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Nutritional effects of different levels of probiotics (*Lactobacillus acidophilus* and *Saccharomyces cerevisiae*) on the major carps (*Labeo rohita* and *Cyprinus carpio*): Comparative analysis

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Abstract

The nutritional effects of *Lactobacillus acidophilus* (L) and *Saccharomyces cerevisiae* (S) on the major carps were evaluated by comparative analysis. Material and Methods. The study was carried out in three replicates in each of the control and treatment groups, trial lasted 90 days in total. Phase-I was done on *C. carpio* (G1) and *L. rohita* (G2) for one month using *S. cerevisiae* and *L. acidophilus*. G1 includes LA1, LA2 and LA3 treatments and control (C) group in triplicates while G2 have SC1, SC2 and SC3 treatments to investigate the influence of different levels (0.15%, 0.30% and 0.60%) of probiotics. Phase II, was extended for 60 days on *Labio rohita* with dosages @ (0.15%, 0.30%, 0.45%, 0.60%, 0.75%, 1.0 % of *S. cerevisiae* administered with basal fish feed to examine growth, hematology, digestive enzyme activity, stress tolerance and survival rate. Better growth performance indicated that the addition of *L. acidophilus* and *S. cerevisae* with basal fish feed was overall significant but supplementation of *S. cerevisae* proved itself to be more effective for the substantial rise in the growth performance ($P<0.05$; 0.01) as compared to the control. The survival rate during growth performance was 100 %. *S. cerevisiae* promoted body crude protein, crude fiber, carbohydrate content, moisture, and ash in all treatments in comparison with the control (C) group. Crude lipid was not significantly increased in any treated group. Hematological parameters were significantly higher (RBC, HB, HCT, MCH, MCHC) in SC3 treatment while MCV was observed minimum.

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Introduction

Aquaculture and Fisheries are progressively increasing sector of food production across the globe. Fishes proved significant species in aquaculture industry as source of income and livelihoods (FAO, 2012, 2016; Addo, 2013). It will fulfil necessary demand of reasonable source of protein for the masses of developing countries like Pakistan (Welker and Lim, 2011). Being a rich source of nutrients, fish is essential for human health (Skonberg and Perkins, 2002). Among the teleost families cyprinidae family is one of the biggest in the world, with approximately 1700 species and 200 genera found in most freshwater ponds, lakes and rivers all over the world. The common carp (*Cyprinus carpio*) and Indian carp (*Labeo rohita*) are dominating species in freshwater fish culture and later is essential food fish. They have been widely used in farming as well-established species by the turn of the 20th century.

There are limited studies reported on physiological investigations of *L. rohita* including haematology except for the earliest findings by Siddiqui and Naseem (1979) and Maftuch *et al.* (2020).

Probiotics and prebiotic are feed additives or microorganisms that affect the host organism by producing inhibitory chemicals, enhancing microbial balance, encouraging immunological action by reducing the use of antibiotics and increasing natural defense in fishes (Gültepe *et al.*, 2011; Amenyogbe *et al.*, 2020). Probiotics are used to avoid the side effects of chemotherapeutic medicines by controlling pathogens thus resulting in high survival rate (Robertson *et al.*, 2000). Antibiotics, hormones and some salts are the growth-promoting additives that are widely used to fulfill demand of animal protein for human consumption (Lara-Flores *et al.*, 2003). The misapplication of these additives can lead to adversative problems such as deposition of residues, immunosuppression in cultivated fish species (Ellis *et al.*, 1988), production of resistant bacterial strains and degradation in aquatic ecosystems (Rao *et al.*, 1992; Mohseni *et al.*, 2012). Therefore, the only alternative is the use of probiotic feed additives and probiotics are thus increasingly becoming an integral part of aquaculture practices to attain high yield (Mohapatra *et al.*, 2014; Mohammadi *et al.*, 2016; Nazeer *et al.*, 2016; Yasin *et al.*, 2018).Various species of *Lactobacillus*, *Bacillus*, *Saccharomyces*, *Shewanella*, *Clostridium*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, *Aeromona* and *Carnobacterius* are the probiotics that are commonly used in the aquaculture industry (Kim *et al.*, 2010).

Lactic acid bacteria (LAB) are Gram-positive, anaerobic and facultative bacteria that have low pathogenicity and are widely dispersed in nature (Klare *et al.*, 2003; Aly *et al.*, 2008; Yisa *et al.*, 2015). These bacteria normally reside in the gut of healthy fish and are regarded as potential probiotics in aquaculture. LAB generate antimicrobial substances such as lactic acid that are adhesive to the mucosal layer, which inhibits pathogen colonization in the gastrointestinal tract (Chen *et al.*, 2017).

Lactobacillus and *Bacillus* have been the subject of several studies on the utilization of probiotics in the diet. They have a significant impact on disease resistance, general health and growth of various species, including fish and shellfish (Liu *et al.*, 2017). Numerous probiotic strains, including *Lactobacillus acidophilus*, *B. licheniformis* and *B. subtilis* have been reported to exhibit antimicrobial and immunomodulatory properties in the host animals (Cutting, 2011) thus improve immune response and survival rates (Lee *et al.*, 2010).

Saccharomyces cerevisiae, known as baker's yeast, is a well-known probiotic. It is a unicellular fungus that is either injected or given orally in fish feed due to its low cost (Tewary and Patra, 2011) to improve the growth rate in culturing of tilapia and carp (He *et al.*, 2009; Korkmaz and Cakirogullari, 2011). In fish diets yeasts can be used as a nutritional supplement as it is a good source of protein and B-complex vitamins. They can easily be produced on industrial scale using various carbon-rich substrate by-products. In aquaculture, several types of yeast, such as autolyzed yeast or probiotic live yeast are used to make various

feed additives (Ferreira *et al.*, 2010). Different immunostimulatory substances such as nucleic acids and chitin-mannan oligosaccharides found in *Saccharomyces cerevisiae*, have advantageous effects on growth parameters, disease resistance and stress tolerance in various fish and crustacean species (Ringo *et al.*, 2012; Ahmadi *et al.*, 2017).

According to the study, fish haematological and biochemical data can be utilized to identify healthy animals from disease-infected animals and to determine an organism's health status. Many authors have discussed the impact of haematological parameters in fish that are fed various types of diet (Ahmadifar *et al.*, 2020). However, there are limited publications on use of probiotics, haematological indices and the immunological response of fish (Ahmadifar *et al.*, 2020; Mani *et al.*, 2021). The aim of current study is to evaluate the effect of various concentrations of probiotics on the nutritional composition of final product (Fish), to analyze the effect of *Lactobacillus acidophillus* and *Sacchromyces cerevisiae* on the growth, body composition, digestive enzymes activity and immune responses of *Labeo rohita* and *Cyprinus carpio* and how these probiotics affected haematological parameters.

Materials and methods

Experimental design and conditions

In the current study similar weight (10±1) g of *Cyprinus carpio* and *Labeo rohita* were selected to conduct the proposed study in fish research lab, Department of Fisheries and Aquaculture, MNS University of Agriculture, Multan.

Experiment design

There were three replicates in each of the control and treatment groups in this research. The trial lasted 90 days in total. The experiment was split into two parts: Phase-I was done on both experimental fish species *Cyprinus carpio* and *Labeo rohita* for one month (30 days) using both probiotic species *Saccharomyces cerevisiae* and *Lactobacillus acidophilus*. Both experimental fishes were divided into two groups, G1 and G2, with equal body weights (10±1gm). Each aquarium contained 12 fish samples.

G1 was divided into three treatment groups: LA1, LA2 and LA3 of the *L. acidophilus* and control (C) group in triplicates, G2 was separated into three *S. cerevisiae* treatment groups: SC1, SC2 and SC3.

The following was fed to all of the treatments: LA1=basal fish feed + *L. acidophilus* @ 0.15 %; LA2=basal fish feed + *L. acidophilus* @ 0.30 %; LA3=basal fish feed + *L. acidophilus* @ 0.60 %; SC1=basal fish feed + *S. cerevisiae* @ 0.15 %; SC2=basal fish feed + *S. cerevisiae* @ 0.30 % and SC3=basal fish feed + *S. cerevisiae* @ 0.60 %. The control (C) group was fed with only basal diet.

In phase II, based on the improved findings of Phase I, the trial was extended for another 60 days on *Labio rohita* with varying dosages of *S. cerevisiae*, depending on better benefits observed in Phase I.

This 60-day experiment was split into six treatment and control groups, each of which was administered a basal fish feed plus probiotic in triplicate @ 0.15% or 0.30% or 0.45% or 0.60% or 0.75% or 1.0 %, respectively. Only the basal diet was provided to the control (C) group. The effects of various amounts of probiotic food supplementation on numerous parameters such as growth, hematology, digestive enzyme activity, stress tolerance and survival rate were assessed. The components for fish feed were purchased and the feed was prepared as Yasin *et al.* (2018).

Determination of nutritional effects (Growth performance)

Using electronic weighing machine (Uni Block D450011585 AUW) the fish was weighed weekly.

IBW: Initial body weight, FBW: final body weight, SGR: specific growth rate, FI: feed intake, FCR: feed conversion ratio, PER: protein efficiency ratio, PPV: protein productive value and survival rates were measured by using the following equations:

SGR = ($\ln W_f$ - $\ln W_i$ × 100) / t Where: ln W_f = the natural logarithm of the final weight, ln W_i= the natural logarithm of the initial weight, t = time (days) between $\ln W_f$ and ln W_i

FI = fish weight × feeding level/100, FCR = Feed consumed/Weight gain, WG = FBW (g) - IBW (g), PER = Weight gain (g)/protein fed (g), PPV = (Protein gain (g)/protein fed (g) × 100.

