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Effects of dietary b-glucan, mannan oligosaccharide, *Lactobacillus plantarum* and their combinations on growth performance, immunity and immune related gene expression of Caspian trout, *Salmo trutta caspius* (Kessler, 1877)

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ABSTRACT

This study investigated the effect of individual and combination of dietary pre- and probiotics (β-glucan, 3 mg/g; mannan oligosaccharide (MOS), 4 mg/g; and *Lactobacillus plantarum*; 10^8 CFU/mg diet) on growth performance, blood immune parameters, expression of immune related genes, and intestinal microbial of Caspian trout (*Salmo trutta caspius*). On the basis of feeding with immunostimulant diets, the fish were assigned into eight groups denoted as: control (basal diet), bβ (basal diet + β-glucan), bM (basal diet + MOS), b*Lp* (basal diet + *L. plantarum*), bβ*Lp* (basal diet + β-glucan + *L. plantarum*), bM*Lp* (basal diet + MOS + *L. plantarum*), bMβ (basal diet + MOS + β-glucan), and bMβ*Lp* (basal diet + MOS + β-glucan + *L. plantarum*). All of the immunostimulant diets, in general, reduced feed intake (FI) and food conversion ratio (FCR) and increased WG, PER, and final weight. Condition factor (CF) demonstrated the lowest level in the experimental group received bMβ*Lp*. Total lipid increased in the fish received the additives, especially bM and bMβ. Ash content demonstrated significant increase in the fish fed on bβ and bMβ*Lp*, whereas moisture content was reduced in the group fed with *L. plantarum*-supplemented diet. All immunostimulant diets enhanced the activity and levels of lysozyme, Immunoglobulin M (IgM), and serum alternative complement activity (ACH50); the highest value for these indices was observed in the groups fed with bMβ, bMβ*Lp*, and bβ*Lp*. bMβ-treated fish group displayed the highest cortisol and glucose levels. bM diet induced the highest mRNA transcription of TNF-α1 in head kidney, whereas bLp, bMβ, and bMβLp showed no effect. IL1β exhibited the greatest up-regulation, about 8.75 fold change, in response to the diet supplemented only with β-glucan. bβ*Lp* and bβ significantly enhanced the relative IL-8 mRNA expression in the head kidney (about 2.75 and 1.9 folds, respectively), yet in response to bMβLp treatment it showed a decrease of about 5.7 times lower than the control group. In addition, intestinal population of *L. plantarum* showed the highest loads in the groups fed on the diets which were treated with the probiotic. Taken together, combinational use of these immunostimulants enhanced humoral innate immune system, whereas their individual and combinational application could increase and decrease the transcription of inflammation-related genes, respectively.

1. Introduction

Development and intensification of aquaculture practices, especially fish culture, has raised concerns over physiological stress and in turn potential risks of disease outbreaks in fish farms [1]. In the past many years, rapid outbreaks of pathogenic microorganisms in aquaculture industry and consequently indiscriminate application of antibiotics have resulted in the emergence of several resistant bacteria, thereby impeding the profitability and sustainability of the aquaculture industry through possible effects on survival, feed utilization and weight gain [2–5]. These limitations or restrictions of antibiotics have recently attracted interest from researchers to develop alternative ecofriendly

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strategies as health promoter and to prevent breaking out pathogenic diseases [5,6].

The concept of functional feed additives, especially immunostimulants such as probiotics and prebiotics, has been recently highlighted in aquafeed industry. These functional ingredients have proved to be biocompatible, biodegradable, and environmental friendly as well as safe for human and animals [7,8]. In aquaculture industry, the application of immunostimulants has opened a promising avenue to develop diets of balanced nutrition for improving the health and disease resistance of cultured fishes [7,9–11].

Prebiotics are non-digestible food ingredients that selectively stimulate the growth and/or activity of one or a limited number of bacteria through altering the intestinal microbiota. β-glucan and mannan oligosaccharide (MOS) are among the most common prebiotics used in aquaculture and have demonstrated promising immunological performances, such as phagocytosis, superoxide anion production, and lysozyme activity. β-glucan binds to several receptors on leucocytes and activate innate immune mechanisms as well as enhances immune responses, such as bacteria killing activity and regulation of cytokine production [12–14]. MOS could promote health and growth performance through enhancing weight gain and feed conversion efficiencies, improving gut structure, and positively regulating the intestinal flora [7].

Probiotics are the live microbial feed supplements that improve intestinal microbial balance and confer health advantages to the host, thereby promoting growth and immune system following stimulating enzyme activity and enhancing disease resistance [15,16]. In the last few years, probiotics have become an integral part of aquaculture practices for increasing growth and disease resistance. Several studies have applied Lactobacillus spp., especially lactic acid bacteria (LAB), in fish feed and showed significant improvments in growth and immunomodulatory responses [17–20]. *Lactobacillus plantarum* is a rod-shaped, gram-positive, catalase-negative, non-spore forming, fermentative, facultative aerobic lactic acid bacterium [21]. When used as a dietary supplement for farmed fish and crustaceans, *L. plantarum* has increased the enzymatic activities of digestive tract, improved the growth performance and feed utilization efficiency, inhibited the adhesion and growth of pathogenic bacteria, increased the levels of immunity, and enhanced disease resistance and survival in the host [21–23].

