**EFFECTS OF SINGLE AND COMBINED SUPPLEMENTATION
OF DIETARY PROBIOTIC WITH BOVINE LACTOFERRIN
AND XYLOOLIGOSACCHARIDE ON HEMATO-IMMUNOLOGICAL
AND DIGESTIVE ENZYMES OF SILVERY-BLACK PORGY
(*SPARIDENTEX HASTA*) FINGERLINGS**

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Abstract

The aim of this study was to evaluate the effects of different levels of dietary *Lactobacillus plantarum* as probiotic (Pro) with bovine lactoferrin (LF) and xylooligosaccharide (XOS) on growth performance, hemato-immune response, body composition, digestive enzymes activity and expression of immune-related and growth-related genes of sobaity (*Sparidentex hasta*) for 8 weeks. Fish were fed with feed including: control diet (no LF, XOS and Pro inclusion), diet 1 (400 mg kg⁻¹ LF + 5000 mg kg⁻¹ XOS), diet 2 (400 mg kg⁻¹ LF + 10000 mg kg⁻¹ XOS), diet 3 (400 mg kg⁻¹ LF + 1 × 10⁶ gr⁻¹ Pro (*L. plantarum*)), diet 4 (800 mg kg⁻¹ LF + 5000 mg kg⁻¹ XOS), diet 5 (800 mg kg⁻¹ LF + 10000 mg kg⁻¹ XOS), diet 6 (800 mg kg⁻¹ LF + 1 × 10⁶ gr⁻¹ Pro (*L. plantarum*)). Growth performance, hematological parameters (except for white blood cell counts), body composition and immune-related gene expression were not affected by different experimental groups (P>0.05). Nonetheless, non-specific immune response (except for total immunoglobulin) and growth-related gene expression of treatments and control group significantly varied (P<0.05). Digestive enzymes activity including total protease and amylase increased by supplementing diets with different combinations of immunostimulants (P<0.05). Our results suggest that diets supplemented with selected levels of LF, XOS and *L. plantarum* could not improve growth performance, body composition and hemato-immune response, but improved digestive enzyme activities in *S. hasta* fingerlings.

Key words: digestive enzymes, growth performance, innate immunity, lactoferrin, sobaity (*Sparidentex hasta*)

Immunostimulants as harmless, environmentally friendly and efficient biological agents have become one of the most promising applied aquafeed supplements

to replace antibiotics (Dawood et al., 2018; