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Dietary administration of

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Introduction

Freshwater fish culture is one of the fast growing sectors among aquaculture practices for the production of food to fulfill the requirement of uncontrolled human that producing food for the growing population of the world (Mert and Bulut, 2014). In Pakistan, freshwater fish culture play significant role in economy where the annual consumption of fish is 2.3 kg per capita, (FAO, 2004). Fresh water fishes, Rohu (*Labeo rohita*), Mrigal (*Cirrihinus mirrigala*) and Thaila (*Catla catla*) are commercially significant in Pakistan (Sheikh et al., 2017). Rohu is commercially cultured at large scale in the South Asia, Japan, Nepal, Philippines, Malaysia and of Africa. In 2014, global production of rohu reached to 1670, 270 tones (FAO, 2014).

Excess use of broad-spectrum chemotherapeutants has led to the expansion of antibiotic-resistant bacterial strains as well as water pollution (Lundén, Lilius, and Bylund, 2002). In aquaculture prevention and treatment of infectious diseases is mainly by vaccines. However, it is costly and cause physiological stress in fish (Misra, Das, Mukherjee, and Pattnaik, 2006). In such circumstances probiotics are considered as an alternative strategy to disease management and prevention in fish (Talpur *et al.,* 2014).

Probiotics in aquaculture show several modes of action like production of inhibitory compounds, enhance the immune response of fish, competitive exclusion of pathogenic bacteria, anticarcinogenic activity and antimutagenic enhancement of nutrition of host species through the production of supplemental digestive enzymes (Harikrishnan, Balasundaram, and Heo, 2010). It has been previously reported that probiotic increase growth and feed efficiency of fish (Mohapatra et al., 2012). The commonly used probiotics in aquaculture are bacteria (e.g. *Lactobacillus* spp., *Bacillus* spp., *Shewanella* spp.) and yeasts (such as *Saccharomyces* spp. or *Debaryomyces*spp.) (Cerezuela, Guardiola, Meseguer, and Esteban, 2012).

Several growth parameters were significantly increased in probiotic (*Bacillus subtilis*+ *Lactobacillus rhamnosus)* incorporated diet fed *L. rohita* and *Cyprinu scarpio* (Parvathi and Karthegaa; Munirasu, Ramasubramanian, and Arunkumar, 2017). Furthermore, yeast also used as a probiotic (Pooramini, Kamali, Hajimoradloo, Alizadeh, and Ghorbani, 2009) in fry of rainbow trout (*Oncorhynchus mykiss*), documented positive on survival, growth parameters and carcass quality Yeast can improve growth parameters by improving lactic acid bacteria and removal of disease producing bacteria especially cell wall constituents (Onifade et al., 1999).

There are new commercially sustainable and environment friendly probiotics need to be investigated. Therefore, in this study we investigated the effects of commercially available probiotic (Magic Grow Plus) on growth performance, activities of digestive enzymes (cellulase, protease and amylase), hematological parameters, proximate composition and immune response, of rohu in a natural earthen ponds in polyculture system.

MATERIALS AND METHODS

Experimental Fish Collection

Twelve hundred healthy fingerlings of rohu were purchased from the Faisalabad Fish Hatchery, Faisalabad, Pakistan and transported to Fisheries and Aquaculture Research Station, Quaid-i-Azam University (QAU), Islamabad, Pakistan. After 3 hours of transportation, 25 fish were randomly selected to and their average length and weight was observed.

Experimental Design

The 1200 fish fingerlings were evenly distributed in 6 earthen ponds (200 fingerlings/pond). The experiments were conducted in earthen ponds (area: 0.065 ha each). Before use, all 6 ponds were dried and outlets were closed, whereas inlets were screened with fine mesh to stop entry of any unwanted fish. Before filling the ponds with natural stream water, each pond was prepared by adding calcium carbonate at the rate of 125 $kgha^{-1}$ for disinfection and stabilization of pH and fertilized with animal manure (solid and semisolid cow dung) at the rate of 3333.33 $kgha^{-1}$ (Javed, Sial and Zafar, 1990) to enhance pond productivity. The water level was maintained at 1.5 m throughout the feeding trial. Semi-intensive culture system (beyond the level supported by food which is naturally available through the use of supplementary feed) was adopted and pond productivity was checked fortnightly using sacchi disc. In case reading was greater than 30 cm, the animal manure was added.