Although there are many publications on the effects of β-glucan, MOS, and *L. plantarum* individually on fish and other aquatic animals, no report is available on the possible interaction and combination effects of these supplements in fish. Therefore, the present research was conducted on Caspian brown trout to compare the combined (i.e., symbiotic) and individual effects of the two commercially available prebiotics β-glucan, MOS, and the probiotic *L. plantarum* on the immune response (lysozyme, ACH50, IgM, IL1, IL8, TNFα), growth performance (SGR, CF, FCR, FI, PER, DGI, WG, and survival), and intestinal microbial of the fish.

2. Materials and methods

2.1. Experimental diets

A basal diet was formulated according to the nutrition requirement of *Salmo trutta caspius*, as previously reported [24]. The ingredients and proximate analysis (Table 1) of the basal diet were determined. To prepare the basal diet with homogeneity of 2 mm, all ingredients were ground and thoroughly mixed with both fish oil and sunflower oil and then some water (about 20%) was added to produce stiff dough. The wet dough was placed into a grinder and sieved using a mincing machine, and then dried via a ventilating oven at 40 °C.

To prepare immunostimulant diets, β-glucan (Angel company, China; extracted from Saccharomyces cerevisiae), MOS (Bio-Mos; Alltech, USA; extracted from *S. cerevisiae*), and *Lactobacillus plantarum* (PTCC 10^{-8} Iranian Research Organization for Science and Technology) were added to the basal diet (i.e., control). The experimental diets were papered as follows: (1) basal diet, (2) basal diet + β-glucan (bβ), (3) basal diet + MOS (bM), (4) basal diet + *L. plantarum* (b*Lp*), (5) basal diet + β-glucan + *L. plantarum* (bβ*Lp*), (6) basal diet + MOS + *L. plantarum* (bM*Lp*), (7) basal diet + MOS + β-glucan (bMβ), and (8) basal diet + MOS + β-glucan + *L. plantarum* (bMβ*Lp*). A total of 3 and 4 g of β-glucan and MOS, respectively, per kg basal diet were used instead of filler. To add *L. plantarum* to the diets, the probiotic was grown for 24 h at 37 °C in MRS broth and then centrifuged. The centrifuged bacteria was diluted ($OD_{600} \approx 0.1$ or 10^8 CFU/ml) and sprayed at concentration of 10^9 CFU/mL on 100 mg of the basal diet (i.e., 10^8 CFU/mg) [21]. The diets (i.e., b*Lp,* bβ*Lp,* bM*Lp,* and bMβ*Lp*) were coated with sunflower oil and air-dried under a laminar flow hood for 30 min. During the feeding trial, the probiotic-supplemented regimes were prepared as needed for every 15 days. All of the diets were kept at 4 °C before the feeding trial.

Table 1
Composition and proximate analysis of the basal diets (as percentage dry weight) for Caspian brown trout, *Salmo truta caspius* (100 g^{-1} diet).

Percentage	Ingredients
55	fish meal[a]
15	soybean meal
11	wheat meal
1	soy lecithin[b]
4.5	fish oil[c]
4.5	Sunflower oil[d]
1	Starch
3	mineral mix[e]
2	vitamin mix[f]
0.01	antioxidant[g]
0.25	antifungi[k]
1.74	Filler[l]
1	calcium phosphate
100	Total
Proximate analysis diet	
48.6	Crud protein (%)
14.56	Crud lipid (%)
15.1	Ash
21.74	Carbohydrate (%)
4.42	Moisture (%)
20.96	Total energy (kj/gr)

[h] Unit (mg kg^1 or IU kg^1)kg^1 of mixture: mineral: Fe, 4500 mg; Cu, 500 mg; Co, 50 mg; Se, 50 mg; Zn, 6000 mg; Mn, 5000 mg; I, 150 mg; choline chloride, 150 000 mg; carrier up to 1 kg. Aras Bazar pharmaceutical Co. Amol, Iran.

[a] Myctophidae meal (Mazandaran Animal and Aquatic feed (Manaqua).

[b] Kilka oil (Manaqua Co. Iran) Khorak-dam Abzian corporation, Sari, Iran.

[c] Soybean lecithin with phosphatidylcholine (Behpak company, Iran).

[d] Sunflower oil (Ladan Co. Iran).

[f] Unit (mg kg^1 or IU kg^1) of mixture: vitamin: retinol acetate (A), 1 200 000 IU; cholecalciferol (D3), 400000; DL-a-tocopheryl acetate (E), 30 IU; menadione sodium bisulphite (K3), 1200 mg; L-ascorbic acid (C), 5400 mg; D-biotin (H2), 200 mg; thiamin mononitrate (B1), 200 mg; riboflavin (B2), 3600 mg; calcium D-pantothenate (B3), 7200 mg; niacinamide (B5), 9000 mg; pyridoxine hydrochloride (B6), 2400 mg; folic acid (B9), 600 mg; cyanocobalamin (B12), 4 mg; antioxidant, 500 mg; carrier up to 1 kg. Aras Bazar pharmaceutical Co. Amol, Iran.

[g] Antioxidant (Gluba Tiox, French).

