Preparation and Characterization of Chitosan Nanoparticles and their Effects on Growth, Immunity and Body Composition of Silver Carp *(Hypophthalmichthys molitrix)*

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Preparation and characterization of chitosan nanoparticles and their effects on growth, immunity and body composition of silver carp *(Hypophthalmichthys molitrix)*

A thesis submitted in partial fulfillment of the requirement for the degree of

Master of philosophy

In

Fisheries and Aquaculture

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Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

NaimaYounus

IN THE NAME OF ALLAH

THE MOST MERCIFUL

THE MOST BENEFICIENT

AND

THE MOST COMPASSIONATE

CERTIFICATE

This dissertation Preparation and Characterization of Chitosan Naanoparticles and their Effects on Growth Immunity and Body Composition of Silver Carp (*Hypophthalmichthys molitrix*). submitted by **Ms. Naima Younus** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in Fisheries and Aquaculture.

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Dedicated

To

My beloved Parents, Teachers

And

Friends

List of Contents

List of Acronym/abbreviations

CNP	Chitosan nanoparticles
FBW	Final body weight
FCE	Feed conversion efficiency
FCR	Feed conversion ratio
IBW	Initial body weight
MS222	Tricaine methane sulphate
PUFAs	Polyunsaturated fatty acids
LDL	Low density lipoproteins
HDL	High density lipoproteins
MP	Moist pellets
AST	Aspartate aminotransferase
COS	Chitooligosaccharides

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Abstract

A 3 months feeding experiment was designed to study the comparative effects of chitosan and its nano form on growth, immunity and body composition of silver carps *Hypophthalmichthys molitrix* under laboratory condition. Chitosan nanoparticles (CNP) of the size ranged between 25.61 to 34.41 nm were prepared through ionotropic gelation method. Uniform sized silver carps, after acclimatization were distributed into three groups. Group 1 was fed 35% protein basal diet; whereas the other two groups, Group-2 and Group-3 were offered chitosan and chitosan nanoparticles supplemented diet respectively at the same rate i.e. 5.0 g/kg. The whole experiment was conducted under semi-static condition in 9 rectangular glass aquaria at a stocking density of 2 g/L. No mortality was observed throughout the feeding trial. At the end of the experiment, fish fed chitosan nanoparticles in the diet showed significantly high ($P<0.001$) % weight gain (WG) and % specific growth rate (SGR) as compared to other groups. Also the % feed conversion efficiency (FCE) was significantly higher ($P<0.01$) in fish fed diet supplemented with CNP. However, no significant difference was observed in growth performances and % FCE of fish fed basal and chitosan enriched diet. Proximate analysis revealed that muscles fats % content was significantly lower ($P<0.05$) in the fish fed chitosan supplemented diet. However, muscle proteins % and moisture% contents were same in all groups while % ash was significantly higher ($P < 0.01$) in muscles of fish fed chitosan and CNP supplemented diets. High red blood cells (RBC's) count and hemoglobin (Hb) level was observed in fish fed chitosan and CNP supplemented diet. An aspartate aminotransferase (AST) and blood glucose level were significantly high in fish fed basal diet as compared to other diets. Moreover, plasma cholesterol level was considerably low ($P<0.05$) in fish reared on a chitosan supplemented diet as compared to CNP enriched and basal diets. Fish fed chitosan and chitosan nanoparticles in the diet showed better immune response after post challenged with *Escherichia coli*. Therefore, WBCs count, lysozyme activity, plasma protein and immunoglobulin were significantly higher in fish fed CNP supplemented diet. The results clearly indicate the positive effect

of CNP as growth and immunostimulant. The Increase in % FCE after CNP supplementation in the diet reduced the feed cost along with that low glucose level, AST activity suggested protective action of CNP against stresses and toxicity in the environment.

INTRODUCTION

Introduction

In this fast growing world, whole global community is facing many problems including economic loss, financial crises and the most important one is the food insecurity and insufficient availability of food resources due to the over exploitation of natural resources (FAO, 2012). Food prices raised up to extreme level in 2008 due to economic crises, led 100 million people at malnutrition level. According to FAO latest reports, about one billion people living worldwide rely on fish as their main protein source.

Fish is considered as the best source of minerals, vitamins and proteins (Hagu, 1992), providing about 20% of total intake of animal protein to about 3 billion people across the world (FAO, 2012). It is considered that it contains all essential amino acids including lysine, tryptophan and methionine as compared to the plant based protein sources (Krajcovicova-kudlackova et al., 2005). Fish meat in human diet, plays an important role in the synthesis of bone marrow, lowering of blood cholesterol level and risk of hypertension, and heart diseases due to the presence of polyunsaturated fatty acids (PUFAs) (Wang et al., 2006), thus keeps the body structure healthy. Worldwide, the fish demand is showing an increasing trend day by day due to increase in human population and the public awareness about the beneficial role of fish in their diet (FAO, 2010).

During the earlier times, the fishing from lakes, rivers, oceans were the main source for fish supply, but due to over exploitation of natural resources, the population of wild fish population falls drastically, leaving the world in situation where captured fish alone could not fulfill the demand of increasing population. Therefore, in 1993, aquaculture start getting importance and efforts were made to increase the fish production by introducing many new trends including increasing stocking density through moving from extensive culture system to intensive or super intensive culture system.

Aquaculture is the rearing of animals and plants living in freshwater, brackish and marine water for the purpose of food. It is believed that aquaculture can restore the wild fish population and relieve the pressure on captured fisheries. Aquaculture is considered as a fastest growing food-producing sector (FAO, 2011), and nowadays, globally it

becomes an industry in many countries. However, in many developing countries, the aquaculture industry is successfully playing a significant role in strengthening of economy and elevating of poverty. During last few decades with the involvement of science and technology, the aquaculture practice now becomes technique and art (Gatlin, 2002). According to the latest report, the annual production of aquaculture increased 12 times at rate of 8.8%. It is estimated that the per capita fish consumption is about 25 kg/year while in some inland countries it is about 50 kg/year. Fisheries and aquaculture activities besides contributing in feeding population also, provided income of about 54.8 million people across the world (FAO, 2012). Hence, apart from the primary production sector, aquaculture is nowadays providing many jobs in different areas like fish harvesting, processing, manufacturing of fish-processing equipment, gears and vessels. Share of aquaculture is dominated by Asia with total 90% of total world's farmed production while China contributes two third of total aquaculture production.

With the advancement of aquaculture, the welfare of farmed fishes has been an area of great attention, because aquaculture activities like handling, capturing, crowding etc induce stress response in fishes which results in increase of mortalities and reduction in productivity (Vazzana et al., 2002). Beside these, in order to increase production, fish farmers are in practice of increasing fish per hector stocking density, therefore switching from extensive towards intensive and super intensive culture system. In such condition fish in captivity are in continuous state of stress that can weaken their immune response.

Nowadays, aquaculture practices were developed by mostly adopting intensive fish farming but unfortunately it is facing many problems mainly due to outbreak of diseases because of alteration in water quality parameters and environmental conditions (Alderman and Hastings, 1998). In 2011, disease outbreak caused a huge loss to the Mozambique shrimp farming, almost wiped out the whole production. Due to the natural disasters in China during 2010, its aquaculture and fisheries industry suffered 17 million tones production loss (FAO, 2012). Thus, wellbeing of farmed fish is influenced by means of certain management factors including feeding.

During intensive culture stresses such as temperature change, handling and poor water quality etc, are directly associated with bacterial infection. These stressors directly interfere with immune system of fish and also disturb hormonal, cellular responses and phagocytosis (Ellis, 1989). During last 20 years, various chemotherapeutics were used to treat bacterial infection (Sakai, 1998). Officially, four antibacterial drugs were accepted in UK for treating bacterial infections in aquaculture. These included oxytetracycline, oxolinic acid, amoxicillin and co-trimazine. These antibiotics were administered through feed, coated on pellets, bath treatment and in some circumstances administered through injections (Alderman and Hastings, 1998). However, usages of these antibiotics sometimes lead to the development of drug resistance in bacterial strain against them (Aoki, 1992) or sometime kill the beneficial bacteria. Different types of immunostimulants such chitin, chitosan, lentinan, levamisole, β-glucan, peptidoglucan and vitamin C are being used to treat bacterial diseases in aquaculture and fisheries. These immunostimulants stimulates natural killer cells, lysozyme and antibody responses through their bactericidal activities (Sakai, 1998).

Immunostimulants activates the lymphocytes as well as stimulates the production of antibodies in fish e.g. when yeast glucan was injected in channel catfish, its showed high serum antibody as compared to the control group in response to the *Edwardsiella ictaluri* (Chen and Ainsworth, 1992). Similarly, there is increased in antibody production against *Aeromonas salmonicidia* in the vitamin C fed group of Atlantic salmon as basal diet (Thompson et al., in 1993). The immunostimulants are not effective against all diseases and their effectiveness depends on dose administration, timings and physiological condition of fish (Sakai, 1998). Thus overdoses, of some of these sometime caused the immune suppression in fish.

Chitin was firstly discovered by French botanist Henri Bracannot (Jung et al., 2008). Structurally chitin consists of N-acetyl glucosamine subunits arranged in anti parallel strands (Romano et al., 2007). The β-1, 4 linkage in chitin makes it rigid and imbalance due to the presence of many hydroxyl groups (1 primary hydroxyl at C-6 and second one at C-3) and amino groups with concomitant tendency for intra- and

intermolecular hydrogen bonds results in formation of linear aggregates with extensive crystallinity (Sanford, 1992 and Li et al., 1992). Although firstly discovered in mushrooms, chitin also occurred in variety of species i.e. amoebae, algae, yeast, fungi, crustaceans, worms etc but absent in vertebrates, plants and prokaryotes (Sandford, 2004). It is colorless, hard, nitrogenous polysaccharide with 5-15% degree of deacetylation (Madhavan, 1992; Kurita, 2001). Molecular weight of chitin is about 1.03×10^6 to 2.5×10^6 Dalton (Lee, 1974). It is non toxic, analgesic, hypocholesteroloemic, antifungal immunologic and anti-tumor (Okamoto et al., 1975).

Chitosan is de-*N*- acetylated analog of chitin consists of linear of β-1, 4 linked GlcN and GlcNAc units. The acetylation process under heterogeneous conditions provides block-wise distribution whereas under homogenous conditions provides random distribution of acetyl groups in chitosan (Tolaimate et al., 2000). Chitosan is fiber like cellulose but unlike plant fiber it has amino group at C2 position instead of hydroxyl group (-OH) present in cellulose that increased its biological reactivity (Rout, 2001). Applications of chitosan depend upon certain characteristics including degree of deacetlylation, molecular mass, viscosity and percentage of dry matter. Solubility of chitosan depends upon the distribution of the acetyl groups, distribution of the monomers along the chain, the flexibility of the chain, branching, charge density, and molecular weight (Kurita, 1986). Chitosan is water soluble when it is prepared from chitin under homogeneous conditions while under heterogeneous conditions; chitosan is soluble only in weak acid i.e., acetic acid (Peniche and Peniche, 2010). Its importance and applications in wastewater treatment, pharmaceutical (Dias et al., 2008), biomedical industries (Carlson et al., 2008), agriculture (Hirano, 1996), (Ratheret al., 2011) and textile (El Tahlawy et al., 2005) are continuously increasing.

Chitosan is used as stabilizer e.g. during frozen storage, 2% chitosan coating enhanced the quality and shelf life of fish, *Ctenopharyngodon idellus* and *Hypophthalmichthys molitrix* for thirty days as compared to controls. The total viable count in chitosan coated group was greatly reduced indicating the inhibitory effect of chitosan on spoilage bacteria (Wenjiao et al., 2009). It was reported that Chitosan helped in the regeneration of skin and healing of wound (Bartone and Adickes, 1988), but α-and

β chitosan showed variability in their activity (Ramesh and Maridass, 2010). Its supplementation improved the wound closure in carp *Cyprinus carpio* but wound was completely healed on 28th day in case of α-chitosan supplemented group while only 40% in case of β-chitosan supplemented group.

