B. X., & Karthikayan, K. (2014). Assess the efficacy of ethanol extract of Aloe vera as immunostimulant to fish silver carp, post challenged with *Pseudomonas aeruginosa*. *International Journal of Advanced Life Sciences*, 7(3), 500-507.

Talpur, A. D., Ikhwanuddin, M., & Ambok, B. A., (2013). Nutritional effects on ginger (*Zingiber officinale* Roscoe) on immune response of Asian sea bass (*Lates calcarifer*) and disease resistance against *Vibrio harveyi*. *Aquaculture*, 400, 46-52.

Tan, B. K. H., & Vanitha, J. (2004). Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs: A review. *Current Medicinal Chemistry*, 11, 1423-1430.

Tang, D., Kang, R., Zeh, H. J., & Lotze, M. T. (2011). High-mobility group box-1, oxidative stress, and disease. *Antioxidants and Redox Signaling*, 14 (7), 1315-1335.

Townsend, E. A., Siviski, M. E., Zhang, Y., Xu, C., Hoonjan, B., & Emala, C. W. (2013). Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. *American Journal of Respiratory Cell and Molecular biology*, 48, 157-163.

Tremacoldi, C. R., & Pascholati, S. F. (2002). Detection of trypsin inhibitor in seeds of *Eucalyptus urophylla* and its influence on the in vitro growth of the fungi *Pisolithus tinctorius* and *Rhizoctonia solani*. *Brazilian Journal of Microbiology*, 33, 281-286.

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Annals of Clinical Biochemistry*, 6, 1-24.

Tsabang, N., Ngah, N., Estella, F. T., & Agbor, G. (2016). Herbal medicine and treatment of diabetes in Africa: case study in Cameroon. *Diabetes Case Reports*, 1-6.

Vahedi, A. H., Hasanpour, M., Akrami, R., & Chitsaz, H. (2017). Effect of dietary supplementation with ginger (*Zingiber officinale*) extract on growth, biochemical and hemato-immunological parameters in juvenile beluga (*Huso huso*). *Iranian Journal of Aquatic Animal Health*, 3(1), 26-46.

Vallejos-Vidal, E., Reyea-Lopez, F., Telas, M., & MacKenzie, S. (2016). The response of fish to immunostimulant diets. *Fish and Shellfish Immunology*, 56, 34-69.

Venkataramalingam, K. J., Christopher, G., & Citarasu, T. (2007). *Zingiber officinalis*, an herbal appetizer in the tiger shrimp *Penaeus monodon* larviculture. *Aquaculture Nutrition*, 13, 439-443.

Verma, S. K., Singh, J., Khamesra, R., & Bordia, A. (1993). Effect of ginger on platelet aggregation in man. *The Indian Journal of Medical Research*, 98, 240-242.

Wallin, R. P. A., Lundqvist, A., More, S. H., Bonin, A. V., Kiessling, R., & Ljunggren, H. G. (2002). Heat-shock proteins as activators of the innate immune system. *Trends in Immunology*, 23(3), 130-135.

Walstab, J., Krueger, D., Stark, T., Hofmann, T., Demir, I. E., Ceyhan, G. O., Feistel, B., Schemann, M., & Niesler, B. (2013). Ginger and its pungent constituents non-competitively inhibit activation of human recombinant and native 5-HT3 receptors of enteric neurons. *Neurogastroenterology and Motility*, 25, 439-447.

Wang, X. W., Gao, J., Xu, Y. H., Xu, J. D., Fan, Z. X., Zhao, X. F., Wang, J. X. (2017). Novel pattern recognition receptor protects shrimp by preventing bacterial colonization and promoting phagocytosis. *Journal of Immunology*, 160-170.

Watson, F. L. (2005). Extensive Diversity of Ig-superfamily proteins in the immune system of insects. *Science*, 309, 1874-1878.

Wei, C., Tsai, Y., Korinek, M., Hung, P., El-Shazly, M., Cheng, Y., Wu, Y., Hsieh, T., & Chang, F. (2017). 6-Paradol and 6-shogaol, the pungent compounds of ginger, promote glucose utilization in adipocytes and myotubes, and 6-paradol reduces blood glucose in high-fat diet-fed mice. *International Journal of Molecular Sciences*, 18, 168-172.

Yagi, K. (1984). Assay for plasma lipid peroxidase. *Methods in Enzymology*, 109, 328-33.

Yeh, H., Chuang, C., Chen, H., Wan, C., Chen, T., & Lin, L. (2014). Bioactive components analysis of two various gingers (*Zingiber officinale* Roscoe) and antioxidant effect of ginger extracts. *LWT-Food Science and Technology*, 55, 329-334.

Yuan, C., Li, D., Chen, W., Sun, F., Wu, G., & Gong, Y. (2007). Administration of a herbal immunoregulation mixture enhances some immune parameters in carp (*Cyprinus carpio*). *Fish Physiology and Biochemistry*, 33, 93-101.

Zhang, Z., Yang, Z., Wang, Y., Liao, M., Wei, X., Yu, Y., Rong, X., & Chen, X. (2020). Effectiveness of garden burnet, *Sanguisorba officinalic*, in controlling acute hepatopancreatic necrosis disease caused by infection of *Vibrio parahaemolyticus* in shrimp farming. *Aquaculture*, 531, 1-36.

VITA

Name of the student	**:**	Priya Rawat
Father's Name	**:**	S. P. S Rawat
Mother's Name	**:**	Anita Rawat
Nationality	**:**	Indian
Date of Birth	**:**	28-01-1992
Permanent Home Address	**:**	R-Z-22 Sita Puri, Street No.- 4 Post Office - Palam, New Delhi-110045
E-mail Address	**:**	priyarawat167@gmail.com
EDUCATIONAL QUALIFICATION		
Bachelor Degree	**:**	B.F.Sc.
University	**:**	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana
Year of Award	**:**	2015
OGPA/OCPA/%marks	**:**	8.19/10
Awards/ Distinctions/ Fellowships/Scholarship	**:**	ICAR-NTS, Gold Medal
Master's degree	**:**	M.F.Sc. (Aquaculture)
University	**:**	Central Agricultural University, Imphal
Year of Award		2017
OCPA	**:**	8.90/10
Awards/ Distinctions/ Fellowships/Scholarship	**:**	Institutional Fellowship
Title of Thesis	**:**	Evaluation of Different Feed Attractants on Seed Rearing Performance of *Ompok bimaculatus* (Bloch, 1794)
Ph.D.	**:**	Aquaculture
OCPA	**:**	8.73 /10.00
Awards/ Distinctions/ Fellowships/Scholarship	**:**	Institutional Fellowship in India SERB-DST sponsored OVDF Fellowship in Sweden