2.2. Diet microbial analysis

To assess bacterial viability of the sprayed bacterium, a total of 1 g of each diet (b*Lp,* bβ*Lp,* bM*Lp,* and bMβ*Lp*) was dissolved and its bacterial count was performed on MRS agar at different time intervals (0, 5, 10, and 15 day). Microbial evaluation showed a decreasing trend in the population of *L. plantarum* added to the experimental diets, about 1.4 log CFU g^{-1}.

2.3. Experimental animals and culture condition

The Caspian trout (*Salmo trutta caspius;* n = 240, with initial weight of 10 ± 0.2 g) were purchased from the Cold Water Fishes Research Center (Mazandaran, Iran) and transferred to laboratory of Marine Science Faculty of Tarbiat Modares University. Prior to the feeding trial, the animals were acclimated to the laboratory condition for two weeks and then randomly were distributed into 24 100-L circular fiberglass tanks (10 fish per each tank) with continuous aeration. The feeding trial was conducted on eight experimental groups with three replicates. The fish groups were fed thrice daily with the experimental diets to satiation. All tanks were cleaned and siphoned to remove the debris and about 80% of water was exchanged. The experimental feeding trail was carried out under a 12 h: 12 h light: dark cycle condition for 56 days, and water temperature, dissolved oxygen, and pH were 15 ± 1 °C, 7.1 ± 1.5 mg L^{-1} and 7–8, respectively.

2.4. Growth performance and survival

At the end of the experiment, biological indices such as feed intake (FI), daily growth index (DGI), condition factor (CF), feed conversion ratio (FCR), specific growth rate (SGR %/day), wet weight gain (WG%), protein efficiency ratio (PER), and survival rate (SR%) for experimental fish were determined as follows [25]:

- FI = (grams feed per animal per day)
- DGI = 100 × ((final body weight)$^{1/3}$ – (initial body weight)$^{1/3}$)/duration
- Condition factor (CF): g/cm^3 = 100 [final body weight (g)/final body length (cm)^3]
- Feed conversion ratio (FCR) = the weight of food consumed by fish during the study period (g)/(final body weight (g) – initial body weight (g))
- Specific growth rate (SGR): % day-1 = 100 [ln (final wet weight)-ln (initial wet weight)]/duration (day)
- Wet weight gain (WG): % = 100 [(final mean wet weight (g)-initial mean wet weight (g))/initial mean wet weight (g)]
- PER = WG (g)/protein intake (g)
- Survival rate (SR) % = 100 [(final number of organism) (initial number of fish)-1]

2.5. Proximate chemical analysis

Proximate composition of the experimental diets and fish body were analysed according to AOAC method (AOAC, 2000). Briefly, moisture was measured following oven-dried at 105 °C to reach a constant weight, and ash through incineration in a muffle furnace at 550 °C for 24 h. The content of crud protein (N × 6.25) and lipid was determined using the Kjeldahl and ether extraction methods, respectively.

2.6. Blood collection and biochemical analysis

A total of 9 fish per treatment were randomly selected and anesthetized using clove powder (500 mg L^{-1}). Blood samples were collected from caudal vien into heparinized plastic syringes, centrifuged at 3000 rpm for 10 min and the plasma samples were kept in a freezer at –20 °C until subsequent analysis.

2.7. Evaluation of immune parameters

2.7.1. Lysozyme activity

Plasma lysozyme activity was measured according to a previous published method [26]. Briefly, *Micrococcus lysodeikticus* suspension (Sigma; 0.375 mg/ml, 0.05 M PBS, pH 6.2) was mixed with 250 µ/L of each plasma sample, and the optical density was determined after 15 and 180 s by spectrophotometer (Perkin-Elmer Lambda Bio 20 UV–Visible) at 670 nm. PBS was used as blank, and the results were expressed according to the amounts of lysozyme (*µg*/mL) of sample calibrated using a standard curve which was determined with hen's egg white lysozyme (Sigma) in sterile sodium phosphate buffer.

2.7.2. Alternative complement activity (ACH50)

ACH50 was determined according to haemolysis of rabbit red blood cells (RaRBC), the method described by Sunyer and Tort [27]. The volume of serum yielding 50% haemolysis (ACH50) was determined and used to calculate the complement activity of the samples.

2.7.3. Immunoglobulin analysis

Total immunoglobulin (IgM) levels were determined according to the method described by Siwicki and Anderson [28]. The Bradford method was employed for determining the protein content. Briefly, an aliquot (0.1 ml) of each serum sample was mixed with an equal volume of 12.0% solution of polyethylene glycol (Sigma), and incubated for 120 min. This solution precipitated the IgM molecules following centrifugation at 5000*g* at 4 °C. The supernatant was diluted 30 times with 0.85% NaCl and the protein content was determined; the difference between the protein values of untreated and polyethylene glycol treated sample corresponds to the total IgM content and is expressed as mg/mL.