Recently it was proved that by changing the size of materials from bulk to nano form, the physiological and chemical properties e.g. solubility, diffusion, reactivity of material also changed (Alishahi et al., 2011; Wang and Li, 2010; Rather et al., 2011; Cha et al., 2008). The U.S. National Nanotechnology Initiative (NNI) defined nanotechnology as the synthesis of materials at nanometer scales i.e. reduction of material size up to less than 100 nm or at atomic and molecular level. The nano form changes the electrical, optical and mechanical properties of material. It is a highly advanced technology possessing huge advancements in nanosciences, nano medicines, food packaging, drug delivery etc. Nanotechnology is not a separate field, instead of this, this scientific field is providing stand for a range of other disciplines i.e. physics, biology, fisheries, chemistry, neurosciences etc (ETC Group, 2003). Nanotechnology also includes the manufacturing of nanoparticles and nano nanofabrication (Maynard, 2006).

Nanotechnology is getting importance even in every field of life. Nano food packing is the most recent and commercialized technique being used nowadays. These techniques involve nanosensors which detect the food spoilage, presence of microorganism and toxic proteins. In agriculture sector, nano pesticides are used which contains nano scale chemical toxins. These nano pesticides are easily available and highly soluble in water and are highly toxic as compared to the bulk form of that molecule (Kuzma and Verhage, 2006). Nano encapsulation caused the controlled release of toxins in the insect's digestive systems under alkaline environment or under specific moisture and heat levels (FoE, 2008). In food processing, nano encapsulation is used for controlled release of nutrients e.g. nano encapsuled fish oils are being developed to enhance the stability, bioavailability and transparency of food components (Zimet and Livney, 2009).

Nanotechnology has greater applications in different fields like fisheries, aquaculture, aquatic environment management, food preservation and medicines (Rather et al., 2011). Nano packaging of fish for preservation for longer time is already being used where natural nanoscale polymers which are biodegradable (De Azeredo, 2009) like chitosan, cellulose and starch are in practice (Thompson et al., 2004). These nano packaging are antifungal and antibacterial in nature e.g. silver nano coating protects the surface from bacterial and fungal attack for longer period of time hence increasing the shelf life of fish for longer period of time during storage and keeping all nutritional values of food during that time (Moraru et al., 2003). In aquaculture, nanoscale mineral supplementations are used which are easily available and absorbed in body without excessive loss in fecal matter (Carriquiriborde et al., 2004). Some plant based feed ingredients in fish feed contains anti-nutritional factors which depress the fish growth and metabolic activities, so nano material can provided the alternative form of feed supplementation (Berntssen et al., 2010). Nanoparticles enhance the growth of fish e.g. when young carp and sturgeon were supplemented with these iron nano particles, they showed better growth performances as compared to the control group. Similarly, when Se- nano particles were supplemented to the crucian carps *Carassius auratus gibelio* in feed, they showed better growth rate, relative weight gain and anti oxidant activity (Zhou et al., 2009). Moreover, Mooney and Cromwell (1995) reported that chromium loaded chitosan nanoparticles enhanced the growth and lipid metabolism in pigs.

Keeping in view of the importance of nano technology in this fast growing world, scientists adopted different techniques to prepare chitosan nanoparticles. These CNP are smaller in size, spherical in shape and due to large surface area they are bioavailable and easily absorbed across the body (Amar et al., 2004; Liu et al., 2007; Paolicelli et al., 2009). Four methods were reported for the preparation of CNP including microemulsion, Ionotropic gelatin, polyelectrolyte complex and emulsification solvent diffusion (Sailaja et al., 2010). In aquaculture, gold and silver nano sensors were used to detect the pathogens (Patolsky et al., 2004).

It was experimentally proved that chitosan enhanced the intestinal absorption by opening the tight junctions of cell membranes (Artursson et al., 1994). Being mucoadhesive and biodegradable the products of enzymatic degradation of chitosan are non toxic in nature (Nagahama et al., 2008). By nature, chitosan possess antibacterial, antiviral antitumor activities. Chitosan nanoparticles are used for encapsulation of drug for better delivery of medicine at targeted location and controlled release of it (Bansal et al., 2011). This process of nano encapsulation of drugs includes ionotropic gelatin, spray drying chitosan coating and emulsion phase separation. For usual drug delivery systems, oral, nasal and pulmonary administrations are used (Thanou and Junginger, 2005). During oral administration of drugs, there is chance of enzymatic degradation while passage through the gastrointestinal tract. Also for the better absorption of medicine across the intestinal membrane, drug has to overcome the mucous layer and epithelial absorption barrier. By encapsulating the drug in chitosan nanoparticles protects it from these hurdles (Peniche and Peniche, 2010).These nanoparticles are mucoadhesive and they increased the residence time of drug in the digestive tract hence increasing the bioavailability of drug (Prashanth and Tharanathan, 2007).

Chitosan nanoparticles were also used for the encapsulation of vitamin C. These Vit-C loaded chitosan nanoparticles are heat stable, spherical in shape with smooth surface (Desai and park, 2006). When CNP were used to carry vitamin C in *Oncorhynchus mykiss*, it was observed that due to bio adhesion property of CNPs, chitosan loaded vitamin C stays longer in contact with the epithelial membrane causing the controlled release of vitamin C in the intestine as compared to the non encapsulated vitamin C (Dorkoosh et al., 2003). The mechanism of their activity is related to the fact that at pH less than 6.5, the positive charge on nanoparticles, due to the amino acids groups on CNPs, makes them able to attach to the negative compounds on the epithelial surfaces. Due to this interaction, CNPs cause to open the tight intracellular junctions at epithelial cell membranes (Dudhani & Kosaraju, 2010; Kean & Thanou, 2010). Innate immunity, lysozyme and complement activity of *Oncorhynchus mykiss* was greatly increased by supplementing vitamin C loaded chitosan nanoparticles as compared to the control group (Alishahi et al., 2011).

Recently, the antimicrobial activities of chitosan nanoparticles were experimentally proved by (Luis et al., 2011). They used chitosan nanoparticles against *Streptococcus mutans* biofilms. Oral bacterial biofilms are commonly naturally occurring in mouth and are associated with diseases especially dental, gum inflammation and degradation of periodontal tissues (Marsh, 2004). Scientist used novel anti microbial approach of chitosan nanoparticles in oral care products. The antimicrobial activity of chitosan depends on its molecular weight (Rabea et al., 2003). Low molecular weight chitosan showed high anti microbial activity against *S. mutans* biofilms. The binding of positively charged nanoparticles with negatively charged *S. mutans* cell membrane leads to the leakage of intracellular constituents leading to death of it (Luis et al., 2011). Chitosan is equally effective against gram-positive and gram-negative bacteria, hence showing its broad spectrum antibacterial activity (Kumar et al., 2005).

In intensive aquaculture practices, one or more species are reared at high densities. Due to overcrowding, infectious diseases and disease causing pathogens are easily transferred. Disease outbreaks cause the economic loss, reducing meat quality and reduced profit margins (Smith et al., 2003). This problem could be overcome by good management practices, nutrition, soil, water and environment management. Due to mismanaged aquaculture practices, pathogens become established in animal, causing diseases. Antibiotics were used earlier for the treatment of bacterial infection but due to antibiotics resistant bacteria and consumer hesitation towards antibiotic treated fish, scientists introduced the use of immunostimulants in fish feed. These immunostimulants enhance the non-specific cellular and humoral defense mechanism in fish (Verlhac et al., 1998; Esteban et al., 2001).

Chitosan is a pseudonatural polymer which is used as immunostimulant to protect the fish against bacterial disease (Anderson and Siwicki 1994; Paramá et al., 2005; Cha et al., 2008). Non-specific defense mechanisms are important in fish and in other vertebrates. Antibiotics, drugs etc are effective against bacterial and other infections but they are effective only for short time (Stoskopf, 1993). Uses of immunostimulants rapidly

activate the fish non-specific defense mechanism to protect fish against pathogens (Siwicki et al., 1994).

In 2008, Cha and his colleagues studied the effect of chitosan coated diet on the innate immunity of olive flounder *Paralichthys olivaceus*. They used 1% chitosan to make moist pellets (MP) along with 1% tamarind gum, 0.5% vitamin, 0.3% glucosamine sulphate, 0.1% chitosan oligosaccharide and 0.5% asthaxanthin. These chitosan coated pellets in feed increased the serum lysozyme activity, total protein, hemoglobin and high density lipoproteins HDL in fish, while lowering the low density lipoproteins LDL and AST value which is indicator of liver toxicity. Chitosan coated diet (1%) also influenced positively on some of the water quality parameters e.g. chemical oxygen demand and suspended solid values were significantly reduced ($P < 0.05$) in chitosan coated group as compared to control. Many other scientists also used chitosan as immunostimulant e.g. *Cyprinus carpio koi* (Maqsood et al., 2010; Gopalalaknnan and Arul, 2006), Cobia *Rachycentron canadum* (Geng et al., 2011) and tiger shrimp *Penaeus monodon* (Niu et al., 2013).

Furthermore, Lin et al. (2012) used chitooligosaccharides (COS) to enhanced the innate immunity of *Trachinotus ovatus*. The COS was produced from chitosan by chemical and enzymatic decomposition. It was observed that COS supplementation in fish enhanced its immunity i.e. highest WBC's, neurophil and monocyte count as compared to control group. By increasing the COS concentration, SGR and FCR were also improved significantly ($P < 0.05$), however no significant difference in survival rate of control and treated group was observed. In another study, *Cyprinus carpio* was offered with 0.2% COS (Lin et.al, 2012) and the effects of COS supplementation in enhancing immunity, growth was compared with control group. It was reported that effects of immunostimulants on growth performances depend on dosage, molecular weight of immunostimulants, duration of dosage given in feed, temperature and species (Dalmo and Bogwald, 2008). Over dosage of these immunostimulants may cause immune suppression i.e. decreasing of immune function (Castro et al., 1999).

The effect of dietary chitosan on fish growth was evaluated by different scientists and they reported variable results. Gopalakannan and Arul (2006) reported that 1% chitosan supplemented diet enhanced the growth performance of *Cyprinus carpio*. Similarly, 10% increased in growth of olive flounder *Paralichthys olivaceus* was observed by supplementing chitosan coated moist pellets (Cha et al., 2008). Furthermore growth rate was enhanced in *Labeo rohita* when supplemented with 1% chitosan in diet (Asthi et al., 2013). According to Niu et al. (2013), chitosan enhanced the growth performance of *Penaeus monodon* in dose dependent manner; therefore, supplementation level up to 0.3% in feed enhanced the growth rate, while further increasing chitosan dose decreased the growth performance i.e. final body weight. This was due to the fact that chitosan enhance the digestion and absorption of nutrients at lower level (Gopalakannan and Arul, 2006). Although many investigators used chitosan supplemented diet but reported variable results like according to Kono et al. (1987) dietary chitosan (10%) exerts no positive effect on the growth performance of red sea bream, Japanese eel and yellowtail, whereas some other studies reported negative effects of chitosan on the growth performance of fish (Shiau and Yu, 1999; Wang and Li, 2011). This depressed growth rate was due to the fact that chitosan interfere with the digestion and absorption of fats in small intestine as a result of which, they are excreted out of the body (Deuchi et al., 1994).

Chitosan is hypocholesterolemic in nature which is due to its high viscosity, polymeric nature, high water binding properties and non-digestibility in upper digestive tract and low water binding in the lower digestive tract (Riccardo and Muzzarelli, 1996). It was observed that some soluble dietary fibers with their increased viscosity cause to increase in the thickness of intestinal lumen boundary those results in reduced lipid absorption (Johnson, 1981; Furda, 1990). Chitosan also affects the metabolism of intestinal bile acids in rats (Fukada et al., 1991). Ammonium ions associated with the polyglucosamine chains shows high anion-exchange capacity and consequently bile acid binding capacity (Sugano et al., 1980). Amino sugar of chitosan interacts with bile acid and cholesterol in the intestinal lumen and stimulating the fecal excretion of steroids (Sugano et al., 1988).