Proximate analysis (Analytical method)

The proximate composition for experimental fish was conducted by using the method of analyses adopted by the Association of Official Analytical Chemists (AOAC, 2005). Moisture contents of samples were dried in 24 hrs at 67-70 °C through the Micro Kjeldahl method adopted to determine Crude protein (CP) and Kjeldahl distillation unit (UDK 127, Velp Scientifica, Milano, Italy) and Soxhlet apparatus was used to extract Crude lipid by adding petroleum ether (60-80 °C). Muffle furnace (Hanau, Germany, model M110) was used to detect ash content at 550 °C for 12 hrs.

Moisture (%)

To examine moisture content by oven drying method (AOAC, 2005) 10 g of sample was kept at 67-70 °C in an oven for 24 hrs. The formula used to determine the moisture contents is: Moisture (%) = Loss in weight (Wt) of sample/weight of sample ×100.

Ash (%)

To determine the ash contents homogenized samples of organic components of known weight were burned with the help of a furnace. 2g sample was kept in in an electric furnace (EHRET TK/L 4105) at 450 °C in pre-weighed crucibles for 12 hrs until the formation of white ash. The contents were calculated through formula Ash (%) = weight (Wt) of ash/weight of sample × 100

Crude protein (%)

% Nitrogen was calculated using Kjeltec machine (Model Tecator Kjeltec System 8000). **S**amples were broken down by adding a mixture of K_2SO_4: $FeSO_4$:$CuSO_4$ @ 100:5:10, respectively until the color was changed to green. These samples were diluted with distilled water. In the distillation apparatus, NaOH (10 ml), Methyl red indicator and 4% boric acid (20 ml) was added with digested samples (10 ml to collect free ammonia in a beaker) and the material was titrated against H_2SO_4 (0.04 N). To calculate protein contents following formula was used: % of Nitrogen = volume of H_2SO_4 used × 0.0014 × volume of dilution / volume of distillate × weight of sample × 100, % Crude protein = Nitrogen × factor (6.25).

Crude lipid (%)

To measure the fat contents a set of Soxhlet system (Soxhlet extractor, thimble, flask, condenser and heating mantle, Behr-lab, D40599) was used. The flask was oven-dried (overnight at 60 °C) and a sufficient amount of petroleum ether was added to it as solvent. The sample (10 g) was kept in a thimble plugged with cotton wool at the top and then extractor was fitted with flask. It was fitted with the condenser and heating mantle. Now flask was heated and the extraction period was continued for 06 hrs until the solvent was mildly boiled. Lastly, the residual solvent was allowed to dry overnight in the oven at 60 °C and retained in a desiccator to cool down. To calculate fat contents following equation was used: TS: weight of thimble with dried sample (g), T: weight of thimble, S: weight of the dried sample (g), S= TS – T

FE: weight of flask with ether extract, F: weight of flask (g), EE: ether extracts (g), E: weight of ether extract (g), E = FE – F, EF (g/kg DM) = E × 100/S

Crude fiber (%)

Crude fiber contents were measured by taking 2 g of the sample and H_2SO_4 (200 ml) 1.25 % was added in a conical flask of 250 ml. After boiling for 30 minutes, the solution was passed for filtration process through the man filter paper. To the remaining filtrate 1.25 % NaOH (200 ml). Now in a digestion apparatus it was boiled for 30 minutes, filtered again and rinsed repeatedly to make it neutral. To rinse the filtrate distilled water was used and its neutralization was confirmed by pH paper. For drying purpose, the residues were shifted into a crucible and kept in an

electric oven at 100 °C for some hrs. Then it was placed in desiccator, allowed to cool and then weighed. It was burned again, allowed to cool and weighed. Following equation was used to estimate crude fiber contents: Crude fiber (%) = (wt. of sample + wt. of crucible)/ wt. of sample × 100.

Carbohydrates (%)

By subtracting the total crude protein, fat and ash contents from 100 Carbohydrate percentage was calculated. Following equation was used to calculate %: Carbohydrates (%) = 100 – (Fat + Crude Protein + Ash)

Haematological parameters

After 30 days of experimental trials, five fish were taken randomly from all aquariums for hematological analysis (Standen *et al.*, 2013).

Collection of the blood sample

During the experimental period blood samples were collected from the caudal vein of both control and probiotic-fed fishes randomly on weekly basis. The syringes of 02 ml were flushed with EDTA (Anticoagulant) and to avoid coagulation 150 to 200µl of EDTA was kept in syringe needles before taking the blood. The blood samples were transferred into Eppendorf (1.5 ml capacity) and stored for analysis.

Haematological analysis

The blood samples were analysed used to calculate Haemoglobin (HB %), Red blood cells count (RBCs count) and Haematocrit (HCT %) (Dacie and Lewis 1975). Red Cell Indices like MCH, MCHC and MCV, values were also calculated by the given formulae (Britton, 1963). The blood samples were centrifuged at 3000 r/mn for 15 min to get plasma for analysis (Casas and Dobrogosz, 2000). Red Cell Indices were calculated using following formulas were use (fl) = Hct /RBC, MCH (pg) = Hb/RBC × 10, MCHC (g/dl) = Hb/Hct × 100.

Digestive enzyme activities

After homogenizing digestive enzymes were extracted from the gut hand held homogenizer was used and cooled phosphate buffer (pH 7.5) with the ratio of 1/10 (w/v) was poured in it, then it was subjected to centrifugation at 4 °C and 5000 rpm for 5 min (Huang *et al.* 1999; Yanbo and Zirong 2006). Protein contents, protease, lipids and amylase enzyme activitty were determined from the extracts. The method of Lowry *et al.* (1951) was used to assess the protein contents. While protease enzyme and amylase enzyme activity were measured by adopting the methods of Anson, 1938 and Smith and Roe, 1949. The titrimetric method described by Essa *et al.* (2010) was used to measure the lipase enzyme activity.

Immunological parameters

Stress resistance and survival rate

To check the survival rate and stress resistance after 60 days' trials, the fish from all treatments were divided into two subgroups and challenge test was performed. For this purpose, the first subgroup was injected (IP) with pathogenic *A. hydrophila* (0.1 mL of 10^7 cells/mL) whereas, the second one as control group was administered with saline (0.1 mL). Salinity stress challenge test was also performed to determine stress resistance following Soleimani *et al.* (2012). From all aquaria, fish were divided in triplicate and then exposed to 15ppt salinity and monitored on daily basis to check the survival rate.

Basal feed preparation

The basal fish feed was prepared using the common ingredients, Fishmeal (25.00%), Soybean meal (6.09%), Corn starch (32.51%), Glutalys (10.00%), Sunflower oil (3.80%), Lysamine pea protein (10.00%), Vitamin-mineral premix (2.00%) and CMC-binder (0.50 %) that were purchased from the local market and its proximate chemical analysis was carried out according to AOAC (2005). A paste or semi-moist dough was made by mixing the ingredients with boiled water, which was passed through an electrical mincer to make pellets by Kenwood Multi-processor and dried for a few days at room temperature following crushing to make fine particles. Further these particles were mixed with proposed doses of probiotics at the time of feeding. During the whole period of experiments feed was

given twice a day (9.00 am and 4.00 pm each day) @ 5% of body weight.

Statistical analysis

To evaluate the data from all parameters Two-way ANOVA (analysis of variance) was used. The data was provided as treatment mean ± Standard deviation and the variation of means across various groups were examined for the significance at the 95% confidence level. $P < 0.05$ were considered significant using Duncan's multiple range test. Software package (SPSS, version 17) was used for statistical analysis.

Results

Phase 1 (Thirty days trial)

Growth performance in 30 days' trial on Labio rohita

In the first 30 days trial, growth performance was investigated by observing various parameters such as initial body weight (IBW), final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), the protein efficiency ratio (PER) and protein productive value (PPV). Analysis of variance for growth performance revealed a non-significant difference among treatments and the control group for initial body weight, a significant difference in final body weight ($P<0.05$) and highly significant ($P<0.01$) differences in specific growth rate, weight gain, feed intake, protein efficiency ratio, feed conversion ratio and protein productive value.

Growth performance was calculated after 7, 15, 21 and 30 days and survival rate of this trial was 100%. The initial body weight of fishes was nearby to each other, as in treatments LA1 (8.65±0.33g), LA2 (8.98±0.54g) LA3 (9.09±0.12g), SC1 (8.94±0.58g), SC2 (8.85±0.75g) and SC3 (8.55±0.69g) and in the (C) control (8.44±0.20g) respectively. Maximum final body weight was observed in SC3, which was 23.97±0.14g and minimum final body weight was 20.07±0.12g in LA1 while final body weight was observed 19.04±0.32g in the control (C) group. All the treatments exhibited higher FBW as compared to the control (C) group after 30 days. Maximum weight gain (WG) was observed 15.54±0.56g in SC3 and a minimum of 11.02±0.49g in LA1, while in the control (C) group it was noted 10.54±0.67g.

Specific growth rate (SGR) was observed maximum in SC3 (2.04±0.25g) and the minimum in LA2 (1.43±0.21g) while it was 1.22±0.13g in the control (C) group. Feed intake (FI) was observed maximum of 70.31±1.04g in SC3 and a minimum of 59.81±1.73g in LA1 while it was observed 56.92±1.66g in the control (C) group. Feed conversion ratio (FCR) was observed maximum in LA2 (6.34±0.50g) and minimum in SC3 (5.66±0.55g), while in control (C) it was observed 5.55±0.30g. Protein efficiency ratio (PER) was maximum in SC3 (0.90±0.13) and minimum in LA2 (0.63±0.21g) while in control (C) it was noted 0.61±0.21g. Protein productive value (PPV) was observed maximum in SC3 (12.33±0.52g) and minimum in LA2 (10.50±0.56g) while in control (C) its ratio was observed 9.88±0.61g.