The experiments were performed in polyculture conditions (Including other fish species Mori (*Cirrhinus mrigala*), Grass carp (*Ctenopharyngodon idella*) between May and August. Completely randomized design was used to conduct the experiments in triplicates. The fingerlings were divided

into two groups, i.e. control group (C) (fed with 35% crude protein (CP) pellets without any supplement) and treated group (T) (the same basal pellets supplemented with probiotics Magic Grow Plus). The feed was given at the rate of 3% of the total body weight twice a day at 09:00 am and 16:00 pm. Every 14 days, 5-6 fish from each pond were randomly weighed and the feeding rate was set accordingly. During rearing period, water quality parameters were maintained optimum using Multi-parameter Hanna HI 9147. Temperature and dissolved oxygen were measured on a daily basis at 09:00 am and 16:00 pm whereas pH and total ammonia (TA) were measured every week. Water temperature (24.5 - 30.5°C), pH (7.5 - 8.0) and dissolved oxygen (5.0 - 7.0 mg L^{-1}) were maintained during the rearing period whereas TA was less than 0.2 mgL^{-1}.

Experimental Feed

Probiotic product Magic Plus contains 1 106^{a6} $CFUg^{-1}$ Bacillus subtilis and 1 106 $CFUg^{-1}$ yeast, *Saccharomyces cerevisiae* with final concentration of 1 10^{12} $CFUg^{-1}$ was obtained from the Department of Microbiology, QAU, Islamabad Pakistan. The dry pelleted 35% CP feed, 2 mm size was purchased from Oryza organic PVT LTD and used as basal and probiotic supplemented diet for the control and experimental groups respectively. Before starting feeding trial, all fish were fed with basal diet for 5 days in order to wean them on this diet. To add probiotic in experimental diet, 1 g Magic Plus powder was dissolved in 10 ml phosphate buffer (pH, 7.2), mixed well to make homogenous solution and sprayed on pelleted feed. Feed was kept overnight at ambient temperature. It was then dried and stored in zip-lock bag at low temperature for further use.

Growth Performance

At the end of the feeding trial, fish were not provided with feed for 24 hours before harvesting. For assessment of the growth performance, at least 20 fish were randomly collected and each fish was weighed individually, whereas for the assessment of total biomass of the pond, the fish were weighed collectively after complete drainage of the pond. Growth performance of the fish were measured using standard formulae for initial body weight (IBW), initial body mass (IBM), final body mass (FBW), final body mass (FBM), weight gain (WG), specific growth rate (SGR) and feed conversion rate (FCR) (Zhou *et al.,* 2013).

Enzymes Analysis

15 fish per pond were collected randomly, and anesthetized using clove oil (0.2 mlL^{-1}). The anesthetized fish were dissected on ice pads and their guts were removed, collected and weighed in bulk. Samples of intestine of 2-3 fish were pooled to take 1 g per sample that was stored at -80°C for quantitative assay of cellulase, amylase, and protease. Each sample was homogenized in 10 ml phosphate buffer (pH = 7.5) on ice and centrifuged at 4500 rpm at 4ºC for 15 min. The clear supernatant was transferred into another tube and later used for determination of different enzyme activities.

Determination of Cellulase Activity

For determining cellulase activity, the protocol described by (Denison and Koehn, 1977) was used with minor modifications. From each of the enzyme solution, 1 ml was mixed with 1% carboxymethyl cellulase solution and citrate buffer. This mixture was incubated for about 30 minutes at 50ºC. 3 mL of 3,5-Dinitrosalicylic acid (DNS) reagent was added and the solution was kept in a water bath for 15 minutes. 1.0 ml of 40% sodium potassium tartrate was added and then was kept for cooling at room temperature. The absorbance was measured against glucose being used as standard at 540 nm. One unit cellulase activity refers to the amount of enzyme in mL^{-1}culture filtrate that release 1 mg glucose min^{-1}.

Determination of Protease Activity

The protease activity was calculated by mixing 1 ml of the protease solution with 5 ml of casein solution (0.65%). The mixture was incubated for 5 minutes at 37ºC. Trichloroacetic acid (110 mM) solution was added to stop the reaction and again incubated at 37ºC for 30 minutes. After cooling at ambient temperature, the mixture was filtered using Whatmann filter paper. From the filtered solution an amount of 2 ml was mixed with the same amount of Na_2CO_3 (500 mM) and 1 ml of folin and ciolcaltea's reagents (0.5 mM). The mixture was once more incubated at 37ºC for 30 minutes and absorbance was measured at 660 nm using UV-visible spectrophotometer.