When administered orally and intravenously in rabbits, chitosan did not accelerate the restoring of normal serum cholesterol level in high serum cholesterol rabbits. Orally administered chitosan depressed the absorption of cholesterol in the intestine showing the hypocholesterolemic action site in the digestive system (Hirano and Akiyama, 1995). Orally administered chitosan significantly reduced the total serum cholesterol level along with low density lipoproteins (LDL). In humans, chitosan dose up to 1.2 g/day (Bokura and Kobayashi, 2003) significantly lowered the cholesterol and LDL, thus lowering the risk of coronary heart diseases (Rackley, 2002). However chitosan supplementation did not reduce the cholesterol synthesis, it only hindered the absorption of fats in the small intestine (Bokura and Kobayashi, 2003). Polycationic nature of chitosan caused the binding of polyglucosamine to lipid micelles in the alkaline fluids in the upper part of small intestine as a result of this; fat absorption was reduced in small intestine (Sugano et al., 1978). The cholic acid is synthesized from blood cholesterol, therefore low serum cholesterol level results in decrease of circulating cholic acid to the liver in grass shrimp *Penaeus monodon* (Shiau and Yn, 2011).

Chitosan influences the movement of nutrients along the gastrointestinal tract. The suitable dietary chitosan might enhance the protein synthesis in the body (Gopalkannan and Arul, 2006) resulting better growth performance while decreased lipid contents, thus effecting the overall body composition of fish. Innate immunity of fish is commonly determined by blood serum lysozyme activity (Cecchini et al., 2000). It was well documented that lysozyme is involved in lysis of bacteria through their lyctic activity. It is measured through Turbimetric assay, based on lysis of *Micrococus lysodeikticus* (Balfry and Iwama, 2004). Many scientists observed that different supplementation levels of chitosan increased the serum lysozyme activity in grass shrimp *Penaeus monodon* (Niu et al., 2013), *Cyprinus carpio koi* (Maqsood et al., 2010; Gopalalaknnan and Arul, 2006) and Cobia *Rachycentron canadum* (Geng et al., 2011)

Keeping in view wide applications of chitosan along with increasing importance of nanotechnology days by day, the present research project was designed to investigate

the importance of chitosan nanoparticles in enhancing fish growth performance, immunity and overall body composition of silver carp, *Hypophthalmichthys molitrix*, under laboratory conditions . Silver carp was supplemented at the same quantity of chitosan and chitosan nanoparticles. The silver carp was selected as model species as it is an exotic and freshwater culturable species in Pakistan and China. This species is popular among farmers due to its faster growth rate, high feed efficiency ratio, high nutritional value as well as easy cultivation. Silver carp has strong odor and taste and cheap in price compared to major carps. Although, it is most popular in culture system but it is more vulnerable to stress during routine aquaculture practice that affects its growth and production. Therefore, efforts are going on to enhance its immunity. The objectives of our research were manifold; (a)To prepare chitosan nanoparticles through ionotropic gelation method and their characterization through X-rays diffraction and scanning electron microscope, (b) To compare the efficiency of chitosan and chitosan nanoparticles enriched diets by growth performance of silver carp , (c) To investigate immune responses of these silver carp i.e. WBC's count, lysozyme activity and immunoglobulin after challenged with *E.coli*, (d) To study the effect of chitosan and its nanoparticles enriched diet on hematological parameters and body composition of fish.

MATERIALS AND METHODS

Silver carp, *Hypophthalmichthys molitrix* fingerlings were obtained from Faisalabad Nursery Unit. The average weight of fish was 6.0±0.5g. They were transported live in aerated plastic bags to the Fisheries and Aquaculture facility in the Department of Animal Sciences, Quaid-i-Azam University, Islamabad. The water from bags was gradually replaced with de-chlorinated water. Initially fishes were kept in circular fiber tank having flow through system. They were acclimated for two weeks and during that period they were offered prepared 35% protein basal diet.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by Ionotropic gelation method, firstly reported by Calvo et al. (1977) and modified by Du et al. (2009). Briefly, 1ml of acetic acid was dissolved in 99 ml distilled water to prepare 1% acetic acid solution. Then 250 mg of chitosan was added to 1% acetic acid solution. The pH of the mixture was maintained at 5.6 with the help of sodium hydroxide (NaOH). Then under magnetic stirring, 0.1% (w/v) sodium tripolyphosphate (STPP) was added drop wise for 35 minutes. At room temperature, bluish color suspension (nanoparticles) was formed quickly. Formed nanoparticles were centrifuged at 6000 rpm for 40 minutes and supernatant was discarded. Nanoparticles were freeze-dried for further use in fish feed.

Characterization of Chitosan nanoparticles

X-rays diffraction technique

XRD of chitosan nanoparticles was carried out from the Department of Chemistry, Quaid-i-Azam University Islamabad. It is non-destructive and analytical technique for identification of different solid and crystalline substances. In XRD technique, normally radiation used has wavelength ranges between 0.7 and 2.3 Å because this range of wavelength is very close to inter planar spacing of most crystalline materials. Collimated beam of X-rays is made incident on a specimen and is diffracted by the crystalline phase present in the specimen. The intensity of the diffracted X-rays is measured as a fraction of diffraction angle, and specimen's orientation. This diffraction pattern is used to identify the specimen's crystalline phases and other structural properties. This technique is used for measuring metal particle size.

Scanning electron microscope (SEM)

Scanning electron microscope (JSM-6490-A, Japan) was used to find out the size, shape and surface morphology of chitosan nanoparticles at National University of Science and Technology (NUST) Islamabad. Scanning electron microscope (SEM) is one of the supreme extensively used diagnostic of present science that permits the study of composition of biological and physical materials and surface. Magnification of SEM was 50,000 X.

Diet preparation

Formulation of basal and experimental diet is presented in Table 1. Chitosan (75% deacylated) was purchased from Sigma, Germany in white powder form. All dry ingredients i.e. rice polish, while fish mal (55% crude protein), wheat bran, 30% gluten, soybean meal (46.2 % crude protein), DCP, CMC and vitamin premix were grinded in a grinder along with chitosan and its nanoparticles in their respective diets at rate of 5.0 g/kg diet.

All ingredients were then mixed with oil and paste was made with water. After that paste was passed through a meat grinder and pellets were formed. Formed pellets were then oven dried, saved in Ziploc bags and then stored in refrigerator. Three experimental diets, basal diet (Control), chitosan supplemented (Ch) and chitosan nanoparticles supplemented (CNP) diets were fed twice daily at 10% body weight.

Experimental design

After acclimatization, 90-day feeding trial was conducted. Healthy and uniformed sized fishes with individual average weight 6g were selected by using an electronic top- loading balance and evenly distributed into 9 glass aquaria (60×30×30 cm) at stocking density of 2 g/L. Experiment was executed in triplicate by dividing fish into following groups.

Group 1: Control fish, fed with 35% protein in diet

Group 2: Fish fed 35% protein basal diet supplemented with Chitosan (Ch) at the rate of 5 g/kg

Group 3: Fish fed 35% protein basal diet supplemented with chitosan nanoparticles (CNP) at the rate of 5 g/kg diet.

Fishes were offered diet daily at the rate of 10% body weight at the start of experiment. The feeding levels were adjusted after every fourteen days. Daily feed intake was calculated by the calculation of FCR i.e. unconsumed feed was subtracted from the total feed given. Daily fish excreta were removed by simple siphoning and water level was maintained at original level by de-chlorinated water. Dissolved oxygen levels were also monitored with the help of Dissolved Oxygen Meter (Oxi 3205, Germany) on regular basis for the maintenance of oxygen level nearly 5.5 mg/l in three different groups.

Sampling and growth measurements

At the end of experiment after 90 days, 7 fishes were removed from each aquarium and anesthetized with MS-222 (60 mg/l). Blood was withdrawn from caudal puncture and fishes were scarified on ice box. Muscle tissues were removed and immediately deep freeze in liquid nitrogen and then stored at -20°C for biochemical analysis. Haprinized blood was centrifuged at 3500 rpm for 15 minutes and plasma was separated which then stored at -20°C for further analysis of hematological parameters.

Growth performances

After 90 days, growth performances of fishes were assessed by using the following growth parameters.

Weight gain = $W_f - W_i$

Where,

$\mathbf{W_f}$ = Final body weight of fish

$\mathbf{W_i}$ = Initial body weight of fish

% Weight gain (%WG): It was calculated by the following formula:

$$\% \text{ WG} = \frac{W_f - Wi}{Wi} \times 100$$

\

Specific growth rate (SGR): It was calculated by using the following formula:

$$SGR\ (\%) = \frac{(\ln \text{final weight} - \ln \text{initial weight})}{\text{No. of days of experiment}} \times 100$$

Feed conversion ratio (FCR): The FCR was calculated by using the formula:

$$FCR = \frac{\text{Total dry feed fed (g)}}{\text{Total wet weight gain (g)}}$$

Feed conversion efficiency FCE (%): It was calculated by the following formula:

$$FCE\ (\%) = \frac{1}{FCR} \times 100$$

Hematological parameters

Blood parameters like RBCs count, hemoglobin and cholesterol levels were determined in all three basal experimental diet groups through chemistry analyzer. At the same time one drop of blood was placed on glucose strip and its level was measured with the help of Glucometer ACCU-CHEK® Softclix.

AST activity

AST Aspartate aminotransferase activity was determined with the help of AST/GOT kit. Briefly 100 µL serum (centrifuged non-haprinized blood at 1500 rpm for 15 minutes) was mixed with 1000 µL of working reagent and incubated for 1 min at 37°C. After that, absorbance was measured with the help of spectrophotometer (Micro-spectro Agilent 8453 by fixing wavelength at 340 nm). Absorbance reading was repeated after every 1 min for 3 times. The AST activity was expressed in U/l.

Challenge test

At the end of feeding experiment, 9 fish from each group were challenged with *Escherichia coli* (ATCC 8739) strain provided by Department of Microbiology, Quaid-i-Azam

University. Strain was inoculated orally by the help of dropper at rate of 100 CFU/gram of fish. All groups were kept under observation for 1 week to record mortalities and clinical signs. At the end of challenge test, blood was collected from the caudal vein and serum was collected by centrifuging blood at 1500 rpm for 15 minutes for immunity test

WBC's counting

WBC's were counted by mixing 400 µL of commercially available WBCs solution with 20 µL of blood. WBC's were counted with the help of hemocytometer.

Lysozyme activity

Lysozyme activity in blood serum was determined by using method previously described by Anderson & Siwicki (1995) with some modifications. Briefly, 100 µL serum was taken in test tube and mixed with 900 µL of a 0.75 mg/ml *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) suspension in phosphate buffered saline(pH 6.2). Absorbance was measured at 450 nm with the help of spectrophotometer after 1 minute intervals for 10 minutes. After mixing with bacteria, rate of change in absorbance was recorded and lysozyme activity was calculated using hen egg white lysozyme (Sigma-Aldrich) as a standard.

Total protein and Immunoglobulin in plasma

Total plasma protein was determined through Lowry's method (Lowry et al., 1951). For determination of protein concentration, standard curve was plotted by taking bovine serum albumin protein's concentration as a standard.

Anderson and Siwicki (1995) method was used for the determination of the immunoglobulin in the plasma. Briefly, 100 µL of plasma was mixed with 0.1 mL polyethylene glycol. The solution was incubated under constant shaking (ISS Innova 43) for 120 minutes, and then centrifuged at 7000 rpm for 10 minutes. Protein content of supernatant and plasma was calculated. Total immunoglobulins were calculated by subtracting the protein content of supernatant from total protein content in plasma.

Silver carp muscles composition

For the determination of Silver carp body composition, samples were analyzed by adopting standard AOAC (2000) protocols. Moisture content (%) was analyzed at National center for physics, Islamabad while ash content, crude fiber, crude protein and lipid contents in fish flesh were determined form poultry Research Institute (PRI), using the micro Kjeldahl method and Soxthlet apparatus.