2.7.4. RNA extraction and relative mRNA expression of immune related genes

At the end of the experiment 50–100 mg of head kidney tissue were collected and immediately kept in liquid nitrogen and then transferred to −80C° until RNA extraction. The samples were homogenized and total RNA was extracted using 1 ml Biozol Rigent (Bio flux; China) and prepared as previously described [27]. The quality and quantity of total RNA was assessed by 1% agarose gel and spectophotometer, respectively.The first strand cDNA was synthesized by SuPrime Script RT Premix (2X) cDNA Synthesis Kit (GeNet BIO Inc; Daejeon, South Korea). TNF-1α, IL1β, IL8 and β-actin specific primers tested by normal PCR. qPCR reactions were run in triplicate using a standard protocol as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation, annealing and extension at 95 °C for 15s, 55 °C for TNF2- α and TNF1-α for 30 min and 72 °C for 15s. The qPCR primers for TNF-1α, IL1β, and IL8 were designed according to the conserved regions of the trout Gene Bank sequences, using Oligo7 (Table 2). To validate primers, qPCR efficiency was also taken into account for choosing the best qPCR primers pair with specific and correct size. B-actin gene was used as the reference gene to normalize the expression of the target genes. The iQ5 optical system software (Bio-Rad) and 2-ΔΔCt method was used for data analysis.

Table 2
The sequences of primers used in the experiment.

Primer sequence (5′-3′)	Accession number	Target gene
F: CAAGAGTTTGAACCTTGTTCAA R: GCTGCTGCCGCACATAGAC	AJ277604	TNF-α1
F: ACATTGCCAACCTCATCATCG R: TTGAGCAGGTCCTTGTCCTTG	AJ223954	IL-1β
F: AGAATGTCAGCCAGCCTTGT R: TCTCAGACTCATCCCCTCAGT	AJ279069	IL-8

2.7.5. Intestinal L. plantarum population

The analysis of intestinal *L. plantarum* was conducted at day 0 (i.e., autochthonous population) and the end of the nutrition trial. A total of 9 fish per treatment (n = 3 per replicate) were sampled and their skin was sterilized before opening the abdomen and dissecting their intestine. The intestine samples were pooled, weighed (≈1 gr), and homogenized in sterile saline (i.e., physiological serum). The suspensions were serially diluted to 10^{-7} and 100 μl of the solution was spread onto DeMan, Rogosa and Sharpe (MRS) media. The cultivated plates were incubated at 37 °C for 48 h, and the number of colonies was counted.

2.7.6. Statistical analysis

Data analysis was performed using SPSS software (version 22). All results are presented as the mean ± standard deviation (S.D.). Kolmogorov–Smirnov test was applied to assess the normality and homogeneity of data. Significant differences were determined using one-way ANOVA, followed by Duncan's test to compare the differences between the experimental groups and the respective control group. Differences were considered statistically significant when $p < 0.05$.

3. Result

3.1. Growth performance and body composition

Data on growth performance and body proximate composition of the experimental fish are presented in Tables 3 and 4, respectively. The highest and lowest value for FI was observed for the fish fed with the basal diet and the diet supplemented with bMβ*Lp,* respectively ($p < 0.05$); that is, generally all of the experimental diets caused a significant reduction in FI index when compared to the control diet. Likewise, CF demonstrated the lowest level in the experimental group received all of the additives (i.e., mannan oligosaccharide + β-glucan + *L. plantarum* (bMβ*Lp*)). In addition, all of the diets containing the additives significantly decreased FCR and increased WG, PER, and final weight ($p < 0.05$); WG showed the highest value in the experimental group fed the diet containing mannan oligosaccharide (bM). No difference was observed in DGI and SGR between the supplemented diets and basal diet.

Table 4 shows the whole body proximate composition of the experimental animals. No difference was observed in the percentage of protein contents between the control group and the ones received the pro- and prebiotic additives. Total lipid content of the fish fed on the regimes treated with bM*Lp* and bMβ*Lp* exhibited no change but increased in the other treatments, especially bM and bMβ ($p < 0.05$). Ash content demonstrated significant increase in the fish fed on bβ and bMβ*Lp* treatments when compared to the control group. The amount of moisture reduced only in the fish group treated with the diet containing *L. plantarum* (i.e., b*Lp* diet) ($p < 0.05$).

3.2. Immunological analysis

Table 5 represents the effects of the supplemented diets on humoral innate immune parameters (IGM, lysozyme and ACH50) as well as blood glucose and blood cortisol. The data revealed that, in general, all of the pro- and prebiotic supplemented diets significantly enhanced the activity and levels of these blood indices when compared to the control group ($p < 0.05$). The highest values for the innate immune indices were observed in the fish groups fed with bMβ, bMβ*Lp*, and bβ*Lp*. Concerning cortisol and glucose, bMβ-treated fish group displayed the highest significant levels ($p < 0.05$).

3.3. Inflammation-related gene expression

The utilised pre- and probiotic suplimented feeds induced conciderable alterations in the expression of TNF-α1, IL-1β, and IL-8 genes (Fig. 1A, B, C). Compared to the control and other treatments, bM diet induced the highest mRNA transcription of TNF-α1 in head kidney tissue ($p < 0.05$), whereas bLp, bMβ, and bMβLp showed no significant effect (Fig. 1A). Similarly, IL1β showed the greatest up-regulation in response to the diet supplemented only with β-glucan (i.e, bβ), with increasing about 8.75 fold change when compared with the control diet. Moreover, the diets supplemented with only one pre- and probiotic also enhanced its transcription ($p < 0.05$; Fig. 1B). Relative IL-8 mRNA expression in the head kidney of the fish treated with bβLp and bβ significantly increased (about 2.75 and 1.9 folds, respectively), yet in response to bMβLp treatment it showed a decrease of about 5.7 times lower than the control group ($p < 0.05$; Fig. 1C).