Determination of Amylase Activity

Amylase activity was determined using DNS method described by (Bernfeld, 1955) and (Areekijseree *et al.,* 2004). The principle of this method was the estimation of reducing sugar at 560 nm using glucose as standard. After incubation for 3-4 minutes at room temperature, 0.5 mL of amylase solution was mixed with 500 L of starch solution (1%). Then, 1 mL of DNS reagent

was added to the solution and incubated for 5 minutes in a boiling water bath and the solution was cooled at room temperature. The next step was the addition of 10 mL of reagent graded water followed by noting the absorbance at 540 nm on spectrophotometer. One amylase unit refers to the amount of enzyme mL^{-1} filtrate that release one microgram reducing sugar min^{-1}.

Proximate Composition

Muscle constituents of both control and treated groups were investigated using the standard protocol (AOAC, 2000) by collecting 3 fish per pond, hence 9 samples per group. Micro Kjeldahl procedure and Soxthlet apparatus were used for the determination of percent moisture, crude protein and lipid contents in fish flesh.

Moisture Content and Dry Matter

Moisture content and dry weight were calculated by taking 5 g of sample in a pre-weighed, washed china dishes and then placing that in the oven for 24 hours at 65ºC. Percent dry weight was calculated using the following formula:

$$\% \text{ Dry matter} = \frac{\text{Dried sample weight}}{\text{Sample weight when dried up}} \times 10\% \text{ moisture} = 100 - \% \text{ dry matter}$$

Crude Protein Contents

Crude protein in the samples was measured using Micro Kjeldahl's procedure. A sample weighing 5 g was put in 30 ml concentrated H_2SO_4. The mixture was heated at 250ºC for 2-3 hours till the appearance of light green color. The final volume (250 ml) was obtained in a volumetric flask by cooling the solution and adding deionized water. 10 ml of 40% (NaOH) was added to the mixture in Kjeldahl's equipment and heated for 3-5 minutes. Finally, NH_3 was collected in 10 ml of boric acid (2%) that contained methyl red drop as an indicator and titration was performed against H_2SO_4 (0.1N) upon the appearance of golden yellow color. The protein concentration was calculated by the following formula:

$$\text{Protein (\%)} = \frac{(1- \quad) \quad 0.0140 \quad .25}{} \quad 100$$

Where, V1- Vº = Volume of HCl, M= Sample weight and C= Concentration of HCl

Crude Lipid Measurement

For measuring lipid contents, 3 g sample was taken in thimble of the Soxthlet equipment and afterward in the extractor. The apparatus was then connected to a receiving flask that was supplied with 150 ml of ether. During this entire process of extraction, 3-4 drops per second were maintained at 100ºC for 10 hours. Finally, the thimble was removed from the apparatus and weighed. Total lipids were calculated using the following formula:

$$\text{Total lipid (\%)} = \frac{\text{Thimble weight after evaporation} - \text{Empty thimble weight}}{\text{Sample Weight}} \quad 100$$

Crude Ash

Crude ash was calculated using a pre-weighed crucible in which 5 g of the sample was taken and heated in a furnace at 550-600ºC for 24 hours. The crucible was then placed at room temperature for chilling. Immediate weighing of the ash was carried out to prevent moisture absorption using the following formula:

$$\text{Crude ash (\%)} = \frac{\text{Ash Weight}}{\text{Sample Weight}} \quad 100$$

Hematological Parameters

Blood samples were drawn from the caudal vein of 8 fish per pond using 3 ml syringes. Blood samples from each 2 fish was pooled together in an EDTA tubes. Hence 4 samples per pond and 12 samples per group were used for analysis. Hematological parameters including WBCs, RBCs, Hemoglobin (Hb), Hematocrit (Hct), Mean corpuscular Volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC) (were analyzed in both control and treated groups of juvenile rohu using Hematology Analyzer, while Glucometer (ACCUCHEK®Softcli) was used for measuring blood glucose level (mg dL^{-1}).

Immunological Parameters

For immunological study, blood was drawn from eight fish of each pond using a heparinized 3 ml syringe and collected in Eppendorf tubes such that each tube contain blood of 2 fish. Centrifugation of the blood at 1500 rpm for 15 min resulted in separation of serum which was then stored in separate Eppendorf and stored at 4ºC for further analysis of aspartate aminotransferase (AST), lysozyme activity, immunoglobulin (IgM) and total serum proteins.

Aspartate Aminotransferase Activity (AST)

AST activity was determined using an AST/GOT kit (AMEDA Laborodiagnostik GmbH, Graz Austria) according to the manufacturer's instructions. The absorbance was taken at 340 nm and AST activity was expressed in UL^{-1}.

Lysozyme Activity

For lysozyme activity, the protocol described by (Anderson and Siwicki, 1995) was followed with minor amendments. The serum (100µl) from fresh blood was taken in tubes and 900µl *Micrococcus lysodeikticus* (0.75mg mL^{-1}) (Lowry, Rosebrough, Farr, and Randall, 1951), Sigma, St Louis, MO, USA suspension in phosphate buffer saline with pH 6.2 were added. The solution was shaken well and absorbance was noted at 450 nm each minute. The change in the rate of absorbance was measured by mixing the bacteria. The lysozyme action was computed by use of white lysozyme of hen's egg (Sigma- Aldrich) as a standard.

Total Protein and Immunoglobulin in Plasma

For the estimation of total protein content in blood plasma, calibration curve was developed using bovine serum albumin as standard (Lowry *et al.,* 1951). Protocol described by (Anderson and Siwicki, 1995) was used for investigation of plasma immunoglobulin. For this purpose, 100 µl of plasma was mixed with 0.1 ml of polyethylene glycol (12%) to separate immunoglobulin from plasma by precipitation. The solution was incubated at room temperature under constant shaking for 2 hours, followed by centrifugation at 7000 rpm for 10 min. Using the same method, protein content was measured from diet and plasma separately. Subtraction of protein contents of diet from total protein content in plasma gave total immunoglobulin.

Statistical Analysis

The growth performance, biochemical indices, hematological parameters, proximate composition and the immunological findings were expressed in mean ± SE. The experimental data were analyzed with paired t- test by using Graph pad prism 5.

Results

Probiotic Enhanced the Growth of L. Rohita

Fish were stocked with approximately the same weight and length. Different growth performance parameters, (i.e., FBW, FBM, WG, SGR, Survival, WGR and FCR) were measured for 90 days and the results are presented in **Table 1**. We have found significant increase ($p<0.05$) in all the above

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Effects of commercial probiotics on immunity response in Rohu (*L. rohita)* after 90 days feeding trial in earthen ponds.

Parameters	Diets	
	Control Group	Probiotics Treated Group
White Blood Corpucles (103 l-)	248.77 ± 2.18	312.03 ± 3.63***
Plasma Protein (mg ml-)	15.73 ± 0.03	28.30 ± 1.03**
Immunoglobin (mg ml-)	7.99 ± 0.31	14.24 ± 0.81 *
Lysozyme Activity (g ml-)	4.91 ± 2.02	15.28 ± 0.02 *

All data represented as Mean ± SEM ns= nonsignificant *= p<0.05 **= p<0.01 and ***= p<0.001

Discussion

Feed additives like prebiotics, vitamins and mineral that work against pathogenic microbes have significantly improved the health of aquatic fauna (Ibrahem, Fathi, Mesalhy, and El-Aty, 2010). Literature suggests the efficiency of different probiotics in developing resistance against aquatic pathogens (Irianto and Austin, 2002) including *Carnobacterium spp.* (Robertson, 2000) and *vibrio spp.* (Planas, Gorina, and Chamorro, 2006). The current study was designed to investigate the consortial effect of two microbial species on growth, digestion and immune response of rohu.

We have found the enhancing effects of probiotic (Magic Grow Plus) on body weight, and decreasing effect on FCR in rohu. Furthermore, our results have also showed increase in SGR, however, statistically insignificant (p>0.05). The growth rate was enhanced with the same probiotic in *C. mrigala* as well (Ullah *et al.,* 2018). Other bacterial species like *Lactobacillus acidophilus* and *Bacillus* species, *Geotrichum candidum* and Biogen® probiotic enhanced growth rate in other fish species (Al-Dohail, Hashim, and Aliyu-Paiko, 2009; Mohapatra *et al.,* 2012). Previous reports have also showed significantly lower FCR in *T. putitora* (Talpur *et al.,* 2014) in probiotic treated fish. Improved growth rate was observed in a study where *Lactobacillus acidophilus* was used as probiotic in the feed of snake head fish (*Channa striata*) (Al-Dohail *et al.,* 2009).