The overall result of the 30-day trial indicated that IBW was approximately similar in control (C) and all treatment groups. Final body weight was maximum in SC3 and overall FBW was observed high in treatments SC1, SC2 and SC3 fed with *S. cerevisiae* as compared to the treatments LA1, LA2 and LA3 fed with *L. acidophilus*. Weight gain was calculated maximum in SC3 and it was observed that all treatments fed with both probiotics have better weight gain as compared to the control (C) group. In the case of SGR maximum value was found in SC3 and better SGR was noted in all treatments fed with probiotics as compared to control (C). Maximum feed intake was 70.31±1.04g /fish in 30 days in SC3 while FCR was observed Minimum in SC3 (5.66±0.55g). PER and PPV were observed maximum in SC3 and were increased significantly due to the probiotics in experimental diets than control.

Growth performance in 30 days' trial on Cyprinus carpio

Trial for growth performance was investigated by observing same parameters mentioned above for *Labeo rohita*. Analysis of variance for growth performance revealed a non-significant difference

among treatments and the control group for initial body weight. A significant difference in final body weight was observed among the control group and various treatments ($P<0.05$). Highly significant ($P<0.01$) differences were observed in specific growth rate, weight gain, protein efficiency ratio, protein productive value, feed intake, feed conversion ratio (Table 1, Fig. 1).

Growth performance was calculated and survival rate of this trail was 100%. The initial body weight values of fishes were close to each other, as in treatments LA1 (7.35±0.23g), LA2 (7.08±0.24g) LA3 (8.01±0.02g), SC1 (7.84±0.57g), SC2 (7.75±0.65g) and SC3 (7.66±0.59g) and in the (C) control (7.54±0.70g) respectively. Maximum final body weight observed was 21.07±0.44g in SC3, wand minimum was 18.89±0.65g in LA1 while final body weight was observed 18.23±0.22g in the control (C) group. After 30 days, All the treatments exhibited higher FBW as compared to the control (C) group. Maximum weight gain (WG) was observed 14.22±0.59g in SC3 and a minimum of 9.92±0.79g in LA1, while in the control (C) group it was noted 10.22±0.02g. Specific growth rate (SGR) was observed maximum in SC3 (1.55±0.25) and the minimum in LA2 (1.06±0.21) while it was 1.09±0.43 in the control (C) group. The maximum value 66.91±1.02g of Feed intake (FI) was observed in SC3 and a minimum of 54.71±1.63g in LA1 while it was observed 55.62±1.76 in the control (C) group. Feed conversion ratio (FCR) in LA2 was observed to be maximum (5.22±0.30g) and minimum in SC3 (4.36±0.35g), while in control (C) has the value 5.25±0.10g. Protein efficiency ratio (PER) was maximum in SC3 (0.65±0.13g) and minimum in LA2 (0.51±0.11g) while in control (C) it was noted 0.55±0.03g. Protein productive value (PPV) has the same trend as (PER) it was found to be maximum in SC3 (10.93±0.42g) and minimum in LA2 (9.31±0.26g) while in control (C) its ratio was observed 10.05±0.31g.

Table 1. Treatment mean±SE of haematological analysis for 30 days trial in *Cyprinus carpio*

Means	Treatments						
	Control	SC1	SC2	SC3	LA1	LA2	LA3
RBC	3.04±0.34B	3.23±0.05B	3.35±0.49B	3.34±0.09A	3.28±0.51B	3.52±0.09A	3.59±0.52A
HB	4.68±0.24C	4.87±0.25BC	4.86±0.25BC	5.23±0.44AB	4.88±0.38BC	4.97±0.17BC	5.87±0.61A
HCT	23.68±0.30A	23.95±0.48A	24.03±0.02A	24.47±0.58A	24.32±0.51A	24.37±0.55A	24.60±0.65A
MCV	8.49±0.79A	8.43±0.67A	8.48±0.75A	7.67±0.67A	8.36±0.84B	7.65±0.67B	7.62±0.64B
MCH	16.43±0.94A	16.59±0.75A	16.73±0.78A	15.99±0.80AB	16.61±1.23A	15.62±0.74B	15.99±0.50AB
MCHC	19.70±0.73C	20.09±0.60BC	19.99±0.54BC	20.96±0.93AB	20.92±0.85BC	20.83±0.82BC	21.81±0.99A

Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Table 2. Treatment mean±SE of haematological analysis for 30 days trial in *Labio rohita*

Means	Treatments						
	Control	SC1	SC2	SC3	LA1	LA2	LA3
RBC	3.24±0.54B	3.43±0.25B	3.55±0.69B	3.54±0.29A	3.48±0.71B	3.72±0.29A	3.79±0.72A
HB	4.88±0.44C	5.07±0.45BC	5.06±0.45BC	5.43±0.54AB	5.08±0.48BC	5.07±0.37BC	6.01±0.81A
HCT	23.88±0.60A	24.95±0.68A	24.33±0.32A	24.57±0.78A	24.62±0.51A	24.87±0.75A	24.80±0.85A
MCV	8.69±0.89A	8.63±0.97A	8.68±0.85A	7.77±0.77A	8.56±0.94B	7.85±0.87B	7.82±0.84B
MCH	16.63±0.84A	16.89±0.85A	16.83±0.88A	16.09±0.90AB	16.71±0.23A	16.42±0.84B	16.09±0.60AB
MCHC	20.70±0.83C	21.29±0.80BC	20.09±0.74BC	21.06±0.03AB	21.02±0.45BC	21.05±0.92BC	22.21±0.99A

Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

The overall result of the 30-day trial indicated that the values of IBW was approximately same in control (C) and all treatment groups. Final body weight was observed high in all treatments with *S. cerevisiae* while it was maximum in SC3 as compared to the treatments LA1, LA2 and LA3 fed with *L. acidophilus*.

It was observed that all treatments fed with both probiotics have better weight gain as compared to the control (C) group while it was observed maximum in SC3 and same is the case of SGR, where maximum value was found in SC3 and better SGR was noted in all treated fish as compared to control (C). Maximum feed intake was 66.91±1.02g /fish in 30 days in SC3 while FCR was observed Minimum in SC3 (4.36±0.35g). The values of PER and PPV were also observed maximum in SC3 and were increased significantly due to the probiotics in experimental diets.

Proximate analysis Labio rohita in 30 days trail

For proximate analysis, for moisture (P>0.05) analysis of variance revealed a non-significant difference among treatments and control (C) group. A significant difference was observed in crude protein, crude lipids and ash contents when compared between various treatments and control (C) group (P<0.05). Highly significant (P<0.01) differences were observed in carbohydrates and crude fiber.

The outcomes of proximate analysis after 30 days' trial described that the moisture contents were maximum in SC3 (80.63±0.81) and minimum in LA3 (77.07±0.53) in fish muscles while, these were recorded (78.92±0.97) in the control (C) group. Crude lipids were observed maximum in LA3 (25.67±0.55), and minimum in SC3 (21.53±0.36), while it was observed 21.16±0.29 in the control (C) group. The results were opposite for crude protein contents that were maximum in SC3 while minimum in LA2. The SC3 has maximum value of ash contents and the minimum value was observed in LA3, while the control (C) group had 16.7±0.93 ash contents. For crude fiber, the maximum value was observed in SC2 (3.04±0.29) while the minimum value was noted in LA1 (2.02±0.16) whereas, in the control (C) group its value was found 1.88±0.27. Proximate analysis revealed the nutritional value of carbohydrate was maximum in SC3 (5.24±0.18) and minimum in LA1 (4.28±0.16) while, the control (C) group has the value 4.02±0.01.

After 30 days, the overall results of the proximate analysis indicated that the yeast, *S. cerevisiae* promoted the chemical composition of fish muscles as compared to control (C) as well as *L. acidophilus* treated groups and this significant change in composition is due to inclusion of dietary probiotics. The tested diets proved to increase significantly the crude protein and other content, as compared to the control (C) group, while crude lipid was decreased.

Proximate analysis Cyprinus carpio in 30 days trail

For proximate analysis, for moisture (P>0.05) analysis of variance revealed a non-significant difference among treatments and control (C) group. A significant difference in crude protein, crude lipids and ash contents were observed among various treatments and control (C) group (P<0.05). The differences were highly significant (P<0.01) for carbohydrates and crude fiber.

After 30 days' trial the results of proximate analysis described that the moisture contents in fish muscles were observed maximum in SC3 (76.03±0.61) and minimum (74.07±0.53) in LA3 while, these were recorded (73.32±0.27) in the control (C) group. It was obvious from the results that crude protein contents were recorded maximum in SC3 while minimum in LA2. Crude lipids were observed maximum (21.37±0.25) in LA3, and minimum in SC3 (19.83±0.16), while in in the control (C) group, the value noted was 20.01±0.29. SC3 has maximum value of ash contents observed and LA3 has the minimum, while the control (C) group contains 16.08±0.33 ash contents. In this study, the maximum value of crude fiber was observed in SC2 (2.04±0.19) while the minimum value was observed in LA1 (1.92±0.16) whereas, in the control (C) group its value was found 1.78±0.17. Nutritional value of carbohydrate value was found to be maximum in SC3 (5.2±0.28) when proximate analysis was performed, and minimum in LA1 (4.68±0.16) whereas, in the control (C) group its value was found 3.22±0.17.

