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Effects of Pre- and Probiotics on Growth, Survival, Body Composition, and Hematology of Common Carp (*Cyprinus carpio* L.) Fry from the Caspian Sea

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Abstract

This study examined the effects of Immunowall® prebiotic and Primalac® probitic (each with 1.0 and 1.5 g kg^{-1} diet, respectively) on common carp (*Cyprinus carpio* L.) fry (n= 4200, 10 ± 0.015 g) from the Caspian Sea. Specific growth rate (SGR), body weight gain (BWG), total length (TL), survival, feed intake (FI), feed conversion ratio (FCR), carcass composition, and hematological parameters were evaluated in triplicate treatments after 8 weeks. BWG, SGR, TL, and survival rate were significantly influenced in the fish fed 1.5 g of mixed Immunowall® and Primalac® ($P < 0.05$). However, none of the growth factors plus FCR and survival of the fish shoed improvements in treatments with 1.0 g of mixed Immunowall® and Primalac® compared to the control ($P > 0.05$). The experimental carp fry displayed marked improvements in some of carcass qualities ($P < 0.05$). Also, the fry fed Primalac® showed greater white blood cell (WBC) counts than the control group ($P < 0.05$). The positive effects of Primalac®, and to a lesser extent, Immunowall®, or their mixture on the parameters examined in the carp fry signify improved growth performance, enhanced body composition, and stimulated fish immune system.

Key words: Caspian Sea, *Cyprinus carpio*, Immunowall®, Primalac®, growth, carcass composition, blood factors.

Introduction

High larval mortality is one of the difficulties in fish larviculture, hence, successful culture of fish depends on the availability of suitable nutritive feeds in order to guarantee the health and growth of fish fry (Girri et al., 2002). Feed additives such as probiotics and prebiotics were, therefore, widely used as active ingredients to maintain and improve the intestinal microbial balance essential for maintaining fish health (Fuller, 1989). Accordingly, research on pro- and prebiotics in fish nutrition have gained ground with the demand for the consumer- and environment-friendly aquaculture (Staykov, Spring, Denev, & Sweetman, 2007).

To reduce mortalities in fish larvae, a food supplement such as the multi-strain probiotic Primalac® containing *Bacillus* species (*Lactobacillus acidophilus, L. casei*, *Enterococcus faecium*, and *Bifidobacterium bifidium*) can be added in order to reinforce farmed larvae through raising the secretion of digestive enzymes that help digestion and absorption of food leading to improved health, disease prevention, and high fish production (Verschuere, Rombout, Sorgeloos, & Verstraete, 2000). Larval feeding can also benefit from the

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inclusion of prebiotic Immunowall® derived from the cell wall of the brewer's yeast *Saccharomyces cerevisiae*. It is indeed a unicellular protein that can replace dietary fish meal up to 25-50% with no negative effects on fish growth (Peng & Gatlin, 2003). Immunowall® consists of mannan oligosaccharide (MOS) and *β*-glucans (40% MOS and 17% b-1,3 glucan), which promote balance and integrity in gut microflora to stimulate the immune system of fish. This prebiotic is also a source of nucleic acids and polysaccharides including a variety of glucans (Dalmo & Bogwald, 2008). These two pro- and prebiotics have been shown to have immunostimulatory properties, increase survival rate and disease resistance, and modulate innate and acquired immunity responses in fish (Dalmo & Bogwald, 2008). For cyprinids, it is also well documented that *β*-glucan can enhance the innate immune response and disease resistance (e.g. Gopalakannan & Arul, 2010; Siwicki et al., 2010), but limited evidence are available about the effects of *β*-glucan on fish growth performance (Kühlwein, Merrifield, Rawling, Foey, & Davies, 2014). For instance, feeding diets containing Immnowall® (1.5 g/kg) and Primalac® (0.5 g/kg) to juvenile common carp for 8 week revealed significant effects on growth parameters and survival rates (Barari, Pakzad Sorki, & Nazari, 2015).