Dry matter and Moisture Content

For the determination of dry matter, a pre weighted and washed china dish was taken and placed in oven at 65^{0}C for 10 minutes. After that, dish was placed in desiccators for cooling. After cooling, weight of empty china dish was noted on electrical top loading balance. Then 5g of sample was taken in that china dish and was placed in oven at 65^{0}C for 24 hours. After heating, china dish was again placed in desiccator, cooled and then again weighted near 0.01g on digital balance. Percentage of dry matter was calculated by following formula:

$$\% \text{ Dry matter} = \frac{\text{Weight of sample after drying}}{\text{Weight of sample before drying}} \times 100$$

% Moisture = 100 – dry matter (%)

Crude ash

For determination of crude ash, clean crucible was placed in a muffle furnace oven at 100^{0}C for 1 hour. Then crucible was placed in desiccators and cooled down at room temperature. Weight of empty crucible was noted. Then 5g of sample was taken in pre-weighted crucible dish and then placed the crucible again in furnace and heated at 550-600 ^{0}C for 24 hours. Then crucible was cooled in desiccators at room temperature. Ash in crucible was quickly weighted as early as possible to prevent moisture absorption.

$$\text{Crude ash (\%)} = \frac{\text{weight of ash}}{\text{Weight of sample}} \times 100$$

Crude protein

Crude protein in sample was determined by micro Kjeldahl's method. Briefly, 2 g of sample was mixed with 5 g of digestion mixture (30 ml conc. H_2SO_4) to digest the samples. Whole mixture was heated for 2-3 hours at 250^0C until the appearance of light green color. After that mixture was cooled and distilled water was added to make the final volume of 250 ml in volumetric flask. Then 10 ml of mixture was taken and mixed with 10 ml of 40 % NaOH in Kjeldahl's apparatus. Funnel was plugged firmly, heated for 2-3 minutes, and then 10 ml of 2% boric acid solution was added. Liberated ammonia was collected in 10 ml Boric acid (2% i.e. by adding 2g of boric acid in 98 ml of water) containing a drop of methyl red as an indicator. After appearance of golden yellow color, titration was done against H_2SO_4 (0.1N).

$$\text{Nitrogen (\%)} = \frac{\text{Normality of } H_2SO_4 \times \text{volume of } H_2SO_4 \text{ used} \times 250 \times 0.014}{\text{Weight of sample} \times 10} \times 100$$

Where,

250 = Dilution of digested mixture

0.014= Standard volume of H_2SO_4 to neutralize 1ml of ammonia

10= used volume of diluted mixture

100= percentage of nitrogen

Crude protein (%) = Nitrogen (%) × 6.25

Where,

6.25 = Assumed factor to calculate crude protein from nitrogen (%)

Fats content

Soxthlet apparatus was used to determine total fat content of sample. Approximately, 3 g of sample was placed in Soxthlet apparatus thimble, positioned in an extractor and placed correctly under the condenser of extraction apparatus. Then 150 ml of ether was added into the receiving flask and connected the flask to the apparatus. After that temperature of Soxthlet apparatus was increased. Whole extraction process was done at 100 °C for 10 hours at rate of 3-4 drops/ seconds. After extraction, thimble was removed from the extractor .Weight of thimble was noted after extraction.

$$\text{Total fats (\%)} = \frac{\text{Wt of thimble after evaporation} - \text{Weight of empty thimble}}{\text{Weight of sample}} \times 100$$

Crude fiber

For crude fiber determination, organic residue, left after sequential extraction for fats determination was used .Mixture was transferred to the flask and pre-heated 200 ml of H_2SO_4 was added gently for 30 minutes. Hot water was added to maintain constant volume of acid. After that Whatman filter paper was fitted with funnel and boiled sample was filtered into the funnel. Residue left was washed several time with hot water until the appearance of neutral color of litmus paper, then transferred back to the beaker. Pre-heated Na_2SO_4 (200 ml) was added and boiled for 30 minutes. Residue left was filtered and dried at 65^0C for two hours, then weighted. Residues were then transferred to crucible and placed in furnace for 4 hours at 400-600^0C, cooled in desiccators and weighted again.

$$\text{Crude fiber (\%)} = \frac{\text{dry weight of residue before ash} - \text{weight of residue after ash}}{\text{Weight of sample}} \times 100$$

Data analysis

Data obtained from the experiment was expressed as mean ± SE. The results were analyzed using one way analysis of variance followed by LSD and Tukey test SPSS statistical package (Version 16.0, SPSS, Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Characterization of chitosan nanoparticles

No peak was observed in XRD pattern of chitosan nanoparticles due to their amorphous nature as shown in Figure 2. Chitosan nanoparticles were also analyzed for the determination of their size, surface morphology and shape through scanning electron microscope (SEM). Scanning electron microscope image of chitosan nanoparticles is shown in Figure 1. They appeared spherical in shape with smooth surface. The sizes of these nanoparticles were 25.61 nm to 34.41 nm.

Growth performance

The growth performance of silver carp *Hypophthalmichthys molitrix* fed basal, chitosan and chitosan nanoparticles enriched diet for 90 days is shown in Table 2.

Final weight gain (FWG) of fish reared on chitosan nanoparticles (CNP) supplemented diet was 13.83±0.15g, significantly higher ($P<0.001$) as compared to the chitosan supplemented diet and basal diet fed groups. Percentage weight gain (%WG) in case of chitosan nanoparticles enriched diet was statistically higher ($P<0.001$) i.e. 129.61±2.29% as compared to the chitosan supplemented group and control group of fish. Although there was no significant difference in %WG between control and chitosan fed *Hypophthalmichthys molitrix* however, chitosan supplemented silver carps showed slight decreased in %WG i.e. 3% as compared to the control group.

The specific growth rate of fish fed chitosan supplemented diet was almost same as compared to fish raised on basal diet. However, silver carps fed diet supplemented with chitosan nanoparticles showed significantly higher ($P<0.001$) specific growth rate i.e. 0.92±0.01% as compared to other groups of fish. Feed conversion ratio (FCR) in case of CNP fed silver carp was significantly lower ($P<0.001$) as compared to chitosan supplemented and basal diet fed silver carps. There was no significant difference in FCR of chitosan enriched and basal diet fed fishes. Similarly, no significant difference was observed in feed conversion efficiency (FCE %) of groups of fish reared on basal and chitosan enriched diet while fish fed diet enriched with chitosan nanoparticles showed significantly higher ($P<0.01$) FCE %

Fillet proximate composition

The effects of chitosan and chitosan nanoparticles on proximate composition of fillet of juvenile silver carp *Hypophthalmichthys molitrix*, fed same level of chitosan and chitosan nanoparticles i.e. 5g/kg are shown in Table 3.

Percentage of fats in fillet of *Hypophthalmichthys molitrix* was statistically different in all three experimental groups of fish. Significantly high ($P<0.001$) % fat content was obtained in the muscles of fish fed chitosan nanoparticles supplemented diet as compared to chitosan supplemented and basal diet fed silver carps. Significantly lower content (fats %) were noticed in the group of fish reared on chitosan supplemented diet.

No significant difference was observed in fillet protein (%) content in all three groups of fish. Same results were obtained in case of % moisture content in fillets of all three groups of silver carp. Moreover, no significant difference between % fiber content was observed among basal and chitosan nanoparticles supplemented diet fed fishes. However, significantly higher ($P<0.01$) % fiber was obtained in the fillets of fish fed chitosan supplemented diet. No significant difference between was observed in % ash content among chitosan and chitosan nanoparticles supplemented diet fed fishes. However, significantly lower ($P<0.01$) % ash was obtained in the muscles of fish fed basal diet.

Hematological indices

Effects of chitosan and its nanoparticles on hematological indices of juvenile silver carp *Hypophthalmichthys molitrix* at same supplementation level i.e. 5g/kg are shown in table 4.

Blood hemoglobin level was statistically same in both chitosan and chitosan nanoparticles enriched diet fed silver carps. Blood hemoglobin level in silver carp fed basal diet was significantly ($P<0.05$) lower than groups of fish reared on chitosan and CNP enriched diets. Same trend was observed in case of red blood cells count of all three groups of silver carps with significantly higher RBC count ($P<0.05$) in silver carp reared on chitosan and chitosan nanoparticles supplemented diet as compared to basal diet.

Blood glucose level was significantly higher ($P<0.01$) in basal diet fed *Hypophthalmichthys molitrix* as compared to the chitosan and CNP supplemented diet fed silver carps. However, no significant difference ($P<0.05$) in the blood glucose levels was observed in group of fish fed chitosan and CNP supplementation at the same dosage i.e. 5g/kg diets.

AST activity was significantly higher ($P < 0.001$) in basal diet fed *Hypophthalmichthys molitrix* as compared to the chitosan and CNP supplemented diet fed silver carps. Significantly lowest ($P<0.01$) AST activity was observed in serum of chitosan nanoparticles supplemented silver carps.

Plasma cholesterol level was significantly ($P<0.001$) low in chitosan enriched diet fed group of fish as compared to other two groups. However, plasma cholesterol levels of silver carp reared on basal and CNP supplemented diet were statistically comparable.

Immunity parameters (Post-challenge)

Effects of basal and experimental diets on immunity response after challenged with *Escherichia coli* in juvenile silver carp *Hypophthalmichthys molitrix* are shown in Table 5.

After 10 days of introduction of *E.coli*, group of fish reared on CNP supplemented diet showed significantly ($P<0.001$) higher WBCs count as compared to other groups of fish. Moreover, significant difference ($P<0.001$) was also noticed between white blood cell count of fish fed basal diet and chitosan enriched diet.

The lysozyme activity after challenged with *E. coli* was significantly ($P<0.001$) higher in silver carp fed diet enriched with CNP as compared to the rest of fish fed basal or chitosan supplemented diets. Significantly lowest lysozyme activity ($P<0.001$) was observed in silver carps raised on basal diet as shown in Table 5.

Plasma protein levels were significantly ($P<0.001$) higher in silver carp fed chitosan nanoparticles supplemented diet (30.84 ± 0.24 mg m/L) as compared to other two experimental groups of fish. Plasma protein level was significantly ($P<0.001$) in group of fish fed basal diet as compared to other experimental diets.

Results

Significantly higher ($P<0.001$) level of total immunoglobulins was observed in silver carp reared on chitosan nanoparticles supplemented feed as compared to basal diet and chitosan enriched diet. The immunoglobulin level was also considerably different ($P<0.001$) among silver carps fed chitosan supplemented diet and basal diet. However, after challenged with *E. coli* significantly lowest immunoglobulin level was observed in *H. molitrix* reared on basal as compared to other diets.

Table: 1 Formulation and composition of experimental basal diet for silver carp *Hypophthalmichthys molitrix.*

Ingredients	Amount (%)
White fish meal (55% crude protein)	10.6
Soybean meal (46.2 % crude protein)	21.3
Sunflower meal (40 % crude protein)	21.3
Canola seed meal (21.3 % crude protein)	21.3
Rice polish (13.2 % crude protein)	5.3
Wheat bran	5.3
Gluten (30 % crude protein)	10
Carboxymethylcellulose (CMC)	1
Di calcium phosphate (DCP)	1
Vitamin premix[a]	2
Canola oil	0.8

[a](Vitamin premix contains vitamins, amino acid and minerals premix kg^{-1})
Vitamin AB.P 40,000,000IU, Vitamin D3B.P 820,000IU, Vitamin EB.P 6200 mg, Vitamin K3B.P 800 mg, Vitamin B2B.P 2500 mg, Vitamin B3B.P 5100mg, Vitamin B12B.P 1000 mg, Vitamin PP B.P 10,500 mg, L. lysine B.P 10,500 mg, DL- Methionine B.P 50,500 mg, Choline chloride USP 125,500 mg. Manganese USP 30,000 mg, Iron 15,100 mg, Zinc USP 17,555 mg, Copper B.P 1000 mg, Cobalt B.P 50 mg, Iodine B.P 300 mg, Selenium B.P 80 mg.

Table 2: Growth performance of silver carp fed basal and experimental diets.

	Diets		
	Control	Ch	CNP
IBW (g)	06.07±0.01^{ab}	06.06±0.02^{a}	06.02±0.007^{b}
FBW (g)	11.33±0.12^{b}	11.13±0.09^{b}	13.83±0.15^{a}
SGR (%)	0.70±0.01^{b}	0.68±0.01^{b}	0.92±0.01^{a}
WG (%)	86.30±2.46^{b}	83.72±2.04^{b}	129.61±2.29^{a}
FCR	2.92 ±0.08^{a}	02.88 ±0.06^{a}	01.86 ± 0.03^{b}
FCE (%)	34.33± 0.92^{b}	34.75± 0.75^{b}	53.86± 1.01^{a}

Data are represented as Mean ± SE. (n=30). Means followed by the different letters within the rows are significantly different ($P < 0.05$). ANOVA followed by Tukey test and LSD.