After 30 days, the overall results of the proximate analysis indicated that the yeast, *S. cerevisiae*

promoted the chemical composition of fish muscles as compared to control (C) as well as *L. acidophilus* treated groups and this significant change in composition is due to inclusion of dietary probiotics. The tested diets proved to increase significantly the crude protein and other content, as compared to the control (C) group, while crude lipid was decreased.

Hematological parameters in Labio rohita in 30 days trail

The analysis of variance showed a non-significant difference between treatments and the control (C) group for HCT (P>0.05). RBC and MCV levels were found to differ significantly between the control (C) group and the different treatments (P<0.05). In HB, MCH and MCHC, very significant differences (P<0.01) were found (Table 2, Fig. 1).

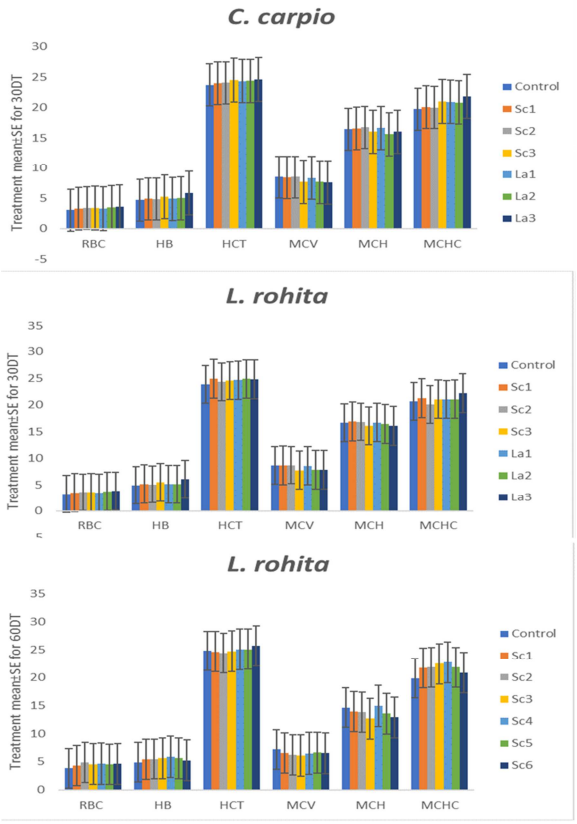

Fig. 1. Comparative analysis of probiotics impact on various haematological parameters in current study in both experimental fish species *Cyprinus carpio* and *Labio rohita*.

LA3 had maximum RBC (3.79±0.72) while minumum count were in control (C) (3.24±0.54) and other treated groups. LA3 (6.01±0.81) had the highest HB, whereas control (C) had the lowest (4.88±0.44). In comparison to the control (C) group (23.88±0.60) and other treated groups, the HCT was highest in SC1 (24.95±0.68). MCH, MCHC and MCV values for Red Cell Indices were also computed. The highest MCV value was found in the control (C) group (8.69±0.89), while the lowest value was observed in SC3 (7.77±0.77). SC1 (16.89±0.85) had the highest MCH value, while LA3 and SC3 (16.09±0.60 and 16.09±0.90) had the lowest. LA3 had the highest MCHC value (22.21±0.99), while LA1 and SC2 had the lowest (21.02±0.45 and 20.09±0.74 and the control (C) group had the lowest (20.70±0.83).

During the 30-day trial, blood samples were obtained at 0, 15 and 30-day intervals. SC2 (3.55±0.69) had a substantially higher RBC count than control (C) (3.24±0.54) and other treatment groups. LA3 (6.01±0.81) had the highest HB, whereas control (C) had the lowest (4.88±0.44). In comparison to the control (C) group (23.88±0.60) and other treatment groups, the HCT was highest in SC1 (24.95±0.68). MCV, MCH and MCHC values for Red Blood Cell Indices were also computed. The SC3 group had the lowest MCV values (7.77±0.77), whereas the control (C) group had the highest (8.69±0.89). The highest MCH value was found in SC1 (16.89±0.85), while the lowest values were found in LA3 and SC3 (16.09±0.60 and 16.09±0.90), respectively). LA3 (22.21±0.99) had highest MCHC value while control (C) group have 20.70±0.83 value. The RBC count, HB, HCT and red blood cell indices all increased significantly, which was a positive indicator.

Hematological parameters in Cyprinus carpio in 30 days' trail

The analysis of variance indicated a non-significant difference between treatments and the control (C) group for HCT (P>0.05). RBC and MCV levels were found to differ significantly between the control (C) group and the different treatments (P<0.05). In HB, MCH and MCHC, very significant differences (P<0.01) were found. LA3 (3.59±0.52) had a higher RBC count than control (C) (3.04±0.34) and other

treated groups (3.04±0.34) (Table 1, Fig. 1). The highest HB was found in La3 (6.31±0.12), while the lowest was found in control (C) (4.68±0.24). In comparison to the control (C) group (23.68±0.30) and other treatment groups, the HCT was highest in SC3 (24.47±0.58). MCH, MCHC and MCV values for Red Cell Indices were also computed. The largest MCV value was found in the control (C) group (8.49±0.79), while the lowest value was found in SC2 (16.73±0.78) had the highest MCH value, while LA2 and SC3 (15.62±0.74 and 15.99±0.80) had the lowest. LA3 had the highest MCHC value (21.81±0.99) and the lowest in SC2, whereas the control (C) group had 19.70±0.73.

During the 30-day trial, blood samples were obtained at 0, 7, 15 and 30-day intervals. The RBC count was considerably higher in La3 (3.59±0.52) compared to the control (C) (3.04±0.34) and other treated groups. The highest HB was found in La3 (6.31±0.12), while the lowest was found in control (C) (4.68±0.24). In comparison to the control (C) group (23.68±0.30) and other treatment groups, the HCT was highest in SC3 (24.47±0.58). MCV, MCH and MCHC values for Red Blood Cell Indices were also computed. The lowest MCV values were found in La3 (7.620.64), while the highest value was found in SC2 (16.73±0.78). SC2 had the highest MCH value (16.73±0.78), while LA2 and SC3 had the lowest (15.62±0.74 and 15.99±0.80, respectively). SC3 had the highest MCHC value (20.96±0.93), whereas the control (C) group had the lowest (19.70±0.73). The RBC count, HB, HCT and red blood cell indices all increased significantly, which was a positive sign.

Digestive enzymes activity in Labio rohita in 30 days trail

Analysis of variance for digestive enzyme activities of protease after 30 days described a non-significant difference in protein content of ($P>0.05$). In amylase and lipase ($P<0.05$) significant differences in protein contents were observed. There were highly significant ($P<0.01$) differences in total and specific activities in protease, amylase and lipase. According to our results, maximum value 14.81±0.31mg/ml^{-1} of protein contents were in SC1 while SC3 has a minimum 13.72±0.19mg/ml^{-1}.Whereas, it was observed 13.46±0.22mg/ml^{-1} in the control (C) group. The total amylase (U ml^{-1}) and specific (U mg protein^{-1}) amylase activities were found significantly higher in *L. rohita* that were fed with different quantities of dietary probiotic as compared to the control one that were not fed with probiotics. The maximum (41.65±0.56U/ml) total amylase activities were found in SC3 and minimum was recorded in LA3 (30.38±0.82U/ml). The value observed in control group was 33.24±0.75U/ml. The results for Specific amylase activities showed the same trend as total amylase. These were observed maximum in SC3 (5.49±0.34U/mg) and minimum 4.04±0.25U/mg in LA1, while in the control (C) group its value was observed 3.71±0.26U/mg. When observed after 30 days' trial Fish fed with *S. cerevisiae* showed the highest total and specific protease activity while fish fed with only a control (C) diet showed the minimum value. Maximum total protease activity (7.17±0.29U/ml) was recorded in SC3 and minimum 5.57±0.40U/ml in LA1, while in control group it was observed 4.44±0.36U/ml. The specific activity has the same trend it was observed maximum in SC3 (1.05±0.12U/mg) and lowest 0.90±0.22U/mg in LA1, while it was recorded 0.62±0.41U/mg for the control (C) group. Results indicated that the addition of different probiotics in different doses enhanced the total and specific activity of protease enzyme in *L. rohita* also the total fatty acids liberated was significantly higher as compared to the control diets. The highest value of total fatty acids liberated were observed in SC3 (5.34±0.40ml) than the total lipase activities of all experimental treatments. Like lipase enzyme, the values recorded in SC3 (0.95±0.33) for specific lipase activity were higher than that of other probiotic fed groups and control (C) group.