Hematological parameters rapidly respond to the environmental conditions, hence, they reflect unfavorable bodily situations and are considered as good indicators widely used in fish health studies (Blaxhall, 1972). Since prebiotics and probiotics contribute to fish health and production, the present study was planned to investigate the effects of dietary probiotic and prebiotic supplementations on growth performance, body composition, hematology, and non-specific immune response of *C. carpio* fry.

Materials and Methods

Common carp fry samples (n= 4200, 10±0.015g) were obtained from a well-known fish culture and restocking center in northern Iran (Mazandaran Province). The larvae were stocked in 21 fiberglass tanks (500 L) and adapted to the new environmental conditions for two weeks. The tanks were separately aerated to maintain optimal oxygen levels. Major physicochemical factors including temperature (using mercury thermometer, 0.1 °C accuracy), pH and dissolved oxygen (DO, with Model Multiline 3/Set, WTW 320I) were daily monitored and recorded during the research. The average values were: T= 23.61±0.2 ºC, pH= 8.03±0.01, and DO= 9.09±0.06 mg/l. A portion (15 percent of total volume) of the tanks water was replaced daily 1.0 h after each feeding time.

After adaptation period, the fish larvae were randomly distributed in tanks (200 fry per tank). Every two weeks, total weight and length of the experimental fry were evaluated for 20 fries from each replicate. The fish fry were not fed 12 h before and after weight and length measurements. The daily ration was based on 10% of body weight (after each biometry), which was weighed by a balance (accuracy: 0.01 g) and fed to the fish in the paste form at different intervals (8 and 15 hrs). A commercial food, SFK, was used as a basal diet. The SKF analysis revealed amounts of 8.8% moisture, 11.36 ash, 34.5% protein, and 10.7% fat.

The experimental plan was a completely randomized design composed of six treatments and a control group each with three replicates as follows: control group (no pre- and probiotics), and treatments named T1 (1g Immunowall®/kg food), T2 (1.5 g Immunowall®/kg food), T3 (1g Primalac®/kg food), T4 (1.5 g Primalac®/kg food), T5 (a mix of 1g Immunowall® and 1g Primalac®/kg food), and T6 (a mix of 1.5 g Immunowall® and 1.5 g Primalac®/kg food). After 8 weeks, three fish from each replicate were randomly

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selected for cupping, anesthetized by clove powder, and blood samples were taken from caudal peduncle using a 2 cc syringe. Because the blood volume taken was low, blood samples of the fry in each treatment group were separately mixed and the mixed blood for each treatment was analyzed three times. Red and white blood cells (RBC and WBC) were counted according to Svobodova et al. (1991) using bubble pipettes (Melangeur) under Neubauer chamber after dilution of non-coagulated blood with a Rees solution. Hemoglobin was measured by a test kit (Pars Azmoon Co., Iran) via cyanmethemoglobin method. A volume (20 µl) of non-coagulated blood was mixed with Drabkin's solution (50 ml) and placed in dark for 5-10 min followed by spectrophotometry (Model 1000RA, Technicon Co., the United States) at a wavelength of 540 nm. Hematocrit percentage was determined using hematocrit centrifuge (CAT.C.E.I capillary-micro USA, 2201). First, over two-thirds of hematocrit tubes were filled with non-coagulated blood samples, placed into micro hematocrit centrifuge (13000 rpm, 3 min), and hematocrit value was read by a graded sheet. According to the results obtained, the RBC indices (MCV, MCH, & MCHC) were calculated as follows:

$$MCV = (Ht/RBC) \times 100$$

$$MCH = (Hb/RBC) \times 10$$

$$MCHC = (Hb/Ht) \times 100$$

Major biochemical factors of the fish body (protein, fat, ash, and moisture) were also assessed at the end of feeding trial. Protein and ash were measured by Kejeldal (BAP40, Germany) according to AOAC (1990). Fat and moisture were estimated with a Soxhlet apparatus (BOHR, Germany).