3.4. Microbial analysis

At the end of the feeding trial, the intestinal population of *L. plantarum revealed* significantly higher loads ($p < 0.05$) in all of the experimental fish groups fed on pre- and/or probiotic supplemented regimes than the control group (Fig. 2). However, the highest loads of *L. plantarum* were observed in the intestine of the fish groups fed on the diets which were treated with the probiotic when compared to the control group and those received MOS and/or β-glucan.

4. Discussion

During the last two decades, the administration of dietary immunostimulants has been highlighted in aquaculture industry. Among these components, probiotics and prebiotics are the most effective additives which possess different health promoting properties and, in

Table 3
Growth performance and nutrient utilization in Caspian trout (*Salmo trutta caspius;* initial weight of 10 ± 0.2 g) fed the experimental diets supplemented with pro- and prebiotics over 8 weeks.

Final weight (g)	PER	WG (% IBW day^{-1})	SGR (%)	FCR	Treatments	DGI (% day^{1})	FI (g fish^{-1})	Experimental diets
					CF			
20.13 ± 1.67^{b}	1.28 ± 0.24^{b}	9.86 ± 1.92^{b}	1.12 ± 0.2	1.65 ± 0.33^{a}	1.03 ± 0.03^{bc}	0.91 ± 0.16	1.75 ± 0.09^{a}	basal diet
25.66 ± 1.61^{a}	2.02 ± 0.18^{a}	15.4 ± 1.44^{a}	1.53 ± 0.2	1.03 ± 0.09^{b}	1.15 ± 0.06^{ab}	1.29 ± 0.14	1.47 ± 0.08^{bc}	bM
25.23 ± 1.04^{a}	1.98 ± 0.28^{a}	14.96 ± 2.25^{a}	1.51 ± 0.29	1.06 ± 0.16^{b}	1.19 ± 0.03^{a}	1.27 ± 0.22	1.47 ± 0.05^{bc}	bβ
23 ± 1.32^{a}	1.65 ± 0.09^{ab}	12.73 ± 0.8^{ab}	1.34 ± 0.09	1.2 ± 0.07^{b}	1.13 ± 0.12^{ab}	1.11 ± 0.06	1.6 ± 0.07^{b}	b*Lp*
25.03 ± 2.06^{a}	1.96 ± 0.28^{a}	14.83 ± 2.25^{a}	1.5 ± 0.21	1.07 ± 0.15^{b}	1.1 ± 0.06^{ab}	1.26 ± 0.17	1.49 ± 0.1^{bc}	bMβ
25.5 ± 0.3^{a}	2.01 ± 0.09^{a}	15.3 ± 0.65^{a}	1.53 ± 0.12	1.03 ± 0.05^{b}	1.09 ± 0.04^{ab}	1.29 ± 0.08	1.48 ± 0.03^{bc}	bM*Lp*
25.73 ± 2.44^{a}	2.07 ± 0.4^{a}	15.53 ± 2.91^{a}	1.53 ± 0.23	1.02 ± 0.18^{b}	0.94 ± 0.08^{c}	1.30 ± 0.21	1.45 ± 0.08^{c}	bMβ*Lp*
23.93 ± 1.13^{a}	1.8 ± 0.13^{a}	13.73 ± 1.13^{a}	1.42 ± 0.07	1.16 ± 0.08^{b}	1.11 ± 0.2^{ab}	1.18 ± 0.07	1.55 ± 0.03^{bc}	bβ*Lp*

bM (basal diet + mannan oligosaccharide), bβ (basal diet + β-glucan), b*Lp* (basal diet + *L. plantarum*), bMβ (basal diet + mannan oligosaccharide + β-glucan), bM*Lp* (basal diet + mannan oligosaccharide + *L. plantarum*), bMβ*Lp* (basal diet + mannan oligosaccharide + β-glucan + *L. plantarum*), bβ*Lp* (basal diet + β-glucan + *L. plantarum*). Data are mean ± SD, n = 3. Letters a, b and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$).

Table 4
Body composition (moisture, lipid, protein and ash) of *S.trutta caspius* juveniles fed on immunostimulant diets for 8 weeks.

Experimental diets								Approximate analysis
bβ*Lp*	bMβ*Lp*	bM*Lp*	bMβ	b*Lp*	bβ	bM	Basal diet	
65.72 ± 1.86	67.89 ± 3.17	63.98 ± 0.6	66.16 ± 4.29	67.02 ± 0.85	64.32 ± 2.06	65.51 ± 0.74	68.98 ± 1.49	Protein (% DM)
27.22 ± 1.67^{ab}	19.31 ± 1.73^{c}	21.16 ± 1.16^{c}	27.76 ± 1.56^{a}	26.14 ± 0.38^{ab}	22.97 ± 0.91^{bc}	27.44 ± 5.5^{a}	19.85 ± 2.16^{c}	Fat (% DM)
6.25 ± 0.04^{b}	8.98 ± 0.35^{a}	6.44 ± 0.04^{b}	5.87 ± 0.07^{b}	6.55 ± 0.3^{b}	8.12 ± 1.84^{a}	6.73 ± 0.36^{b}	6.57 ± 0.1^{b}	Ash (% DM)
74.33 ± 0.23^{abc}	75.08 ± 1.38^{ab}	74.51 ± 0.22^{abc}	74.77 ± 0.53^{abc}	72.71 ± 1.7^{c}	76.42 ± 1^{a}	73.42 ± 1.72^{bc}	75.72 ± 1.11^{a}	Moisture (%)

bM (basal diet + mannan oligosaccharide), bβ (basal diet + β-glucan), b*Lp* (basal diet + *L. plantarum*), bMβ (basal diet + mannan oligosaccharide + β-glucan), bM*Lp* (basal diet + mannan oligosaccharide + *L. plantarum*), bMβ*Lp* (basal diet + mannan oligosaccharide + β-glucan + *L. plantarum*), bβ*Lp* (basal diet + β-glucan + *L. plantarum*). Data are mean ± SD, n = 3. Means without letter are not significantly different. Letters a, b and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$).

Table 5
Immunological parameters of *S.trutta caspius* fed the experimental diets for 8 weeks.

bβ*Lp*	bMβ*Lp*	bM*Lp*	Treatments	b*Lp*	bβ	bM	Basal diet	Blood parameter
			bMβ					
45.33 ± 3.21^{a}	49.33 ± 2.2.51^{a}	39.66 ± 2.08^{b}	45.66 ± 2.51^{a}	37 ± 2^{bc}	37.33 ± 1.52^{bc}	34.66 ± 2.08^{c}	26.33 ± 2.51^{d}	IgM (mg mL^{-1})
40 ± 1^{a}	42 ± 4.35^{a}	38.66 ± 3.51^{ab}	40.66 ± 2.08^{a}	26.33 ± 7.37^{c}	31.66 ± 2.08^{c}	33 ± 3^{bc}	17.33 ± 2.08^{d}	Lysozyme activity (μg mL^{-1})
137 ± 5.29^{a}	139.66 ± 4.04^{a}	127.66 ± 4.04^{b}	137.33 ± 3.51^{a}	120.66 ± 4.72^{c}	116.66 ± 3.78^{c}	134 ± 2.64^{ab}	107 ± 3^{d}	ACH50 (U mL^{-1})
63.33 ± 12.74^{b}	35.66 ± 3.51^{c}	44.66 ± 7.23^{bc}	97.66 ± 22.18^{a}	44 ± 6.08^{bc}	37.33 ± 5.68^{c}	61.66 ± 11.93^{b}	32 ± 4^{c}	Cortisol (ng mL^{-1})
92.33 ± 4.04^{b}	67 ± 8.54^{c}	66 ± 6.55^{c}	115 ± 13.52^{a}	75.66 ± 8.14^{bc}	65.66 ± 6.02^{c}	91.33 ± 16.44^{b}	59.66 ± 4.04^{c}	Glucose (ng mL^{-1})

bM (basal diet + mannan oligosaccharide), bβ (basal diet + β-glucan), b*Lp* (basal diet + *L. plantarum*), bMβ (basal diet + mannan oligosaccharide + β-glucan), bM*Lp* (basal diet + mannan oligosaccharide + *L. plantarum*), bMβ*Lp* (basal diet + mannan oligosaccharide + β-glucan + *L. plantarum*), bβ*Lp* (basal diet + β-glucan + *L. plantarum*). Data are mean ± SD, n = 3. Letters a, b and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$).

turn, enhance the growth efficiency in farmed aquatic animals [29,30]. The present study compared the individual and combined effects of dietary immunostimulants (MOS, β-Glucan, and *L.plantaru*) on growth performance indices (SGR, CF, FCR, FI, PER, DGI, and WG), immune related genes (IL1β, IL8, and TNFα1), and blood biochemical parameters (lysozyme, ACH50, IgM, glucose, and cortisol), as well as on the intestinal population of *L.plantaru* in Caspian brown trout.

Daily feed intake (FI) decreased in all of the experimental groups received the additive immunostimulants, especially in the group fed with the regime supplemented with all of the pre- and probiotics (i.e., bMβLp). These data are similar to those reported previously by Mansour's (2012) findings which showed a significant reduction in daily feed intake in giant sturgeon (*Huso huso*) juvenile treated with 4 g kg^{-1} MOS [31], suggesting lower palatability of the supplemented diets. In contrast, FI of red sea bream (*Pagrus major*) was increased by a diet supplemented with both heat-killed *L-plantarum* (HK-LP) and β-glucan [29].

The observed lower CF in the fish fed with the bMβ*Lp* diet could be attributed to the reduction in FI, suggesting antagonist interaction among the utilized pre- and probiotics when utilized together. The finding is in accord with the study of gilthead sea bream by Dimitroglou (2010) who found lower CF in fish fed 0.2% MOS when compared to the control group [7]. In addition, the applied immunostimulants in the present feeding trial caused lower FCR and also higher WG%, PER, and final weight in all of the experimental groups than in the control, indicating that both individual and combined preparations of MOS, β-glucan, and *L. plantarum* exerted a positive effect on growth parameters. These results are agree with and differ from the findings of other studies; the combination of two customised prebiotics (Bio-Mos® and β-1,3-D-glucan) and two probiotics (*Pseudomonas synxantha* and *P. aeruginosa;* 10^5 CFU/mL at 50:50 v/v) showed no significant effect on FCR when juvenile western king prawns (*Penaeus latisulcatus*) were fed either any prebiotics or probiotics [8]. Instead, lower FCR [7,8] and higher WG%, SGR%, and PER [23,32] were observed when immunostimulants such as MOS, β-glucan, and *L. plantarum* individually were used in fish and crustacean feeds. Another research reported that dietary supplementation of *Bacillus licheniformis* Dahb1 (10^5 and 10^7 cfu g^{-1}) improved the growth performance of tilapia (*Oreochromis mossambicus*) in term of final weight, SGR, and FCR [33]. These controversies could be associated with differences in compatibility of the immunostimulants composition among fish of crustacean species.

In this study, proximate composition analysis indicated that the diets supplemented with only MOS (i.e., bM) and/or with combination of β-glucan and MOS (i.e., bMβ) elevated the total lipid content of the fish carcasses, and also ash content increased in the groups treated with bβ and bMβLp. The lipid findings seems to be consistent with other research which noted a significant increase in crude lipid of giant sturgeon juvenile treated with 2 g kg^{-1} MOS; however, the ash, moisture, and protein contents remained unaffected [31].

Blood parameters are of high importance for indication of physiological and general health condition of fish reflecting nutritional and environmental changes [34]. Finding of the present study revealed that the innate immune parameters (lysozyme, ACH50, and IgM) were significantly enhanced in all of the immunostimulant treated fish groups, especially those received β-glucan (bMβ, bMβLp, and bβLp). In accordance with the present results, previous studies have demonstrated that the applied immunostimulants are capable of enhancing non-specific immune factors such as lysozyme and complement activities [29,32,35], and plasma IgM level [21,36]. Other supportive studies evaluated two dietary forms of modified β-glucan, including β-1, 3 glucan binding protein based selenium nanowire [37] and β-1, 3 glucan binding protein based zinc oxide nanoparticles [38], and reported enhancement of both cellular and humoral immune status. However, the stress indicators in the fish fed on the treated diets showed higher levels when compared with the control, and exhibited the order of bMβ > bM and bM*Lp* > bβ and bM*Lp* for cortisol as well as bMβ > bM and bM*Lp* > bLp > bβ, bM*Lp*, and bMβ*Lp* for glucose. Therefore, the finding indicated that feeding MOS combined with β-glucan induced highest stress status, whereas the fish fed with individual β-glucan as well as combined MOS and *L.plantarum* revealed an optimal