Digestive enzymes activity in Cyprinus carpio in 30 days trail

Analysis of variance for digestive enzyme activities of protease after 30 days described a non-significant difference in protein content of ($P>0.05$). In amylase and lipase ($P<0.05$) significant differences in protein

contents were observed. There were highly significant (P<0.01) differences in total and specific activities in protease, amylase and lipase. According to our results, the maximum protein contents were observed 13.13±0.31mg/ml in SC1 while they were a minimum 11.88±0.28mg/ml in SC3 and 12.03±0.42mg/ml in the control (C) group. The total amylase and specific amylase activities were found significantly higher in *Cyprinus carpio* that were fed with different quantities of dietary probiotic as compared to the control one that were not fed with probiotics. The maximum total amylase activities (38.55±0.46U/ml) were found in SC3 while it was recorded minimum (30.28±0.22U/ml) in LA3 and in the control (C) group fed without probiotics, its value observed was 31.04±0.75U/ml. Specific amylase activities with the same trend as total amylase has maximum observed in SC3 (4.59±0.14U/mg), minimum 4.11±0.25U/mg in LA1 and the control (C) group has observed value as 3.81±0.16U/mg. When observed after 30 days' trial Fish fed with *S. cerevisiae* showed the highest total and specific protease activity while fish fed with only a control (C) diet showed the minimum value. In SC3 maximum total protease activity was recorded (5.17±0.19U/ml) and minimum 4.97±0.30U/ml was recorded in LA1, while it was observed 4.04±0.26U/ml in the control (C) group. The highest Specific activity (1.09±0.22U/mg) in SC3 was observed and lowest 0.80±0.22U/mg in LA1, while it was recorded 0.42±0.21U/mg for the control (C) group. Results indicated that the addition of different probiotics in different doses enhanced the total and specific activity of protease enzyme in *C. carpio* also the total fatty acids liberated was significantly higher as compared to the control diets. The total fatty acids liberated of *C. carpio* fed with supplementary probiotic diet were significantly higher as compared to the control (C) group that were given normal diet. The highest total fatty acids liberated were observed in SC3 (5.34±0.30ml) than the total lipase activities of all experimental treatments. Like lipase enzyme, the values recorded in SC3 (0.95±0.23) for specific lipase activity were higher than that of other probiotic fed groups and control (C) group.

Table 3. Treatment mean±SE of haematological analysis for 60 days trial in *Labio rohita*

Means	Treatments						
	Control	SC1	SC2	SC3	SC4	SC5	SC6
RBC	3.84±0.78D	4.29±0.62C	4.90±0.41C	4.57±0.48AB	4.63±0.74A	4.54±0.57BC	4.64±0.55C
HB	4.93±0.53D	5.46±0.61BC	5.48±0.60BC	5.62±0.55AB	5.86±0.56A	5.64±0.65BC	5.25±0.60CD
HCT	24.81±0.71A	24.63±0.70A	24.39±0.58A	24.71±0.57A	25.04±0.60A	25.08±0.65A	25.65±0.38A
MCV	7.20±0.55A	6.54±0.59B	6.20±0.31BC	6.10±0.39C	6.49±0.72BC	6.64±0.63B	6.51±0.49BC
MCH	14.59±0.08A	13.88±0.84ABC	13.81±0.73BC	12.63±0.51C	14.91±1.26AB	13.51±0.84ABC	12.87±0.58BC
MCHC	19.83±0.45D	21.70±0.90BC	21.83±0.93ABC	22.43±0.88AB	22.74±0.78A	21.81±0.73BC	20.82±0.74CD

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean.

Phase 2 (Sixty days trial)

Growth performance in 60 days' trial

60 days' trial was conducted to evaluate the effect of various levels of dietary supplementation of *S. cerevisiae* as a feed supplement on growth performance of *Labio rohita*. In this trial growth performance was examined by observing various parameters such as initial body weight (IBW), final body weight (FBW), weight gain (WG), feed intake (FI), feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER) and protein productive value (PPV). After 60 days' trial analysis of variance for growth performance revealed non-significant differences among treatments and control (C) group for initial body weight (P>0.05). In final body weight; weight gain, feed intake, feed conversion ratio specific growth rate, protein efficiency and protein productive values, highly significant (P<0.01) differences were observed.

The initial body weight of fishes was nearby to each other, as in treatments SC1 (9.82±0.66g), SC2 (9.86±0.39g), SC3 (10.80±0.47g), SC4 (9.73±0.37g), SC5 (9.92±0.18g) and SC6 (9.88±0.41g) and in

control (9.62±0.36g). Maximum final body weight in SC_3 after 60 days was observed which was 42.83±1.62g while minimum in SC_4 which was 31.59±0.81g whereas, in control (C) group final body weight was observed 28.83±1.47g. All the treatments exhibited higher FBW compared to control (C) group after 60 days. Maximum Weight gain (WG) 42.83±1.62g in 60 days was observed in SC_3 and minimum 31.59±0.81g in SC_4 while in control (C) it was observed 28.83±1.47g. Specific growth rate (SGR) was observed maximum in SC_3 (2.02±0.01) and minimum in SC_4 (0.85±0.12) while in control (C) it remained 0.98±0.13. Maximum 235.88±3.64g Feed intake (FI) was observed in SC_3 and minimum 195.54±5.38g in SC_4 whereas; in control (C) group was observed 166.13±8.47g. Feed conversion ratio (FCR) noted was maximum in SC_4 (9.99±0.35), minimum in SC_6 (8.99±0.21) and in control (C) group it was (8.03±0.13). Protein efficiency ratio (PER) was maximum in SC_4, SC_5 and SC_6 (0.88±0.12) and minimum in SC_3 (0.42±0.21) while in control (C) group it was observed (0.46±0.11). SC_3 has maximum (7.44±0.22) Protein productive value (PPV) and SC_4 has minimum (6.98±0.21) while control (C) has 6.14±0.20. After 60 days of trial survival rate was observed 100%.

Over all outcomes of 60 days' trial indicated that IBW was approximately similar in all treatments and control (C) groups. SC_3 fed with 0.60% dose of *S. cerevisiae* has maximum Final body weight. Weight gain was calculated maximum in SC_3 and it was observed that all treatments fed with probiotics have better weight gain as compared to control (C) group. Better SGR was noted in all treated fish fed with probiotics as compared to the control (C) and SGR maximum value was found in SC_4. Maximum feed intake was 235.88±3.64g /fish after 60 days in SC_6. FCR was observed Minimum (8.99±0.21) in SC_6. PER was observed similar and maximum in three treatments SC_4, SC_5, SC_6 and was significantly increased due to use of probiotic as supplement in experimental diets as compared to the control (C) group. PPV was maximum (7.44±0.22) in SC_3 and gradual increase was observed from control (C) to SC_6 (Table 2). The survival rate in 60 days' experiment was observed 100 %.

Proximate analysis after 60 days

Analysis of variance for proximate composition analysis after 60 days revealed that significant differences were observed among treatments and control (C) group for moisture and crude protein ($P>0.05$) whereas, highly significant differences were observed in crude lipids, ash contents, crude fiber and carbohydrates ($P<0.01$).

After 60 days the outcomes of proximate analysis described that SC_6 has the maximum (82.66±0.36 %) moisture contents, it was observed minimum in SC_3 (80.78±0.80 %). The results presented that moisture contents were recorded 79.77±0.53 % in the control (C) group. Results that protein contents were maximum in SC_6 while these were minimum in SC_3. Crude lipid was observed maximum in SC_3 (23.42±0.61 %) and these were minimum in SC_5 (21.22±0.17 %) while in control (C) it was 21.60±0.38%. It was indicated from the results that the maximum value of ash content was observed in SC_3 and minimum value was observed in SC_5. In this study, after performing experiments on fish the maximum value (3.25±0.16 %) of crude fiber was observed in SC_3 and minimum value was observed in SC_5 (2.55±0.19) while in control (C) it was 1.66±0.15 %. After performing analysis on experimental fish, the maximum (5.25±0.17 %) nutritional value of carbohydrates was found in SC_6 whereas it was minimum in SC_2 (3.44±0.36). In control (C) it was observed 3.21±0.02 %.

The proximate composition analysis of fish muscles indicated that by addition of dietary probiotics the proximate composition was significantly changed. The tested diets proved to increase significantly the selected parameters of proximate compositions, including moisture, ash contents, crude protein, crude lipids and carbohydrates as compared to control (C) group. In contrast, the groups fed with *S. cerevisiae* produced low crude lipid so the value decreases for crude lipid content, as compared to control (C) group.

Hematological parameters in Labio rohita in 60 days' trail

After 60-day trial, an analysis of variance for hematological parameters revealed a non-significant difference between treatments and the control (C) group for HCT ($P>0.05$). RBC, HB, MCV, MCH and MCHC which showed very significant ($P<0.01$) differences. Initial readings for the hematological parameters of *Labio rohita* at 0-60 days of the 60-day experiment. All groups had RBC counts ranging from 3.84±0.78 to 4.90±0.41, with SC2 (4.90±0.41) having the highest and SC1 (4.29±0.62) having the lowest. In the control group, the RBC count was 3.84±0.78 (Table 3, Fig. 1). SC4 had the highest HB (5.86±0.56) and control had the lowest (4.93±0.53). The HCT of the control (C) was found to be 24.81±0.71 while the maximum was in SC6. The MCV, MCH and MCHC Red Cell Indices were also computed; the highest MCV value was recorded in control (7.20±0.55) and the lowest value was observed in SC3 (6.10±0.39). SC4 had the highest MCH value (14.91±1.26) while SC3 had the lowest (12.63±0.51). The maximum MCHC value was computed in SC4 (22.74±0.78), while the value in control was 19.83±0.45 (Table 3, Fig. 1).

Digestive enzymes activity

Digestive enzymes extraction after 60 days

Protein content

According to present study results SC6 has maximum (18.02 ±0.25 mg/ml) protein contents and SC4 has minimum (15.37±0.28mg/ml) while in control (C) group it was observed 15.07±0.26mg/ml. After 60 days, analysis of variance for digestive enzyme activities described highly significant difference in protein content of Amylase and protease ($P>0.01$). Significant differences ($P<0.05$) in protein contents of lipase were also observed ($P<0.05$). Highly significant ($P<0.01$) differences were observed in total and specific activities in protease, amylase and lipase.