The growth and nutritional parameters determined at different treatments were calculated as below:

Feed conversion ratio (FCR) = F/ [Wt (g) – W0 (g)], where F: amount fed, W0: initial weight, Wt: final weight (Ronyai et al., 1990).

$$\text{Specific growth rate (SGR \% d)} = (\text{Ln Wt} - \text{Ln W0}) \times 100/t$$

$$\text{Body weight increase (BWI \%)} = (Bwf - Bwi)/Bwi \times 100 \text{ (Hung et al., 1989),}$$

where: Bwf and Bwi = average initial and final weight, respectively, per tank;

$$\text{Daily growth rate (GR, g/day)} = (Bwf - Bwi)/n \text{ (Hung et al., 1989),}$$

where: Bwf and Bwi = average initial and final weight, respectively, per tank, n= number of cultivation days;

$$\text{Condition factor (CF)} = (Bw/TL^{3}) \times 100 \text{ (Hung et al., 1989),}$$

where: Bw= Average body final weight (g), TL= Average final total length (cm);

$$\text{Survival rate (\%)} = 100 \times (Nt/N0) \text{ (Hung et al., 1989),}$$

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where Nt= No. of larvae at the end, N0= Initial No. of larvae.

Statistical Analyses

The normality and homogeneity of data were first verified among treatments by Kolmogorov–Smirnov test. Then, data were analyzed by one-way ANOVA (SPSS software, version 17) and different treatments were compared by Duncan's test. All data are expressed as mean ± SD.

Results

The results of ANOVA and Duncan's test (Table 1) showed that the weight and length of the carp fry were significantly different among the treatments.

The utmost survival rates were observed in T2, T4, and T6 compared with the least rates in the control. The FCR value was lowest in T6 among the supplemented treatments and the greatest FCR was estimated in the control (Table 2).

The examined treatments were statistically different in the estimates of SGR, BWI, and GR ($P < 0.05$). Duncan's test revealed that T2, T3, T5, and T6 were significantly dissimilar with the control and that T6 and control larvae gained the highest and lowest values of the above parameters (Table 2).

The carcass compositions of the experimental fish larvae exhibited marked differences among the treatments and the control (Table 3). T4 and T3 had the highest and least moisture contents, respectively ($P < 0.05$). The ash percentages in T2 and T3 were significantly different from the control with the highest and lowest contents in T3 and T6, respectively ($P < 0.05$). The fat percentages of T2 and T3 were dissimilar with the control with the greatest fat percentage in T2 and the smallest in the control ($P < 0.05$).

Some of the resultant hematological parameters measured at different treatments were significantly affected by the Immunowall® and Primalac® supplementations (Table 4). WBC count in T6 was significantly different from the control with the largest and lowest counts in T6 and T2, respectively ($P < 0.05$). RBC level marked the greatest value in the control being statistically dissimilar with that in T3 showing the least RBC level ($P < 0.05$). The estimations of Hb, Hct, MCV, and MCH were all lowest in T2 as opposed to the highest levels assessed in the control ($P < 0.05$). On the other hand, T2 contained the highest percentage of MCHC but T5 had the least amount, which were different from that in the control ($P < 0.05$). The fry in T3 held the greatest heterophil percentage and were dissimilar with T5 having the smallest percentage ($P < 0.05$). The recorded levels of monocytes were greatest in T2, T3, T4, and T6 and lowest in T1 being different from the control ($P < 0.05$). Finally, eosinophil values in T5 (largest) and T3 (smallest) were markedly dissimilar with each other with T5 being different from the control ($P < 0.05$).

Discussion

The use of pro- and prebiotics probably stimulates fish appetite and improves nutritional conditions through biosynthesis of vitamins, proteases, and also by degradation of indigestible compounds rendering enhanced nutrient assimilation and meat production (Irianto & Austin, 2002). Increased assimilation of dietary nutrients resulting from pre- and probiotic supplementations reduces feed consumption by fish followed by

relatively lower FCR ratios, which economically favors fish culturists and reduces farming expenses (Ghosh, Sen & Ray, 2002; Yanbo & Zirong, 2006).

Prebiotics containing oligosaccharides like *β*-glucane and mannone ameliorate feeding efficiency and conversion and reduce mortality leading to improved growth (Gibson, 1999). Similar to our results in *C. carpio* fry fed dietary Primalac® probiotic, body weight and survival of both common carp and Persian sturgeon significantly improved by feeding dietary Primalac® compared to control group (Faghani Langroudi, 2010, Salaghi, Imanpuor, & Taghizadeh, 2013). Likewise, grass carp (*Ctenopharyngodon idella*) fingerlings and the Indian major carp *Labeo rohita* fed dietary *Bacillus* and *Lactobacillus* species displayed marked growth improvements (Kumar, Mukherjee Prasad, & Pal,

2006, Wang, 2011). Probiotics such as Primalac® containing *Bacillus* bacteria increase feed consumption desire by producing vitamins and diet detoxification and/or degradation of indigestible components; this is most probably related to the production of proteolytic and peptidolytic enzymes by the bacteria found in the probiotic, which hydrolyze macromolecular compounds to peptides and amino acids (Irianto & Austin, 2002).

Moreover, research has shown that dietary administration of prebiotic Immunowall® (10 g kg^{-1} diet) to great sturgeon *Huso huso* juveniles for 8 weeks and also a similar prebiotic Organoferm (containing MOS and *β*-glucans; 2.5 g kg^{-1} diet) to *C. carpio* fry for 12 weeks significantly increased growth performance (Ta'ati, Soltani, Bahmani, & Zamini, 2011; Eleraky, Yahya, Reda, & Eletreby, 2014). Although the causes of growth improvements observed with dietary *β*-glucans supplementation are not clear (Kühlwein et al., 2014), it is suggested that the effects may depend on dietary concentration a solubility of *β*-glucan, fish species, water temperature, and duration of feeding period (Dalmo & Bøgwald, 2008).

The levels of SGR in our carp fry were utmost in treatments received 1.0 g kg^{-1} of Primalac® and also with a mixture with 1.5 g kg^{-1} of each of Primalac® and Imunowall®. The same finding was reported in common carp fingerling fed Primalac (1.0 %) in diet (Faghani Langroudi, 2010). In addition, our results revealed dissimilar FCR values being lowest in the fries with greatest weight gain (T3, T5, and T6) fed 1.0 g kg^{-1} of Primalac® and a mixture of Primalac® (1.0 g/kg) and Imunowall® (1.5 g/kg). These are in line with those reported in wild *C. carpio* fry (Faghani Langroudi, 2010) and common carp fed lyophilized photosynthetic bacterial cells and *Bacillus* sp. (Yanbo & Zirong, 2006). They further found the best results from mixed probiotics as were also detected in here. Correspondingly, Nile tilapia and rainbow trout fries administered *Bacillus* bacteria yielded enhanced digestion of food and improved growth including low FCR and high SGR values (Khattab, Shalaby, Sharaf Saffa, El-Marakby, & RizlAlla, 2005, Bagheri , Hedayati, Yavari, Alizade, & Farzanfar, 2008).

The percentages of final carcass protein in T4, T5, and T6 fed probiotic Primalac® were statistically higher than that in the control. *Bacillus* bacteria contained in this probiotic are able to secret such extracellular enzymes as protease, which, as a digestive enzyme, leads to better digestion and assimilation of food protein reflected in the larger carcass protein content. The same result was observed in rainbow trout and *C. carpio* fed a variety of probiotics (Farzanfar, Lashto Aghaei, Alizadeh, Bayati, & Ghorban, 2007). In mirror carp, on the other hand, no significant differences were observed in carcass parameters, including protein content, between control and the fish received a yeast *β*-glucan preparation (Kühlwein et al., 2014).

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Total WBC count in treatments with Immunowall® and Primalac® were insignificantly higher than control group ($p > 0.05$). A study showed that the use of Levamisole and Ascorbic acid could increase WBC count (Sahoo & Mukherjee, 1999). The results of our study demonstrate that Immunowall® had no effects on WBC count and the related factors, but Perimalac® could increase these factors. The rises in RBC, WBC, lymphocytes, monocytes, eosinophils, and heterophil counts following dietary Perimalac® feeding indicate the immunostimulant effects of this probiotic, which can improve fish immune system and potential disease resistance. However, the use of prebiotic diets containing a yeast *β*-glucan did not reveal significantly altered haematoimmunological parameters in *Labeo rohita* and mirror carp after 8 weeks (Misra, Das, Mukherje, & Pattnaik, 2006, Kühlwein et al., 2014).

The current study showed a significant rise of heterophil percentage in T3 (1.0 g kg^{-1} of Primalac) followed by T5 (1.5 g kg^{-1} of each of Immunowall® and Perimalac®). Cyprinid heterophils are implicated in the processes of acute inflammation and antibacterial defense (Lieschke, Oates, Crowhurst, Ward, & Layton, 2001). Hence, the applied amounts of probitic and/or prebiotic in here could enhance the fish immunity status probably indicating an immunomodulatory response, which is in agreement with those reported in pre- and probiotic-fed fish (Zhu, Liu, Yan, Wang, & Liu, 2012) and chicken (Farnell et al., 2006). Administration of prebiotic Immunoster, on the other hand, led to no significant effects on heterophil levels in *C. carpio* (Jafari, Baboli, & Alishahi, 2013).

In conclusion, the preliminary results of this study signify that dietary inclusions of Primalac® and, to a lesser extent, Immunowall® or their mixture positively affect most of the parameters examined in the experimental *C. carpio* fry leading to improved growth performance, enhanced body composition, and stimulated fish immune system. Nonetheless, it is necessary to compare other fish farms at different regions in order to improve the outcomes observed. Altogether, both pro- and prebiotics have been proven to be highly operative in the promotion of efficiency and sustainability of aquaculture production.

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Table 1. Average length and weight of the Caspian carp fry at different treatments with prebiotic Immunowall (T1: 1 g/kg & T2: 1.5 g /kg), probitic Primalac (T3: 1 g/kg & T4: 1.5 g /kg), and their mixture (T5: 1 g/kg & T2: 1.5 g /kg of each in combination). Different superscript letters in the same column denote significant differences ($P < 0.05$)

Treatment	Weight (g)	Length (cm)
Control	$59400.00^{a} \pm 0.06$	$11.2^{a} \pm 0.00$
T1	$69500.00^{ab} \pm 0/08$	$11.20^{a} \pm 0/00$
T2	$67700.00^{c} \pm 0.07$	$11.9^{bc} \pm 0.04$
T3	$77650.00^{f} \pm 0.06$	$11.6^{d} \pm 0.00$
T4	$72750.00^{bc} \pm 0.07$	$11.28^{ab} \pm 0.04$
T5	$77650.00^{d} \pm 0.07$	$11.4^{c} \pm 0.00$
T6	$85350.00^{a} \pm 0.01$	$11.54^{d} \pm 0.02$

Table 2. Comparison of prebiotic Immunowall, probiotic Primalac, and their combined effects on growth and nutritional parameters in Caspian carp fry. Treatments (T) are as in Table 1. FCR: feed conversion ratio; SGR: specific growth rate; BWI: body weight increase; GR: daily growth rate; CF: condition factor. Different superscript letters in the same column denote significant differences ($P < 0.05$)