Probiotic strains act as food supplements and cause movement of vital nutrients in gastrointestinal tract (Balcázar *et al.,* 2006). Increased digestive enzymes' activities have been reported in different teleosts by administration of various probiotics. These actively participate in production of a variety of digestive enzymes (De Schrijver and Ollevier, 2000) and can improve digestion with the help of digestive enzymes (Buts *et al.,* 1999). We have found significant increase in cellulase, protease and amylase activities in fish fed with probiotics. Previous studies have also reported increased levels of extracellular digestive enzymes after use of probiotics in different species (Ullah *et al.,* 2018; Gómez and Shen, 2008). Furthermore, probiotics also elevates the amylase level (Gómez and Shen, 2008) in *Nile tilapia* by yeast (Daboor, Esmael, and Lall, 2010).

Hematological parameters are important for determination of health condition of a fish (Ayoola, Ajani, and Fashae, 2013). We have found significantly higher levels of hemoglobin, MCH, HCT, glucose and AST in probiotics treated group of *L. rohita* compared to control group. Previous studies also reported increase in Hb, MCH, HCT and RBC by probiotics in other fish species (Sipra Mohapatra, Chakraborty, Prusty, PaniPrasad, and Mohanta, 2014). Blood glucose level was reduced in the probiotics treated group however remained in normal range. It is established that basal diet supplemented with *Bacillus subtilis* and *Lactococcus lactis* decreases the glucose level in *L. rohita* and with Magic Grow Plus in *C. mrigala* (Mohapatra et al., 2012).

AST level is an indicator of the fish health as it is secreted by the liver when against toxicity. We have found significantly decreased levels of AST in probiotic treated group indicating less toxicity and low activity of the liver. However, previous studies reported an increased AST level under probiotic treatment in tilapia, *C. mrigala* and *L. rohita* (Marzouk, Moustafa, and Mohamed, 2008; Mohapatra *et al.,* 2012; Ullah *et al.,* 2018).

Fish body composition is an important fish health indicator which varies specie in species, place and changes with diet and age (Quinton, Kause, Ruohonen, and Koskela, 2007). In the current study, proximate composition like muscles lipid and protein were significantly higher whereas fat and moisture were significantly lower in the group fed with probiotic. The findings of (Gong *et al.,* 2013) was in line with the results of our study, who chose chitosan as probiotic in the feed of shrimp *Penaeus monodon.* However, the difference observed in moisture was not significant among control (fish fed with basal diet) and treated groups (fish fed with probiotic enriched diet).

Parameters like Lysozyme, IgM, total protein contents and WBCs are the indicators of healthy immune system of the organisms (Rao, Das, Jyotyrmayee, and Chakrabarti, 2006;). We have found that the probiotics treatment increased the lysozyme activity, IgM, total protein contents and WBCs counts. Immune system of an organism is activated by the entry of any foreign antigen. Furthermore, increase in lysozyme and IgM activity has been reported in probiotics treated different fish species (Sipra Mohapatra *et al.,* 2014). Almost same findings have been reported by other studies in other fish species like in *Sparus aurata* (Salinas *et al.,* 2008), *Catla catla* (Krishnaveni and Thambidurai, 2013), *C. mrigala* (Ullah *et al.,* 2018) and *C. striata* (Talpur *et al.,* 2014). Rasha (2010) suggested, *Saccharomyces cerevisiae* and *bacillus subtilis* to elevate IgM level in fishes. Protein level in serum also indicates immunity, we have found increased level of the total protein in treated group compared to the control group. In other fish species like *C. gariepinus* the enhanced serum proteins have been found as a response to probiotics treatment (Al-Dohail *et al.,* 2009). We have found the enhanced level of WBC's in the *L. rohita* fed with probiotics enriched diet group. Almost similar results are reported in O. *niloticus* treated with probiotics containing *Bacillus subtilus* and yeast (Marzouk *et al.,* 2008).

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