Amylase activity

The total amylase (U ml^{-1}) and specific (U mg $protein^{-1}$) amylase activities were significantly higher in those *Labio rohita* which were fed with different levels of dietary probiotic (*S. cerevisiae*) as compared to those that received the control (C) diet. The maximum (50.22±1.38 U/ml) total amylase activities were observed in SC6 and minimum was observed in SC1 while it was recorded in the control (C) group fed with control (C) diet. Specific amylase activities showed the same trend with maximum in SC6 (7.32±0.20U/mg Protein) and minimum in SC1(4.54±0.13U/mg) like total amylase. In control (C) group it was observed 4.27±0.21U/mg Protein.

Protease activity

When observed after 60 days, Fish fed with *S. cerevisiae* along with normal diet showed the highest total and specific protease activity as compared to fish fed with only control diet. Maximum (7.88±0.16U/ml) total protease activity was recorded in SC6 and it was minimum 5.66±0.22U/ml in SC1 while 5.33±0.21U/ml in control (C) group. The highest Specific activity was observed in SC6 (1.07±0.13U/mg Protein) and it was recorded (0.69±0.11 U/mg Protein) in SC1 while for control (C) group it was noted (0.54±0.11U/mg Protein). Results indicated that addition of different probiotic in different doses enhanced the total and specific activity of protease enzyme in *L. rohita* as compared to the control diet.

Lipase activity

L. rohita fed with supplementary probiotic along with control (C) diet liberated significantly high fatty acids as compared to control (C) group. The SC6 (6.31±0.18ml) was observed with highest total fatty acids liberated than the total lipase activities of all experimental treatments. The minimum total fatty acids liberated were observed as 4.77±0.15 (ml) in SC1 while it was 4.19±0.17 (ml) in control (C) group. Like lipase enzyme similar pattern was observed for Lipase specific activity, value recorded for SC6 was higher (0.92±0.12) than that of other treatments and control (C) group. It was recorded minimum as (0.35±0.01U/mg Protein) in SC1 while 0.29±0.21U/mg Protein in control (C) group.

Immunological parameters

Stress resistance and survival rate

Two subgroups of fish were made after 60 days' trial, to assess the stress resistance and survival rate of treated and control (C) groups feed with various doses of *S. cerevisiae*. IP (Intra Peritoneal) injection of 0.2 ml of sterile saline was given to the first subgroup of each treatment and control (C). At the end of the 60 days' trial 20 fish were kept in each tank and subjected to salinity stress challenge and up to 7 days survival rate was observed daily. The second subgroup of each treatment and control (C) group was inoculated IP with pathogenic bacterial suspension *A. hydrophila* (0.2 ml of $108x10^8$ CFU ml^{-1}) and were observed for 7 days to record the survival rate. The results in both subgroups revealed group other then treatments which were supplemented with different doses of *S. cerevisiae* showed higher mortality rate. Analysis of variance for stress resistance, blood total leucocytic count and leucocytic differential count after 60 days described highly significant differences ($P<0.01$) in lymphocyte, monocytes and granulocytes.

Results of salinity challenge test revealed that after 7 days of post stress 85 % fish the control (C) group died while of fish fed with probiotics showed improved resistance against salinity stress challenge ($P<0.05$). The highest survival rate (95 %) was observed in the treatment SC_5 supplemented by *S. cerevisiae,* which was which was significantly higher than all other treatments and control (C) groups ($P<0.05$). In control (C) group, the survival rate was observed 15 % which was minimum while in SC_1, SC_2, SC_3, SC_4 and SC_5 it was observed as 70 %, 80 %, 85 %, 85 % and 80 %, respectively.

Results of challenge test with *A. hydrophila* revealed that after 7 days of post stress 80% of experimental fish were died in control (C) group while the other fish treated with probiotics showed significant resistance against bacteria ($P<0.05$). The maximum survival rate (95 %) was detected in th SC6 which was fed by *S. cerevisiae*, proved significantly higher than all other treatments and control (C) groups ($P<0.05$). In SC_1, SC_2, SC_3, SC_4 and SC6 the survival rate was observed as 75 %, 85 %, 80 %, 90 % and 85 %, respectively.

After 60 days' analysis of variance for stress resistance and blood Immunity parameters described non-significant differences in HCT ($P>0.05$). Highly significant ($P<0.01$) differences were observed in RBC, HB, MCH, MCHC and MCV. Hematological parameters were decreased slightly when observed after challenge test after 60 days' trial as compared to the parameters observed after growth performance trial before challenge test. This may be due to the implication of bacterial or salinity stress. The hematological parameters of *Labio rohita* after challenge test are as follows. The RBC count was observed highest in SC_2 (4.90±0.41) as compared to the control (C) (4.51±0.24) and other treated groups. The maximum HB (5.86±0.56) was recorded in SC_4 and minimum (4.93±0.53) in control (C) group. The HCT in control (C) group was 24.81±0.71. The Red cell indices like MCH, MCHC and MCV were also calculated, minimum value (6.10±0.39) for MCV was recorded in SC_3 while in control (C) it was observed 7.20±0.55. Maximum MCH values (14.91±1.26) were recorded in SC_3 group and minimum in SC_1 (12.91±1.26). Maximum MCHC value was calculated in SC_4 (22.74±0.78) while in control (C) it was 19.83±0.45.

After 60 days' experiment showed total leucocyte counts was increased significantly in group supplemented with *S. cerevisiae* as compared to control (C) group**.** It was observed maximum in SC_5 (46.0±1.73) and minimum in SC_1 (36.88±0.19) while in control (C) group it was 33.62±0.89. Differential leucocyte counts when observed indicated significant increase in monocytes, lymphocytes, and granulocytes in treated groups ($P<0.05$) as compared to control (C) which indicate improved immune response. Results indicated that lymphocytes were recorded higher (6.89±0.21) in SC_5 and minimum (4.12±0.13) in SC_3 while in control (C) it was 3.88±0.20. Monocytes were also recorded higher in SC_5 (3.66±0.14) and minimum in SC_3 (3.06±0.14) while in control (C) group it was 3.01±0.16. Higher granulocytes (29.33±1.55) were observed in SC_5 and minimum in SC_3 group (25.44±0.50) while in control (C) it was 24.55±0.98.

Discussion

In 30 days trial all treated fish fed with both probiotics *L. acidophilus* and *S. cerevisae* had better weight gain and significant differences (P<0.05) in growth parameters like weight gain (WG), Specific Growth Rate (SGR) were observed while Survival Rate (SR %) remained constant. Our results were similar to the results earlier reported where probiotic diet supplementation resulted in better growth performance and feed utilization than in controls and also reduced the culture cost (Yanbo and Zirong, 2006; Adineh *et al.*, 2013; Abumourad *et al.*, 2014; Mohammadi *et al.*, 2016). These results were similar for freshwater and marine fish species such as Nile tilapia, *O. niloticus* and *C. carpio* (Wang and Xu, 2006), *S. ocellatus* (Li *et al.*, 2005), *P. olivaceus* (Taoka *et al.*, 2006) and *S. aurata* (Avella *et al.*, 2010). Our results also coincided with Arığ *et al.* (2013) and Mohapatra *et al.* (2012) where the mixture of three probiotic bacteria *Bacillus* sp., *Lactobacillus* sp. and *S. cerevisiae* were administered to *L. rohita* fingerlings resulted in significantly higher growth over other treatment groups. Our results were contradictory to Aubin *et al.* (2005) and Li *et al.* (2005) where the applications of probiotic *P. acidilactici* did not improve weight gain. Similarly, Günther and Jiménez-Montealegre (2004) observed that *B. subtilis* failed to improve growth in *O. niloticus*. Yeasts in our present study also improved the growth performance, FBW, SGR of fish as reported by former workers in Nile tilapia and other fish species (Tovar-Ramı´rez *et al.*, 2004; Taoka *et al.*, 2006; Pooramini *et al.*, 2009; Essa *et al.*, 2010). According to Kafilzadeh *et al.* (2013) *S. cerevisiae* was documented to have the potential effect to replace fish meal for Nile tilapia (Korkmaz and Cakirogullari, 2011; Abdel-Tawwab *et al.*, 2008), rohu (Tewary and Patra, 2011) while no significant effects were observed in case of Oscar fish (Kafilzadeh *et al.*, 2013). The difference in results from various studies depend on intraspecific differences (Aubin *et al.*, 2005), type and method of adding *S. cerevisiae* to diet (Kafilzadeh *et al.*, 2013).

After 30 days, the overall results of the proximate analysis indicated that the yeast, *S. cerevisiae* promoted levels of body crude protein in experimental treatments in comparison with the control group and *L. acidophilus* treated groups, while the crude lipid was not significantly increased in any specific group. The highest value of crude fiber was recorded in SC2 which was fed with *S. cerevisiae* compared to control and other experimental groups. The highest carbohydrate was found in SC3 but it was not statistically significant. The proximate compositions analysis indicated that the compositions were significantly changed by the inclusion of dietary probiotics. The tested diets proved to increase significantly the crude protein and other content, as compared to control. In contrast, the decrease for crude lipid content was observed because the *S. cerevisiae* fed group produced low crude lipid in the fish muscle as compared to control and other treated groups. Our results are similar to the findings of EL-Haroun *et al.* (2006), where significant improvement in basic carcass biochemical constituents was observed after feeding Biogen *E. faecium, B. subtilis* and *B. licheniformis* (Merrifield *et al.*, 2011), *L. acidophilus, S. faecium* and *S. cerevisiae* (Lara-Flores *et al.*, 2003), *B. subtilis, L. plantarum* and *S. cerevisiae* (Essa *et al.*, 2010), *Bacillus* spp (Seenivasan *et al.*, 2012; Bagheri *et al.*, 2008), *L. sporogenes* (Seenivasan *et al.*, 2012). Our results are contradictory to the findings of Silva *et al.* (2015) who described that Nile tilapia supplemented with probiotics had no significant difference in proximate analysis between treatment and control groups. Similar results were found in Nile tilapia fed with diets containing *B. cereus, B. subtilis* and *B. amyloliquefaciens* reared in laboratory conditions (Reda and Selim, 2015). Similar results were described by Noveirian and Nasrollahzadeh (2012), there were no significant differences (P>0.05) in body composition between the treatments which received probiotic.