Parameter / Treatment	FCR	SGR (%)	BWI (%)	GR (%)	CF	Survival (%)
Control	$2.44^{c} \pm 0.05$	$5.83^{a} \pm 0.03$	$25.42^{a} \pm 0.04$	$10.22^{a} \pm 0.03$	$0.42^{bc} \pm 0.01$	55.77
T1	$2.158^{bc} \pm 0.03$	$6.05^{ab} \pm 0.07$	$28.78^{ab} \pm 0.02$	$12.23^{ab} \pm 0.07$	$0.49^{bc} \pm 0.01$	55.77
T2	$2.163^{b} \pm 0.02$	$6.14^{b} \pm 0.07$	$30.64^{b} \pm 0.02$	$11.18^{bc} \pm 0.01$	$0.4^{b} \pm 0.01$	58.66
T3	$1.183^{a} \pm 0.07$	$6.17^{c} \pm 0.03$	$30.81^{c} \pm 0.03$	$13.36^{d} \pm 0.03$	$0.49^{ab} \pm 0.00$	58.22
T4	$1.931^{bc} \pm 0.02$	$6.16^{ab} \pm 0.01$	$30.47^{ab} \pm 0.02$	$12.38^{ab} \pm 0.01$	$0.5^{c} \pm 0.02$	58.66
T5	$1.801^{b} \pm 0.02$	$6.15^{b} \pm 0.08$	$30.81^{b} \pm 0.02$	$13.34^{bc} \pm 0.07$	$0.52^{abc} \pm 0.01$	58.44
T6	$1.5^{b} \pm 0.04$	$6.27^{b} \pm 0.01$	$32.67^{b} \pm 0.02$	$14.48^{c} \pm 0.01$	$0.55^{a} \pm 0.00$	58.66

Table 3. Analysis of the final carcass composition of the Caspian carp fry carcasses at different treatments. Treatments (T) are as in Tables 1 and 2. Different superscript letters in the same column denote significant differences ($P < 0.05$)

Treatments	Moisture (%)	Ash (%)	Protein (%)	Fat (%)
Control	23.68^{ab}	13.91^{a}	58.81^{a}	14.35^{ab}
T1	24.24^{a}	14.11^{a}	60.81^{a}	17.71^{a}
T2	23.33^{c}	15.89^{ab}	60.81^{a}	21.06^{c}

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T3	23.07^{abc}	18.15^{a}	61.73^{ab}	20.54^{bc}
T4	25.28^{bc}	12.63^{ab}	62.35^{b}	18.39^{bc}
T5	25^{abc}	13.15^{c}	62.88^{b}	16.58^{bc}
T6	23.2^{abc}	10.15^{bc}	63.74^{b}	17.59^{c}

Table 4. Blood analysis of the Caspian carp fry at different treatments. Treatments (T) are as in Tables 1, 2, and 3. Different superscript letters in the same row denote significant differences ($P < 0.05$)

Treatments / Parameters	Control	T1	T2	T3	T4	T5	T6
WBC (10^3/μL)	10700^{d}	9800^{b}	8100^{a}	11300^{e}	12400^{f}	10500^{c}	13400^{g}
RBC (10^3/μL)	1.12^{a}	1.04^{a}	0.83^{a}	1.09^{a}	0.88^{a}	1.04^{a}	0.95^{a}
Hb (g/dL)	7.9^{e}	6.8^{c}	5.2^{a}	7.1^{cd}	5.9^{b}	7.3^{d}	6.2^{b}
Hct (%)	24^{g}	21^{d}	15^{a}	22^{e}	18^{b}	23^{f}	19^{c}
MCV (fL)	214.3^{d}	201.9^{b}	180.7^{a}	201.8^{b}	204.5^{c}	221.3^{e}	200^{b}
MCH (pg/cell)	70.5^{d}	65.4^{b}	62.2^{a}	65.1^{b}	67^{c}	70.2^{d}	65.3^{b}
MCHC (%)	32.9^{a}	32.4^{a}	34.7^{b}	32.3^{ab}	32.8^{a}	31.7^{a}	32.6^{a}
Heter (%)	12^{b}	15^{d}	14^{c}	20^{g}	16^{e}	10^{a}	18^{f}
Lymph (%)	83^{f}	80^{d}	79^{b}	75^{a}	78^{c}	82^{e}	76^{b}
Mono (%)	2^{b}	1^{a}	5^{f}	4^{d}	4^{c}	3^{d}	4^{d}
Eos (%)	3^{c}	4^{d}	2^{b}	1^{a}	2^{b}	5^{e}	2^{b}