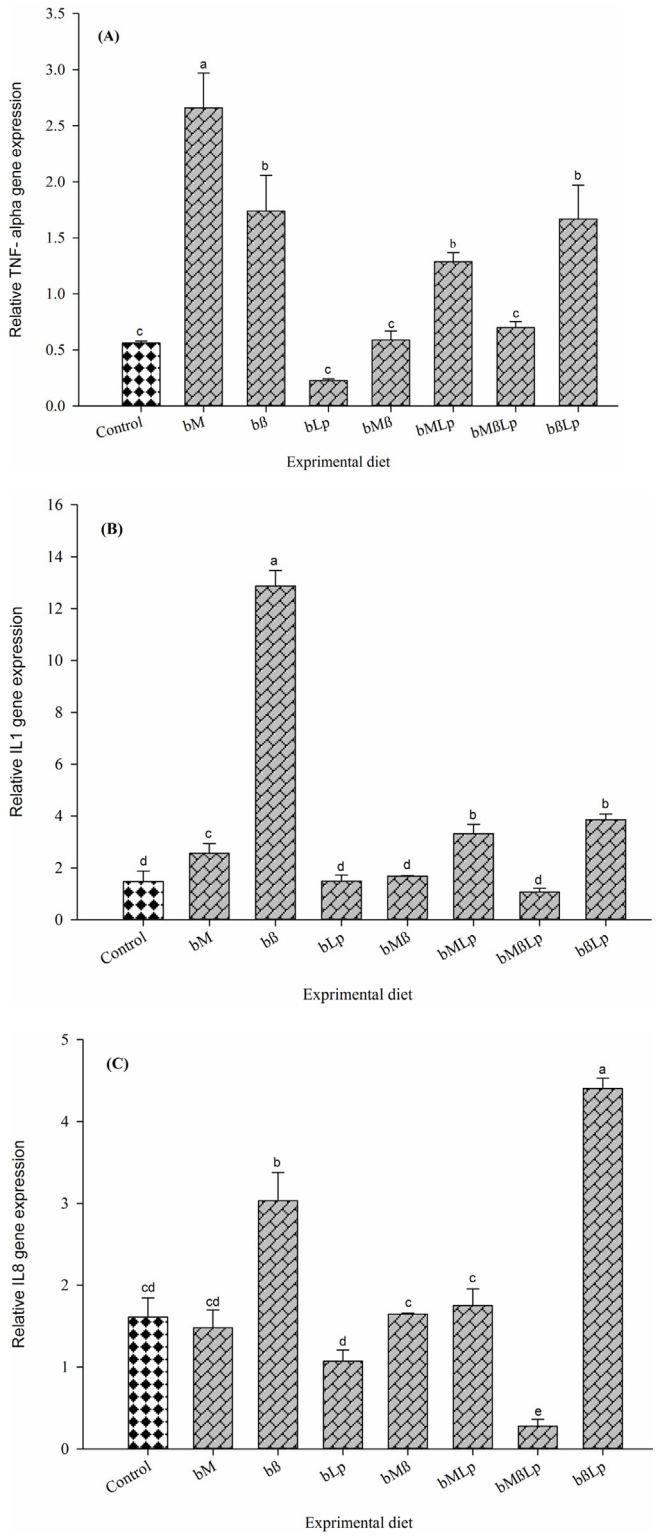

Fig. 1. Expression of immune-relevant genes [(A) TNFα1, (B) IL1β, (C) IL8] determined by real-time PCR in head kidney of *S. trutta caspius* fed with the experimental diets: Control (basal diet), bM (basal diet + MOS), bβ (basal diet + β-glucan), b*Lp* (basal diet + *L. plantarum*), bMβ (basal diet + MOS + β-glucan), bM*Lp* (basal diet + MOS + *L. plantarum*), bMβ*Lp* (basal diet + MOS + β-glucan + *L. plantarum*), and bβ*Lp* (basal diet + β-glucan + *L. plantarum*). Data are mean ± SD, n = 3. Bars sharing the same alphabet are statistically not significant ($p > 0.05$).

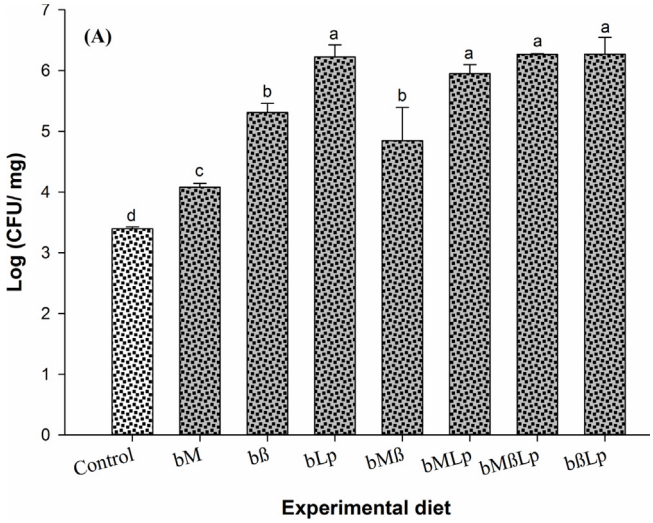

Fig. 2. Intestinal load of *L. plantarum* in Caspian trout (*Salmo trutta caspius*) fed with diets supplemented with individual or combined pre- and probiotics: Control (basal diet), bM (basal diet + MOS), bβ (basal diet + β-glucan), b*Lp* (basal diet + *L. plantarum*), bMβ (basal diet + MOS + β-glucan), bM*Lp* (basal diet + MOS + *L. plantarum*), bMβ*Lp* (basal diet + MOS + β-glucan + *L. plantarum*), and bβ*Lp* (basal diet + β-glucan + *L. plantarum*). Data are mean ± SD, n = 3. Bars sharing the same alphabet are statistically not significant ($p > 0.05$).

physiological condition.

This work demonstrated that feeding the pre- and probiotic-supplemented diets to Caspian brawn trout affect the modulation of the assessed inflammation-related genes (TNF-α1, IL-1β, and IL-8). Individual treatment of MOS and β-glucan, respectively, induced the highest expression of TNF-α1 and IL1β in the head kidney. In addition, relative IL-8 expression up-regulated following feeding with diets supplemented with β-glucan individually and in combined with *L-plantarum*, yet significantly down-regulated in response to diet supplemented with all of the three pre- and probiotics (i.e., MOS + β-glucan + *L.plantarum*). There are similarities between the findings and other researches, which showed that administration of beneficial probiotics and/or prebiotics induced up-regulation [39–41] and down-regulation of inflammation-related genes [42–45]. These differences may be explained by different fish species, prebiotics, probiotics, dose of immunostimulants, and length of the feeding trials.

The immunomodulating activity of probiotics has been suggested that microorganisms confer beneficial effects on the digestive processes [46]. Interaction between beneficial microorganism cells and the intestinal epithelial cells improves mucosal immunity in host gut, thereby tightening the epithelial junction, producing antimicrobial peptides and mucosal immunoglobulin, modulating inflammatory reaction and consequently stimulating immune response [29]. In this study, the use of *L. plantarum* maintained a high bacterial load in the intestine of fish groups administered with the probiotic, indicating possible viability and proliferation of *L. plantarum* during the feeding trial.

5. Conclusion

The current findings add to a growing body of literature on how to design and develop an immunostimulant diet in aquaculture industry. In general, the pro- and prebiotic supplemented diets positively influenced the growth performance parameters, except CF in the experimental group combinationally received all of immunostimulants (MOS + β-glucan + *L. plantarum*). Furthermore, combinational use of these immunostimulants more effectively enhanced humoral innate immune system, suggesting increasing immune capacity and disease resistance ability, whereas their individual and combinational

application could increase and decrease the transcription of inflammation-related genes, respectively.

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