The moisture content and ash were recorded highest in SC3 in our results that are similar to findings of Ayoola *et al.* (2013) who showed chemical composition of African Catfish *C. gariepinus* after feeding a probiotic diet, the data indicated that moisture contents were found higher as compared to control. The highest crude protein was recorded while the lowest lipid contents were obtained in probiotic

supplementation. Our results are contradictory to Essa *et al.* (2010), where moisture content showed no significant differences in experimental diets. However, the body composition was improved i.e. more deposition of protein and fat with less deposition of moisture and ash. Therefore, it is concluded that the chemical composition analysis described by Soltan and Laithy (2008), Essa *et al.* (2010), Pooramini *et al.* (2009) and Silva *et al.* (2015) are highly similar to the present study.

Hematological parameters fluctuate due to the size, age, physiological status, environmental conditions and other parameters like quality and quantity of dietary ingredients like protein sources, vitamins and probiotics (Ayoola *et al.*, 2013; Osuigwe *et al.*, 2005). The results of the present study showed significantly higher RBCs count in 30 days' trial as compared to control and other treated groups. The present study showed significant ($P<0.05$) results which showed significant increase in the blood parameters of fish treated with dietary supplements as compared to the control group which is favorably similar with the previous (Kumar *et al.*, 2006; Marzouk *et al.*, 2008; Rajikkannu *et al.*, 2015). The present study is also similar to Marzouk *et al.* (2008), who reported a significant increase in HB and RBCs values in fish groups treated with *S. cerevisae* and *B. subtilis* while a minor decrease was observed when probiotics concentration was increased in the diet for rainbow trout. Positive effects were represented by Krishnaveni *et al.* (2013) with the significant increase on the blood parameters of *C. catla* due to the probiotics used which increased the blood parameter values as a result of hemopiotic stimulation. All these results indicated a positive effect shown by significant increase in red cell indices, RBCs count, HCT% and Hb which could be credited to the fact that, the probiotics used as dietary supplement resulted in hemopiotic stimulation and increased the blood parameter values (Kamgar and Ghane, 2014).

Our results are opposite to the findings of Kumar *et al.* (2006) and Ghodratizadeh *et al.* (2011) who fed *B. subtilis* and *S. cervisae* to *L. rohia* and *C. carpio* and analogous effect of these probiotics on the hematological parameters were found, while Imanpoor and Roohi (2015) obtained positive results after using Primalac probiotic in Caspian Roach (*R. rutilus*). Silva *et al.* (2015) also did not observe differences in hematological indices, red blood cells (RBC) and hematocrit (HCT) value but he observed higher hemoglobin level after using lower concentrations of probiotics. This increase in hemoglobin level was attributed to enhanced iron absorption in the gut which increased the availability of iron to produce hemoglobin in fish (Silva *et al.*, 2015). The present study did not agree with outcomes of Giri *et al.* (2013) who fed *L. plantarum* to *L. rohita* and Eissa and Abou-ElGheit (2014) who fed *M. luteus* and *P. fluorescens* as probiotics to *O. niloticus* and reported reduction in hematological parameters.

The results of the present study presented high levels of amylase, protease and lipase in *Labio rohita* as compared to control when fed with *L. acidophilus* and *S. cerevisiae* which may be due to the use of probiotics in the gastrointestinal tract. Similar results had been described for other fishes by using different probiotics (EL-Haroun *et al.*, 2006; Essa *et al.*, 2010). Results of the present study also revealed that different probiotics have a different effect on enzyme activities as like previously described by Renuka *et al.* (2013). The same results were also recorded by Yanbo and Zirong (2006) for common carp, *C. carpio* fed with *Bacillus* species. Ayo Olalusi *et al.* (2014) described that lipase levels were better in *C. gariepinus* fed with *L. plantarum* as compared to control. A significant reduction in amylase ($P<0.05$) was recorded in fish fed without probiotics as compared to probiotic treated groups.

Amylase and lipase values were higher due to good performance of the probiotic as described by present study and also supported by various authors on different fish species: *E. coioides* (Son *et al.*, 2009), *E. bruneus* (Harikrishnan *et al.*, 2010) and *O. niloticus* (Ngamkala *et al.*, 2010). The results gained were not in agreement with Salinas *et al.* (2005, 2008) described that the use of two different bacteria *L. lactis* and *B. subtilis* as single dose probiotics were less effective than a double dose of that probiotics. Therefore, this study revealed that the use of probiotics increased digestive enzyme activity and

resulted in an increase in the specific activity of protease, amylase and lipase.

In phase 2, the study was carried out for 60 days to evaluate the effects of *S. cerevisiae* on hemato-biochemical, physiological, stress resistance and growth performance of *Labio rohita*. The supplementation with probiotics has shown advantageous effects and resulted in higher growth and feed utilization of fish species. Results of 60 days' trial shown that the body weight and SGR was increased in all treatments fed with *S. cerevisae* as compared to control (C) group, all treatments fed with probiotics have better weight. Similarly, maximum feed intake and minimum FCR was observed as compared to control (C) group. PER and PPV were also gradually increased in treated groups as compared to the control (C) group. The significant differences ($p \leq 0.05$) in growth parameters like weight gain (WG), Specific Growth Rate (SGR) were observed while Survival Rate (SR%) remained constant similar to the results reported by Mohammadi *et al.* (2016). Survival rate in present experiment was observed 100% due to maintenance of good physio-chemical parameters. Our results are similar to the findings of Abumourad *et al.* (2014); Lara-Flores *et al.* (2003) and Bogut *et al.* (2000) who reported significant increase in body weight of *O. niloticus* by adding supplement *Enterococcus faecium* to fish diet that improved nutrient absorption and utilization. Our results are also in line with the studies conducted by Ghosh *et al.* (2003); Bairagi *et al.* (2004); Yanbo and Zirong (2006); Adineh *et al.* (2013) where supplementation with probiotic resulted in better growth performance and feed utilization than in control (C) group.

In this study, the results of growth performance of *Labio rohita* are in agreement with the findings of Lara-Flores *et al.* (2003), who used *S. cerevisae* for *O. niloticus*. and observed significant increase in growth performance. Our results are also in line with the findings of Ayoola *et al.* (2013) who described that growth performance, specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR) and survival rates were significantly higher ($P<0.05$) in fish treated with probiotic supplemented diets. In our present study, yeast has improved the Growth parameters, FBW and SGR of fish as reported by former workers in tilapia and other fishes (Taoka *et al.,* 2006; Pooramini *et al.,* 2009; Essa *et al.,* 2010). Different results obtained from various studies depend to intra specific differences (Lara-Flores *et al.*, 2003), type and method of adding *S. cerevisiae* to diet (Tovar-Ramirez *et al.*, 2002; Kafilzadeh *et al.*, 2013; Agboola *et al.*, 2020).

Overall results of proximate analysis After 60 days' trial, indicated that SC6 group showed high levels of crude protein in treatments than control and other treated groups while the crude lipid level showed the highest value in control (C) group instead of any treated group. The results of Pooramini *et al.* (2009) are comparable with our results in all parameters like crude protein, fat and ash contents ranged similar to the chemical composition analysis of the carcass of rainbow trout larvae. According to Ayoola *et al.* (2013) the data from chemical composition of African Catfish *Clarias gariepinus* fed with probiotic diet, indicated that moisture contents were comparatively high as compared to control. The proximate compositions observed in the current study is in line with the previous study where significant changes in the crude protein content, the highest was found in probiotics fed groups as compared to the control. So, probiotics additives increase more crude protein and less lipid contents which proved good for food fish (Wee, 1982) but body composition analysis showed no significant differences between dietary groups (Merrifield *et al.* 2011). Only crude protein and lipids contents were comparable with current study while only moisture and lipid contents are comparable in the present study with Mian and Siddiqui (2014). Our results are contradictory to the findings Noveirian and Nasrollahzadeh (2012), who reported no significant differences ($P>0.05$) in body composition between the treatments which received probiotic. Our results are also opposite to previously described work by Diab *et al.* (2002); Lara-Flores *et al.* (2003); Gafarian *et al.* (2007) and Mohamed *et al.* (2007).

The results of the present study showed that RBCs count, Hb %, Hct % were significantly higher in SC6 as compared to the control (C) group. The red cell indices showed maximum RBCs count and minimum MCV value in SC6 after 60 days' trial. Maximum MCH and MCHC values were recorded in SC6 which were fed with the probiotics as compared to control. The significant ($P<0.05$) results of present study favor the previous studies conducted by Rajikkannu *et al.* (2015) and Firouzbakhsh *et al.* (2012). The present study is also similar to Marzouk *et al.* (2008), who found significant increase in RBCs and Hb values in rainbow trout fish groups treated with *S. cerevisae* while a minor decrease was observed when probiotics concentration in diet for were increased. Our results are not in agreement with Silva *et al.* (2015) who reported no variations in red blood cells (RBC), hematocrit (HCT) value and hematological indices.