Ch : chitosan supplemented diet

CNP: chitosan nanoparticles supplemented diet

Table 3: Proximate composition of fillets of juvenile silver carp fed basal and experimental diets.

	Diets		
	Control	Ch	CNP
Fats (%)	15.24 ±0.13^{b}	13.97 ±0.20^{c}	16.17 ±0.04^{a}
Proteins (%)	61.16 ± 0.55^{a}	61.89 ±0.80^{a}	61.06 ±0.47^{a}
Fiber (%)	02.12 ±0.06^{b}	02.65 ±0.04^{a}	02.24 ±0.13^{b}
Ash (%)	16.70 ±0.37^{b}	18.23 ±0.08^{a}	18.03 ±0.03^{a}
Moisture (%)	03.67± 0.72^{a}	02.78 ±0.19^{ab}	02.11 ±0.16^{b}

Data are represented as Mean ± SE. (n=10). Means followed by the different letters within the rows are significantly different ($P < 0.05$). ANOVA followed by Tukey test and LSD.

Ch : chitosan supplemented diet

CNP: chitosan nanoparticles supplemented diet

Table: 4. Hematological indices of silver carp fed basal and experimental diets.

	Diets		
	Control	Ch	CNP
Hb (g d/L)	8.02±0.31[b]	9.96±0.22[a]	9.70±0.60[a]
RBC(10^6 µL)	1.94±0.04[b]	3.19±0.20[a]	3.31± 0.32[a]
Glucose (mg d/L)	97±2.31[a]	78.33± 2.61[b]	76± 3.06[b]
AST activity (U/L)	18.48± 0.52[a]	9.89± 0.38[b]	6.98± 0.91[c]
Cholesterol (mg d/L)	153.67± 2.89[a]	103.22±2.48[b]	161.56±4.32[a]

Data are represented as Mean ± SE. (n=10). Means followed by the different letters within the rows are significantly different ($P < 0.05$). ANOVA followed by Tukey test and LSD.
Ch : chitosan supplemented diet
CNP: chitosan nanoparticles supplemented diet

Table: 5. Effects of basal and experimental diets on immunity response after challenged with *Escherichia coli* in silver carp.

	Diets		
	Control	Ch	CNP
WBC ($10^3 mm^{-3}$)	206.33± 1.45^c	223.4± 2.08^b	235.33± 1.20^a
Lysozyme activity (µg m/L)	2.46± 0.19^c	4.50± 0.05^b	7.54± 0.15^a
Plasma protein (mg m/L)	13.95± 0.48^c	24.60± 0.20^b	30.84± 0.24^a
Total immunoglobulin (mg m/L)	10± 0.69^c	19.7± 0.45^b	24.1± 0.37^a

Data are represented as Mean ± SE. (n=10). Means followed by the different letters within the rows are significantly different ($P < 0.05$). ANOVA followed by Tukey test and LSD.
Ch : chitosan supplemented diet
CNP: chitosan nanoparticles supplemented diet

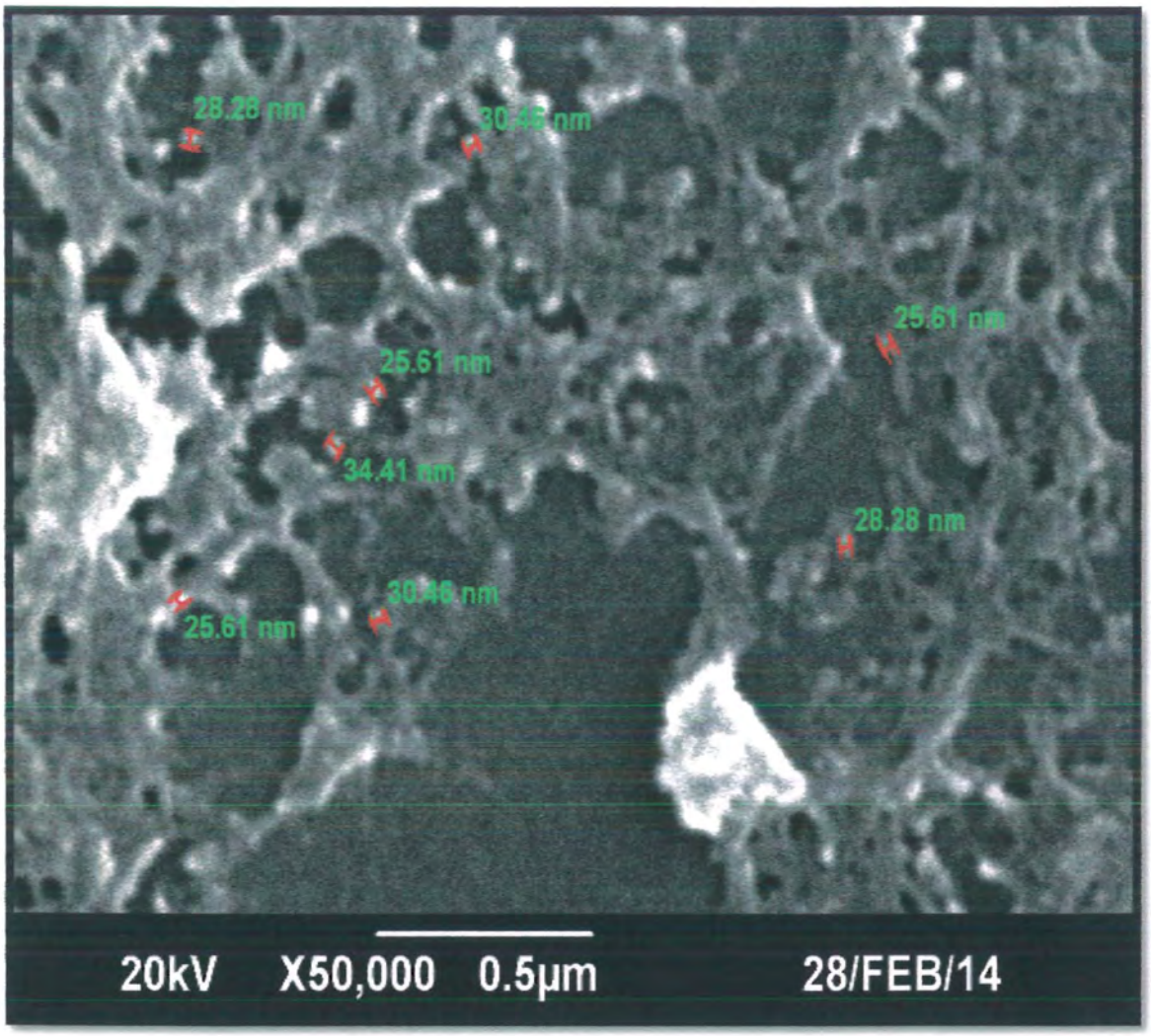

Figure: 1. Scanning electron microscope image of chitosan nanoparticles

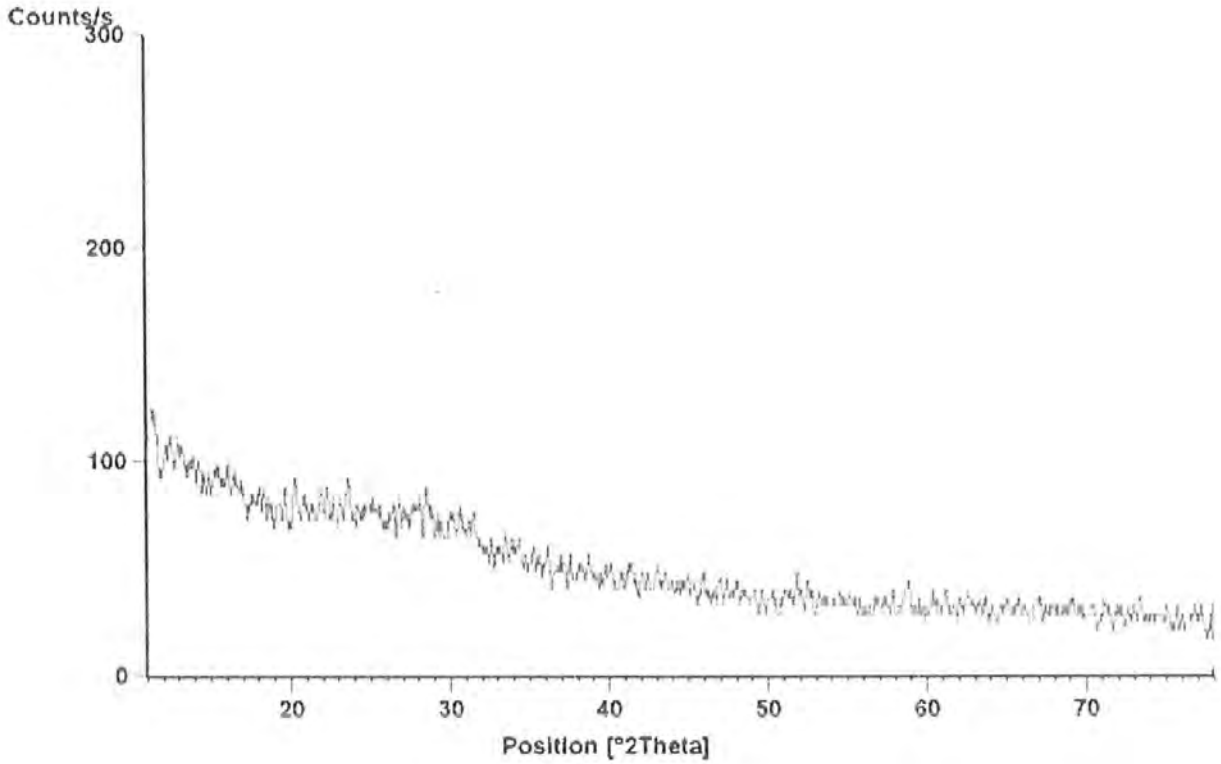

Figure: 2. XRD pattern of chitosan nanoparticles

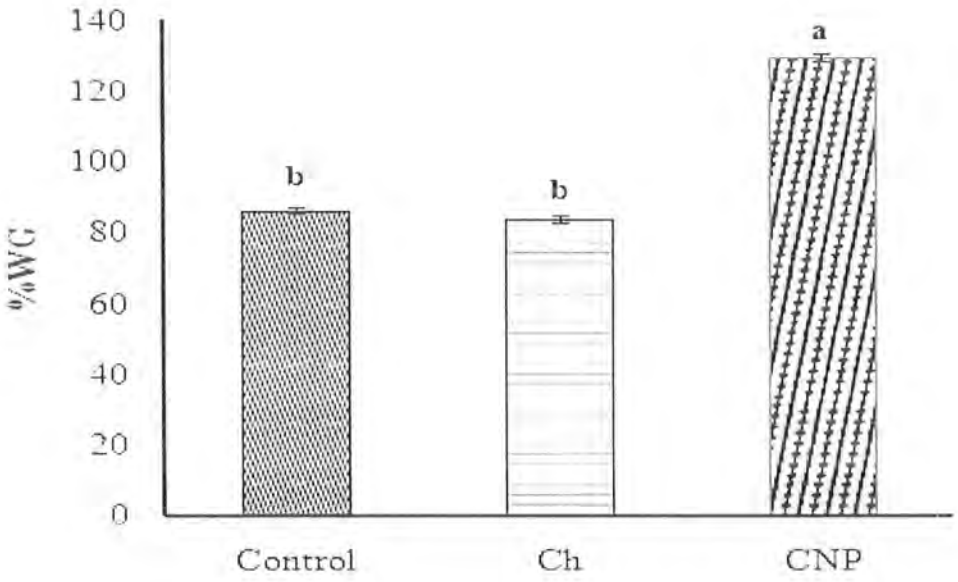

Figure: 3. Weight gain (%) of sliver carp fed different diets after three months.

Each bar represent the values as Mean ± SE, (n=30). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

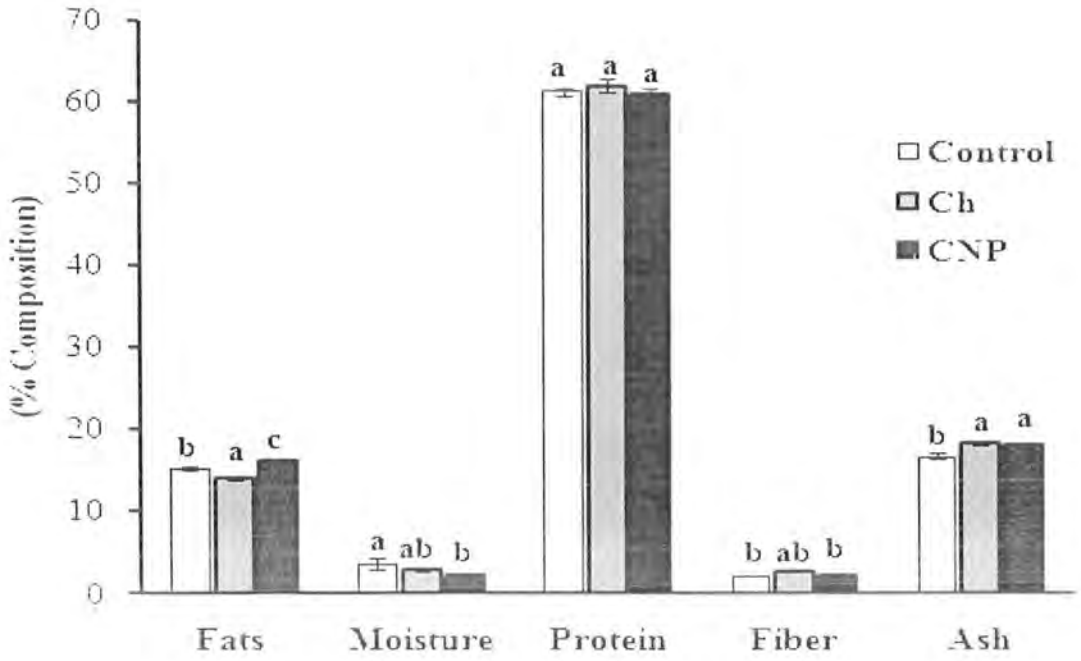

Figure 4: Fillets proximate composition of silver carp fed basal and experimental diets

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

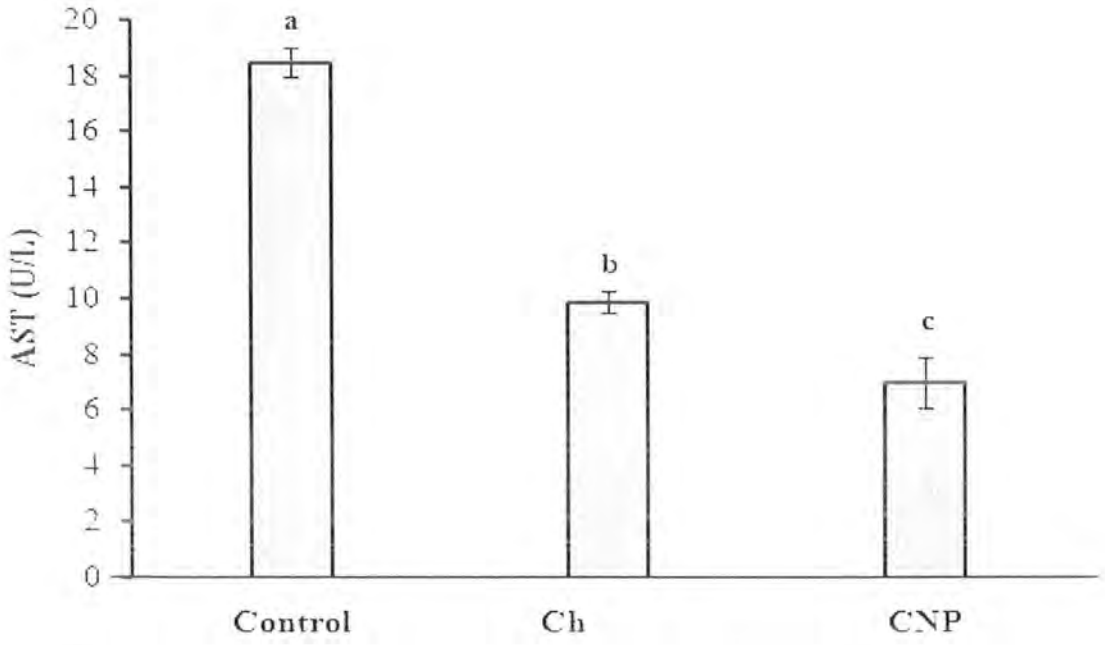

Figure: 5. AST activity of silver carp fed basal and experimental diets.

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

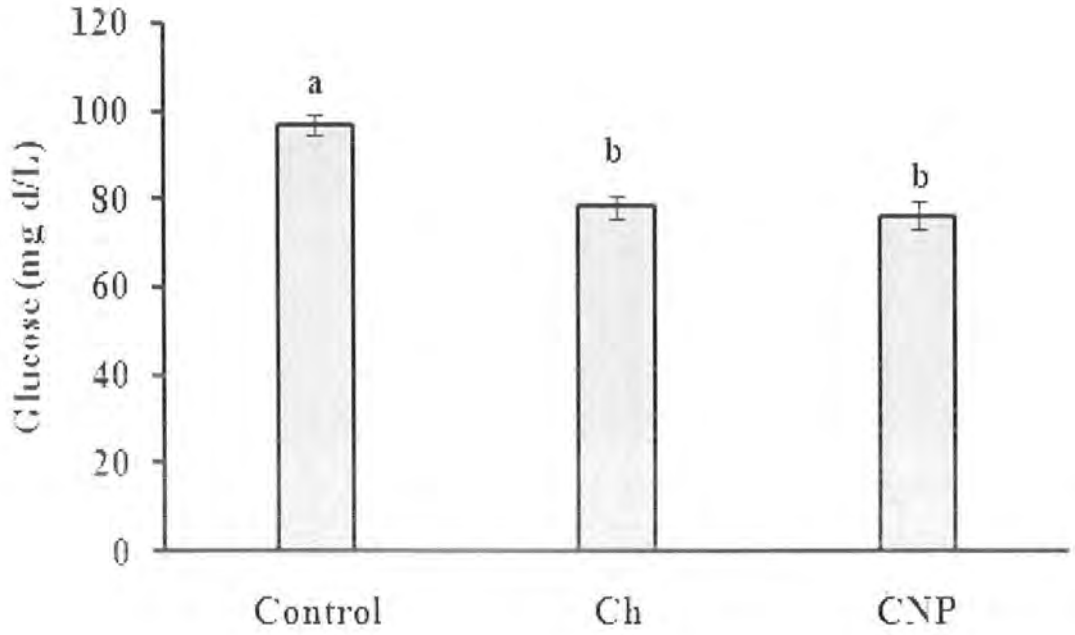

Figure: 6. Blood glucose level of silver carp fed basal and experimental diets.

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

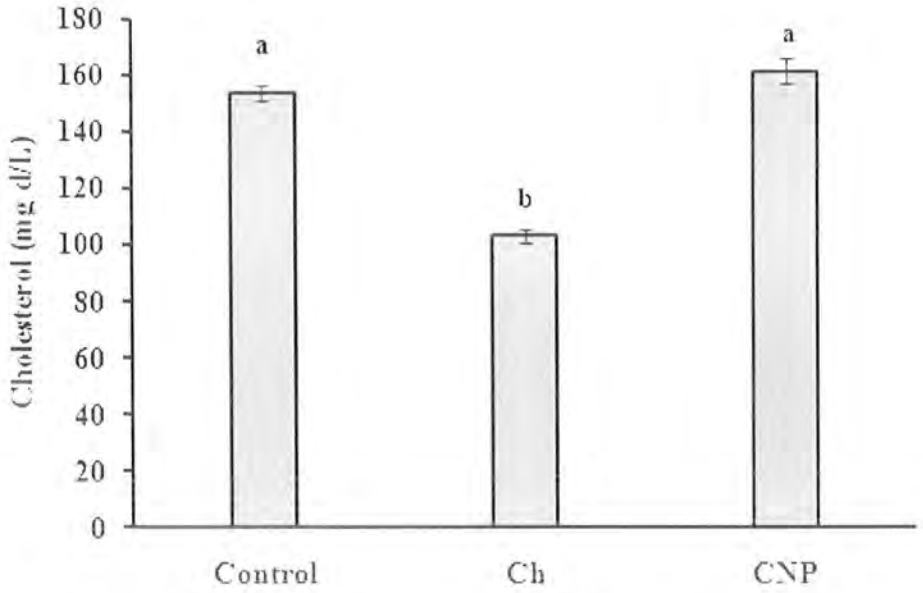

Figure: 7. Plasma cholesterol level of silver carp fed basal and experimental diets.

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD)

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

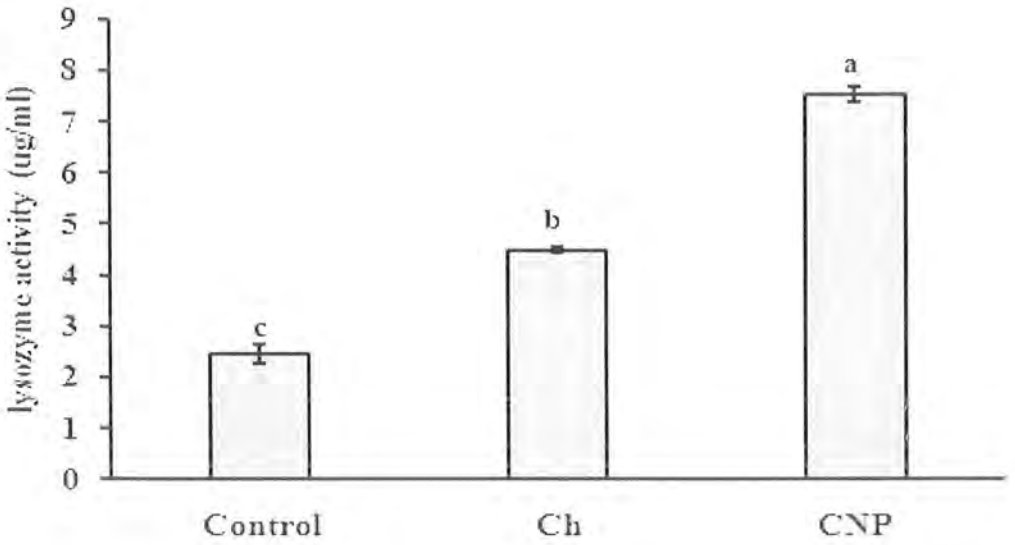

Figure: 8. Serum lysozyme activity of post challenged *Hypophthalmichthys molitrix* raised on basal and experimental diets.

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

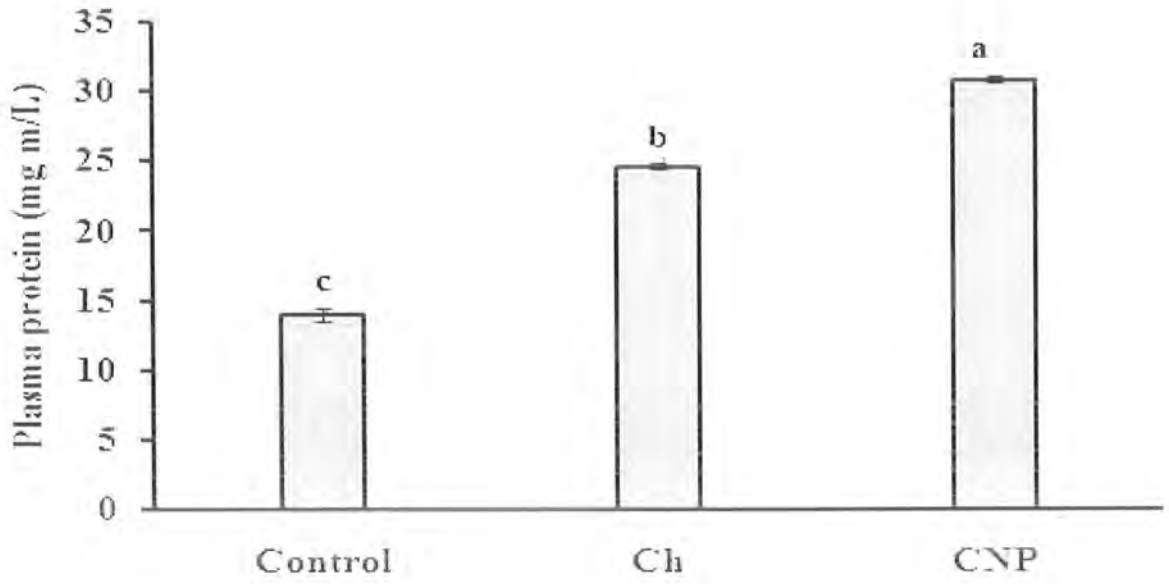

Figure: 9. Post challenged total plasma protein level of *Hypophthalmichthys molitrix* raised on different diets.

Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

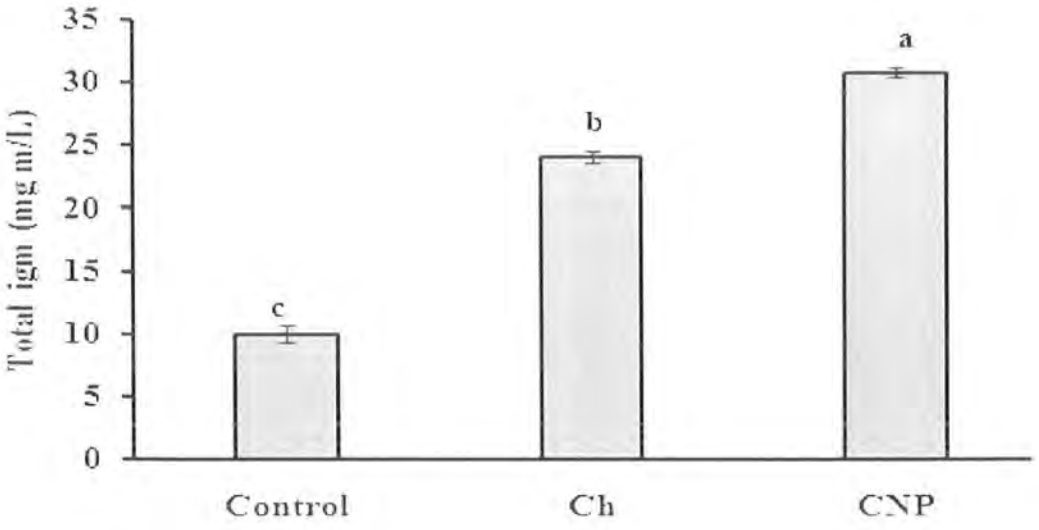

Figure: 10. Post challenged plasma Immunoglobulin level of *Hypophthalmichthys molitrix* raised on basal and experimental diets. Each bar represent the values as Mean ± SE, (n=10). ANOVA followed by Tukey test and LSD.

CNP: chitosan nanoparticles supplemented diets

Ch: chitosan supplemented diet

DISCUSSION

Discussion

Over the past century, advancement in technology played an important role in transforming and reshaping of products. Nanotechnology is the most recent field involves directly manipulating materials at a scale of less than 100 nm. In our study, we used this technique to prepare chitosan nanoparticles through Ionotropic gelation method. According to Rao et al. (2007), the physical, optical and functional properties of particles changed, when material converted to nanometer scale.

Chitin is the most abundant polysaccharide after cellulose with annual production of 10^{10} – 10^{12} ton per year. It is the major component of the exoskeleton of arthropods and shells of crabs and shrimps, also the main constituent of the cell wall of yeast and fungus (Rinaudo, 2006). Chitosan is prepared from chitin (Kumar, 2000) when the degree of acetylation of it is less than 50% and becomes soluble in weak acidic solution e.g. acetic acid (pH < 6.0). Chitosan is 75-95% de-acetylated (Kurita, 2001) it is eco friendly and non-toxic having no antigenic property. Nowadays, chitosan is being used in every field of science including medical i.e. in making artificial skin, treatment of burn and tumors (Kim and Rajapakse, 2005), genetic engineering for delivery of DNA vaccines (Tasker et al., 1997) and also protecting DNA from degradation. In food industry, chitosan is used as a food preservative, as gelling agent, flavor extender and in aquaculture it is used as fish-feed additive (Shahidi et al., 1999).

In our study chitosan nanoparticles were made through interaction between positively charged chitosan and negatively charged tri polyphosphate (TPP) which were further used as feed additive. The actual size, surface morphology and shape of these nanoparticles were analyzed through different techniques i.e. through X-rays diffraction method and through scanning electron microscope (SEM). Clark and Smith in 1937 were the first scientists who make crystal studies of chitin and chitosan by using XRD. In the present study, chitosan nanoparticles did not show any peak in the XRD pattern, thus reveals that all chitosan is converted in to nano forms which are amorphous in nature. Same result i.e. no peak was observed by Lifeng et al. (2004) when he took the XRD pattern of chitosan nanoparticles for characterization.

Scanning electron microscope (SEM) is extensively used for determination of morphology and physical state of surface and for the characterization of chitosan and its nano form (Varma et al., 2004). Scanning electron microscope image of chitosan nanoparticles is

shown Figure 3. The sizes of these nanoparticles were ranging between 25.61 nm to 34.41 nm, and they were spherical in shape. Vimal et al. (2013) also prepared chitosan tripolyphosphate nanoparticles by using a similar Ionotropic gelation technique and used them for gene delivery in different tissues of shrimp through oral route. Although, like our nanoparticles, they were amorphous and spherical in shape, but their size ranges between 30-60 nm as compared to observed, 25.61 nm to 34.41 nm in the present study. This variation may be related to the degree of deacetylation of chitosan used for the preparation of nanoparticle. The same smooth and spherical shape chitosan nanoparticles were also prepared by many other scientists and used them as carriers for the delivery of Vitamin C through the gastrointestinal tract of rainbow trout in order to enhance the immunity (Alishahi et al.,2011) or for coating of bovine serum albumin (BSA) (Gan et al., 2005).

In our experiment, three diets were offered to silver carp in triplicate groups i.e. basal/control diet, chitosan supplemented diet and chitosan nanoparticles supplemented diet (CNP). The Initial body weights of all three groups were approximately. It was observed that the final body weight (FBW) of silver carp fed chitosan nanoparticles (CNP) supplemented diet was about 24% higher as compared to the chitosan enriched and basal diet fed groups, while chitosan supplementation showed a somewhat negative effect, therefore, the final weight of fish after 90 days were about 2% less as compared to control group (Table 2). Similar results were observed in case of tilapia *Oreochromis nilotica* where chitosan and chitosan nanoparticles were used at the same supplementation level, 5 g/kg feed (Wang and Li, 2010). Initial stocking density and culture condition were same in our all groups of fish fed either basal or supplemented diets; therefore, it appears that the increased or decreased in weight is only due to the effect of CNP and chitosan. The % survival of silver carp in the present study and tilapia in the previous study were statistically similar in all experimental and control groups of fish indicate that chitosan had no profound negative effect on the survival of fish. The SGR (%) and FCE (%) in our study followed the similar trend as observed in case of %WG. Therefore, silver carp reared on a diet supplemented with CNP showed better specific growth performance ($P < 0.001$) and higher ($P < 0.01$) feed conversion efficiency (%) as compared to the fish fed chitosan and basal diet. Similar improved SGR% and FCE% were also observed in tilapia in response to a similar dose of chitosan and CNP (Wang and Li, 2010; Shiau and Yu, 1999). The beneficial effect of CNP on growth performance of silver carp similar to tilapia may be due to the enhancement of digestion

and absorption of nutrients across the gastrointestinal tract at lower levels (Gopalakannan and Arul, 2006). Hence, increased feed efficiency in case of chitosan nanoparticles supplementation in feed indicated reduced feeding cost.

The higher efficiency of CNP as compared to chitosan in the present study and reported by many other scientists may be related to the size and physical property of material. Physiochemical properties of matter changes with the reduction of size of material up to nano scale i.e. less than 100 nm. Chitosan is heteropolymer and crystalline in nature, obtained from chitin through its deacetylation while CNP are amorphous in nature and smaller in size, 25.61 nm to 34.41 nm in the present study and 30-60 nm reported by Vimal et al. (2013). Changing the size of chitosan increases its efficiency, bioavailability and enhanced absorption across the gastrointestinal tract (Kumar et al., 2008). Nowadays, chitosan nanoparticles gained their importance due to their better stability, low toxicity and easy preparation methods i.e. through Ionotropic gelation, polyelectrolyte complex, micro emulsion methods (Tiyaboonchai, 2003). Bio availability of drugs depends upon the particle size and residence time of drug carrier in the gastrointestinal tract (Takeuchi et al., 2001). Being mucoadhesive in nature, chitosan nanoparticles are now being used in drug delivery system as carriers such as antifungal, antibacterial and anti parasitic drugs (Soma et al., 2000). The mucoadhesive property of these nanoparticles are due to the presence of positive charge on them which interacts with the negatively charged sialic acid of mucin, thus increasing the contact time between drug and intestine resulted in increased absorption of drug (Soane et al., 1999).

The percentage weight gain of silver carps fed chitosan supplemented diet was same as compared to the basal diets fed fish. The same results were reported by Lin et al. (2011) when they supplemented chitosan to *Cyprinus c*arpio *koi* and observed final body weight and specific growth rate almost similar to the control group of koi. Several investigators reported the positive effect of chitosan when it is converted into its nano form e.g. in tilapia (Wang and Li, 2010), rainbow trout (Alishahi et al., 2011) and in Asian sea bass *Lates calcarifer* (Kumar et al., 2008).

The growth promoting and inhibiting effect of chitosan sometimes depends on dosage of supplement. For example, in case of Grass shrimp, final body weight gain, decreased with the increase in supplementation of chitosan in feed (Shiau and Yu, 1998). Shrimps on 2% and 5%

chitosan supplemented diet showed better growth performance as compared to 10% chitosan in the feed. However, when *Trachinotus ovatus* were raised on a diet supplemented with chitosan oligosaccharide (COS), which was produced from chitosan through the enzymatic decomposition method (Li et al., 2007), showed better growth performance with increasing COS from 0.0 g/kg to 6.0 g/kg diet (Lin et al., 2012). Similarly, Geng et al. (2011) reported enhanced growth performance, improved specific growth rate and feed conversion efficiency of cobia *Rachycentron canadum* when diet supplemented with 6.0 g/kg chitosan and 1.0 g/kg *Bacillus subtilis*.

Similar positive effects of chitosan on growth performance of Indian major carp *Labeo rohita* and olive flounder *Paralichthys olivaceus* were observed in response to 1% chitosan supplementation in feed (Aathi et al., 2013) and chitosan coated moist pellets (MP) respectively. These chitosan coated moist pellets not only increased the average performance i.e. 10% increased in weight as compared to a control group of fish, but also they improved the water quality parameters i.e. reduced chemical oxygen demand and suspended solids in fish tank (Cha et al., 2008). The effects of chitosan on growth performances of aquatic animals are controversial. The variability of results may be due to the degree of deacetylation, molecular weight, size of chitosan i.e. bulk and nano form and dosage of supplementation. It was reported that chitosan in encapsulated form increase their availability and absorption across the gastrointestinal tract (Aranaz et al., 2009). The difference in growth performance of chitosan and CNP raised silver carps may be due to the change in particle size that leads to change in physiochemical properties of chitosan at the nanoscale (Alishahi et al., 2011).