Our results are in line with the studies conducted by Kumar *et al.* (2006) and Ghodratizadeh *et al.* (2011) who after feeding *B. subtilis* and *S. cervisae* to *L. rohia* and *C. carpio*, reported a similar effect of probiotics on haematological parameters. The results of this study are comparable to those of Marzouk *et al.* (2008), who showed a significant increase in RBCs and HB values in fish fed with *B. subtilis* and *S. cerevisae*. This increase in haemoglobin was caused by increased iron absorption in the intestines, which raised the amount of iron available for fish to generate haemoglobin (Silva *et al.*, 2015). The results of the current study are contradictory to those of Abd El-Rhman *et al.* (2009) who fed *M. luteus* and *P. fluorescens* as probiotics to *O. niloticus* and reported a reduction in haematological parameters.

According to Kamgar and Ghane (2014), adding *B. subtilis* to rainbow trout feed had no effect on the rate of hematocrit, haemoglobin, number of red blood cells, MCV, MCH, and MCHC and similar results were also obtained in previous studies (De Carla Dias *et al.* 2010).The current study proved that probiotics had a positive effect, as evidenced by significant increases in RBC count, HB percent, HCT percent and red cell indices, which could be attributed to the fact that hemopiotic stimulation enhanced blood values (Kamgar and Ghane, 2014).

By adding the probiotics in diet Digestive enzymes like amylase, protease and lipase could be enhanced (Ziaei-Nejad *et al.* 2006; Taoka *et al.* 2007; Wang, 2007; Gomez and Balcazar, 2008). This improvement of feed utilization is attributed to improvement in intestinal microbial biota which leads to improved digestion of nutrients, better absorption quality and higher enzyme activities (Lara-Flores *et al.*, 2003; Balcazar *et al.*, 2006; Renuka *et al.*, 2013). The results of the present study when *Labio rohita* fed with *S. cerevisiae* presented enhanced levels of amylase, protease and lipase as compared to control due to the use of probiotics in the gastrointestinal tract. Similar results had been described for other fishes by using different probiotics (Lara-Flores *et al.*, 2003; Tovar-Ramırez *et al.*, 2004; El-Haroun *et al.*, 2006; Essa *et al.*, 2010). Similar results for common carp, *Cyprinus carpio* fed with photosynthetic bacteria and *Bacillus* species were also recorded by Yanbo and Zirong (2006).

Results of present study are in line with the findings of Soleimani *et al.* (2012) who assesed the digestive enzyme activity in Caspian roach (*Rutilus rutilus*) by using Fructooligosaccharide probiotic for 7 weeks and found highest digestive enzyme activity. As compared to probiotic treated groups there was significant reduction in amylase ($P<0.05$) in fish fed without probiotics. Amylase and lipase values were higher due to good performance of the probiotic as described by present study and also supported by various authors on different fish species *E. coioides* (Son *et al.*, 2009), *E. bruneus* (Harikrishnan *et al.*, 2010) and *O. niloticus* (Ngamkala *et al.*, 2010). Trial 2 (60 days' trial) treated with *S. cerevisiae* have exhibited better results for digestive enzyme (protease, amylase and lipase) as compared to trial 1 (30 days' trial) in which *L. acidophilus* was used as probiotic which indicated that *S. cerevisiae* had significantly enhanced the digestive ability than that of trial 1.

Salinity stress test is commonly used to estimate fish quality or fitness after feeding them with probiotics (Dimitroglou *et al.*, 2010; Soleimani *et al.*, 2012). After 60 days' trial Salinity challenge test after 7 days of post stress indicated that 85 % fish died in the control (C) group while the probiotic supplemented diet significantly improved the resistance of fish fed against salinity stress challenge ($P<0.05$). The maximum survival rate was noted in the treatment SC6 (95 %) supplemented by *Saccharomyces cerevisiae* ($P<0.05$) and was found significantly higher than the control and other treatments. Some previous studies conducted also revealed significant increase against salinity stress resistance (Hernandez *et al.*, 2010; Varela *et al.*, 2010) after feeding *L. acidophilus* and *L. lactis* probiotics. Sheikhzadeh *et al.* (2012) also reported similar results after dietary supplementation of *S. cerevisiae*. The results of challenge test with *A. hydrophila* after 60 days' trial 80 % of experimental fish was died in control (C) group after 7 days of post stress while the other treatment fed with probiotics showed significant resistance against bacteria ($P<0.05$). The maximum survival rate was detected in the treatment SC6 (95%) which was supplemented by *S. cerevisiae*, which was significantly higher ($P<0.05$) than other treatments and control (C) groups.

Shoemaker *et al.* (2006) observed significantly higher reflection of immunity after challenging *O. niloticus* with *Streptococcus iniae*. Previous studies revealed that Nile tilapia provided better results against challenge test with *A. hydrophila*. Similar results were indicated by Taoka *et al.* (2006) in Nile tilapia against *Edwardsiella tarda* and *A. hydrophila*. Venkatesan *et al.*, (2012) demonstrated that single probiotic can also play effective role against bacterial pathogens like *Bifidobacterium* sp. had higher inhibitory effect against *Salmonella* sp. While Abd El-Rhman *et al.* (2009) described that *Pseudomonas* did not offer sufficient defense against *A. hydrophila* challenge. Nayak (2010) described that there is need to regulate the dose of any probiotic used for a specific host because higher doses of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* (Nikoskelainen *et al.,* 2001) were failed against challenge in study of *Oncorhynchus mykiss* and high mortality was observed.

Taoka *et al.* (2006) reported that the probiotics are also effective to enhance fish immunity and resistance against the infection of *Edwardsiella tarda* and other bacteria (Eissa and Abou-ElGheit, 2014). Differential leucocyte counts have also shown significant increase in monocytes, lymphocytes and granulocytes in treated groups ($p< 0.05$) as compared to control. Results indicated that lymphocytes, monocytes and granulocytes were recorded higher in SC6. Similar results were observed in previous studies by various researchers after using different probiotics where improved immunological parameters were observed (Ferguson *et al.*, 2010). Few studies have described that use of probiotics have the ability to eliminate the pathogens by stimulating nonspecific immune responses (Gomez and Balcazar, 2008; Ferguson *et al.*, 2010). Several researchers reported low mortality rates and improved immunity when fed *S. cerevisiae* to *Cyprinus carpio* (Mazurkiewicz *et al.*, 2005; Dehghan *et al.*, 2011); *Epinephelus coioides* (Chiu *et al.*, 2010); *Channa striatus* (Dhanaraj and Haniffa, 2011); *Oreochromis niloticus* (Asadi *et al.*, 2012) and *Cichlasoma trimaculatum* (Mohammadi *et al.*, 2016). Markad and Rane (2015) reported Yeast *S. cerevisiae* to be effective for better survival in the Zebra fish, *Danio rerio* which coincides with the present study. Chelladurai *et al.* (2013) reported infected groups of fish challenged with *A. hydrophila* maintained on probiotic diets produced better hematological parameters than the control and the same findings are observed in this study.

Conclusion

Growth performance indicated that the addition of *L. acidophilus* and *S. cerevisae* in basal fish feed gave an overall significant increase in weight gain, survival rate, final body weight, PER and PPV. But supplementation of *S. cerevisae* proved to be more effective for substantial rise in growth performance. In the case of SGR better value was noted in all treatments fed with probiotics and FCR was observed

minimum in treatments while PER and PPV were increased due to supplementation of both probiotics in experimental diets as compared to the control group. The survival rate (%) during growth performance trials remained constant and it was 100 %. The proximate compositions analysis indicated that the body compositions were significantly changed by the inclusion of dietary probiotics. Proximate analysis revealed that *S. cerevisiae* promoted the body crude protein, crude fiber, carbohydrate content, moisture, and ash in all treatments fed with *S. cerevisiae* in comparison with the control (C) and *L. acidophilus* treated groups. Crude lipid level showed opposite trend, it was not significantly increased in any treated group. The Hematological parameters indicated significantly higher RBC, Hb, HCT while the Red Cell Indices indicated maximum MCH, MCHC values in *S. cerevisiae* treated SC3 as compared to the control (C) group. MCV was the only blood parameter that was recorded with minimum value in the treated group. The present study showed significant ($P<0.05$) results by increasing the blood parameters. Total leucocyte count showed significant increase, similarly differential leucocyte counts indicated highly enhanced immune response with significant increase in lymphocytes, monocytes and granulocytes in treatments supplemented with *S. cerevisae* compared to the control (C) group in *Labio rohita*. The digestive enzymes (amylase, protease and lipase) activity indicated that *S. cerevisiae* exhibited better results. The results presented high levels of amylase, protease and lipase in *Labio rohita* as compared to control when fed with *S. cerevisiae*. When fish was given controlled diet without probiotic significant reduction in amylase ($P<0.05$) was recorded. The salinity challenge test described that after 60 days trial, in 7 days of post stress 85% fish died in the control (C) group while in the treatment groups the dietary probiotic significantly improved the resistance against salinity challenge test ($P<0.05$). The challenge test with *A. hydrophila* indicated 80 % mortality of fish in control (C) group after 7 days of post stress while, the other treatments fed with *S. cerevisae* showed significant resistance against bacteria ($P<0.05$). The maximum survival rate was detected in the treatment SC6 (95%) which was supplemented with *S. cerevisiae*. Current project produced good quality of fish after using different probiotics and is beneficial to establish guidelines for the fisheries sector about the use of different level of probiotics in Pakistan. It is recommended that further research on other fish species and probiotics should be conducted in future.

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