Body composition of fish is used as its quality indicator that varies with age, strain and diet (Austreng and Refstie, 1979). Sometime, the fish body composition varies from habitat to habitat and species to species (Quinton et al., 2007). In our experiment, we used silver carp as experimental fish which is filter feeder and mostly fed phytoplankton and zooplankton (Santhanam et al., 1990). Fish flesh is tasty, digestible and contains all essential amino acids, minerals, fatty acids and superior quality protein (Hossain, 1996). In our study, moisture (%) and protein (%) content of muscles of all three experimental groups remained same after 90 days feeding trial. The same results were reported by Niu et al. (2013) when they offered chitosan

supplemented diet to tiger shrimp *Penaeus monodon*. The statistically no significant difference was also observed in (%) moisture of group of shrimps fed basal and chitosan enriched diet. However, whole body muscles fats (%) were significantly different ($P<0.001$) in all three experimental groups of fish. The group of fish raised on chitosan enriched diet showed less fat content in their muscles as compared to fish fed CNP enriched and basal diet. Wang and Li (2010), also reported the similar decrease in muscles fats (%) contents of tilapia in response to a similar dose of chitosan supplementation. The Grass shrimps also showed lower muscles fat % contents when they raised on chitosan (1.68% fats) as compared to chitin (2.28% fats) and basal diet (2.59% fats) (Shiau and Yu, 2011). The % fat content in chitosan fed fish was statistically lower as compared to CNP fed silver carp. This difference in muscle fats contents may be due to difference in particle size as reported earlier by Wang and Li (2010). Lower muscles % fat content in fish may be correlated with lower cholesterol levels in the blood sera of our experimental fish. As reported earlier by many scientists, chitosan is hypocholesterolemic in nature which is due to its water binding capacity and non-digestibility in the upper digestive intestinal tract (Muzzarelli, 1996). When chitosan was supplemented in feed, it developed HCl layer around itself in the stomach. Therefore, while passing through the upper part of digestive tract i.e. small intestine, this HCl layer got diluted and chitosan made agglomerates with cholesterol and fatty acids, hence reducing lipid absorption in the digestive tract and caused their released directly out of the body without being absorbed (Ebihara and Schneeman, 1989). Chitosan fed rats showed increased fecal output of natural sterols. Kannauchi et al. (1995) also reported the same effects of reduced fat absorption in response to diet supplemented with 7% chitosan and fed for a month and suggested that the reduced fat absorption may be due to the reduced chitosan viscosity in the stomach, increase fat holing capacity and their reduced leakage in the intestinal tract.

Cholesterol is the precursor of steroid hormones and structural component of cell membranes along with that it also forms the outer layer of plasma lipoproteins (Yang and Chen, 2003). It was reported by many researchers that chitosan reduced the blood cholesterol level in humans (Mhurchu et al., 2004), rats (Chiang et al., 2000), fishes (Cui et al., 2012) and shrimps (Niu et al., 2013). In our research, chitosan significantly ($P<0.001$) lowered the cholesterol level in fish, while no change in blood cholesterol level was observed in groups of silver carps fed

basal and CNP supplemented diet. In humans, chitosan capsules reduced body weights and total cholesterol level as compared to the placebo group after 24 weeks (Mhurchu et al., 2004; Kobayshi and Boukara, 2003). Same lower cholesterol level in the chitosan fed group was also reported by Sango et al. (1988) and suggested that cholesterol lowering property of chitosan may be due to the amino sugar of chitosan which interacted with bile acid in the small intestine, hence interfering with the absorption and caused the excretion of steroids in feces. According to Chiang et al. (2000) low plasma cholesterol was due to low level of VLDL cholesterol level. Chitosan exerted its effects on liver LDL receptors hence, affect LDL cholesterol level. Hossain et al. (2007) observed the decrease in cholesterol and triacylglycerol concentration along with decreased muscles fat content after providing chitosan containing diet to shrimps.

In our study, it was noticed that the muscles ash content (%) was significantly higher in both chitosan and CNP supplemented silver carps as compared to basal diet fed fish (Table 3). The same results were reported by Yongi and Hong-qi (2012) when they supplemented 0.25% and 0.50% chitosan in the diet of *Carassius auratus gibelio*. They reported that chitosan improved the ash content in *Carassius auratus gibelio* muscles. Relatively higher muscle % ash content was obtained when chitosan oligosaccharide complex with rare earth was supplemented in the diet of turbot *Scopthalamus maximus* (Cui et al., 2012). Muscles % fiber content was slightly higher in silver carp fed chitosan enriched diet as compared to control and CNP supplemented diet. This higher fiber may be due to biochemical composition chitosan as it is insoluble dietary fiber in nature (Furda, 1999).

In aquaculture, fish production could be increased by identifying and providing the proper environmental conditions and through good management practices (Kamal and Omar, 2011). During aquaculture practices, many biological, physical and chemical factors affect the physiological health of fish, thus impairing its resistance against disease, retarding fish growth and affect reproductive success (Wedemeyer et al., 1990). Nowadays, fish hematology has gained much importance in aquaculture practices and use to check fish health status (Hurbec et al., 2000). Changes in fish's blood biochemistry may indicate the unsuitable environment and culture conditions (Barcellos et al., 2004).

Discussion

In aquaculture, level of blood glucose is used as an indicator of fish's stress condition (Kavitha et al., 2010), although it is a less reliable source for determining the stress level (Mommsen et al., 1999). Under stress conditions, blood glucose level rises as reported by Tintos et al. (2006) in gilthead sea bream. In our study, blood glucose level was significantly lower ($P < 0.05$) in both treated groups i.e. in group of fish reared on chitosan and CNP supplemented diets as compared to basal diet, while no significant difference was observed in glucose level of both treated groups. The same results were reported by Cha et al. (2008), when they fed chitosan coated moist pellets to olive flounder. These chitosan coated MP reduced the blood glucose about 17.2% as compared to non-coated moist pellets. Our results suggest that chitosan and CNP enriched diets provide protection against stressful condition and thus positively influenced the health status of silver carp.

Aspartate aminotransferase (AST) is the main enzyme which is involved in the metabolism of proteins to carbohydrate in mammals as well as in fish tissues (Gaudet et al., 1975). AST activity is commonly related to lethal damage to certain organs like liver and kidneys (Benedeczky et al., 1984). Any increase in activity of this enzyme in extracellular fluid or fish's plasma indicates liver damage (Palanivelu et al., 2005). In our study AST activity was significantly lower ($P < 0.01$) in silver carp reared on CNP enriched diets as compared to chitosan supplemented and basal diet. Highest AST activity was observed in a group of fish fed basal diet. Our results are in agreement with Nie et al. (2013) where chitosan supplemented diet to shrimps *Penaeus monodon* significantly lower AST activity as compared to control group. Same low AST activity was also observed in olive flounder when fed chitosan coated moist pellets as compared to non-coated moist pellets (Cha et al., 2008).

Hemoglobin is the iron containing protein involved in the transport of oxygen to all parts of the body. It was reported that chitosan supplementation does not depress serum hemoglobin and iron level in the body (Muzzarelli, 1996). In our experiment, fishes fed basal diet i.e. didn't contain either chitosan or CNP had low serum hemoglobin as compared to the treated group. The same results were obtained in case of RBC count in a group of fish fed basal diet while comparatively similar serum Hb and RBC count was observed in both groups of fish raised on chitosan and CNP enriched diet. Our results may indicate that RBCs were destroyed by

erythroblastosis and leukocytosis in silver carps that did not received chitosan or CNP in their diets. Aithi et al. (2013) reported that RBC count and serum Hb level was significantly higher ($P < 0.05$) in Indian major carp *Labeo rohita* when fed a diet enriched with different levels of chitosan. The same results were reported by (Cha et al., 2008) in olive flounder and in common carp *Cyprinus carpio* (Victor et al., 2004).

With the advancement in aquaculture, a range of bacterial infections emerged and caused major problems in fish production (Alderman and Hastings, 1998). These infections are commonly due to interaction between host, pathogen and the surrounding environment (Toranzo et al., 2005). For the treatment of fishes, antibiotics were used and four antibiotics were approved (Alderman and Hastings, 1998) for use in fish against bacterial infections. However, due to the development of antibiotics resistance in bacteria (Aoki, 1992), these antibiotics are not much useful against them. Besides antibiotics, vaccines are also commercially available which are effective against bacterial infections, but these vaccines are also not too effective against all bacterial infections (Sakai, 1999). Nowadays, immunostimulants are used along with antibiotics and vaccines. These immunostimulants are quite useful for the disease control. They enhance the fish non-specific defense mechanisms. Different types of immunostimulants are used in aquacultures which are harmless to human health and the environment, biocompatible and biodegradable (Esteban and Ortuno, 2001). These immunostimulants increase serum lysozyme activity, caused the stimulation of natural killer cells and increased antibodies production in fishes and protect them against bacterial infection (Harikrishnan, 2010). Chitosan as an immunostimulant showed bactericidal activity against many bacteria (Tsai and Su, 1999). The positive charge on chitosan caused the binding of chitosan on the bacterial cell surface, and leading to the gradual shrinkage of the cell's outer surface that result in cell death (Prashanth and Tharanathan, 2007). Binding of chitosan to bacteria's cell membrane also results in leakage of intracellular components due to the changes in the permeability barrier; causes hindrances for nutrients to enter into the bacteria's cell. Chitosan showed broad-spectrum bactericidal activity against both gram negative and positive bacteria (Kumar et al., 2005).

In our research, we challenged some fish from both control and experimental group with *E.coli* after 90 days feeding trail in order to compare the immune responses. The white blood cell

count in fish fed diet enriched with CNP was significantly higher ($P < 0.001$) as compared to other fish. Same results were reported by Lin et al. (2011) when they challenged *Cyprinus carpio* with *Aeromonas veronii* after feeding chitosan in the feed. Post challenged WBC count was significantly higher ($P < 0.05$) in fish fed chitosan in diet as compared to the controls. Similarly, *Labeo rohita* reared on chitosan enriched diet and challenge also showed significantly enhanced number of WBCs (Aithi et al., 2013).

Lysozyme is a cationic enzyme which is involved in the breakage of glycosidic bonds between N-acetlymuramic acid and N-acetlyglucosamine present in the cell wall of bacteria (Alexander and Ingram, 1992). High serum lysozyme activities directly indicate high activity of phagocytic and bactericidal activity, protecting fish from bacterial infection (Robertsen, 1994). Aithi et al. (2013) studied the effects of different dosages of chitosan on the serum lysozyme activity of Indian major *carp Labeo rohita* and reported high serum lysozyme activity in fish fed 1% chitosan in feed after 90 days feeding trial. The Similar results were observed in silver carps reared on chitosan and CNP enriched diet as compared to basal diet. However, significantly ($P < 0.001$) highest lysozyme activity was observed in silver carps fed CNP supplemented diet. Antimicrobial activity of chitosan nanoparticles was reported by Luis et al. (2014) on *Streptococus mutans* biofilms. High serum lysozyme activities in fishes after providing diet enriched with chitosan were reported in cobia *Rachycentron canadum* (Xu et al., 2011), *Cyprinus carpio* (Gopalkannan and Arul, 2006), olive flounder *Paralichthys olivaceus* (Cha et al., 2008) and *Trachinotus ovatus* when fed a diet enriched with chitooligosaccharides (Lin et al., 2012).

In fish, serum proteins play an important role in immunity. The higher levels of serum protein indicate strong immunity (Rao et al., 2006). In our study, CNP enriched diet significantly increased the serum protein along with chitosan enriched diet as compared to the basal diet. Our results were in agreement with Cha et al. (2008) where high serum protein was observed in olive flounder when fed chitosan coated moist pellets as compared to basal diet.

The main component of humoral system is serum immunoglobulin M and it is most abundant in fish (Wilson et al., 2006). In fish, immunoglobulin production is the result of the fish immune response. Bony fishes have just one class of immunoglobulin i.e. IgM, which is almost similar to that of mammalian IgM. Ming et al. (2007) reported significantly ($P<0.05$) higher

serum IgM level in obscure puffer *Fuga obscures* when fed a diet enriched with a combination of chitosan and probiotics whereas Cuesta et al. (2004) reported statistically ($P<0.05$) increased serum IgM level in sea bream *Sparus aurata L.* in response to different immunostimulants and reported that their effect depends on dosage and time of administration of particular immunostimulant. The same results were obtained in our study, where high serum IgM level was obtained in fish fed diet enriched with CNP followed by chitosan enriched diet as compared to basal diet.

Keeping in view the results, the present study revealed that chitosan nanoparticles enriched feed improve the growth performance, enhance the fish immune response and improve biochemical indices, thus showing its importance in aquaculture.

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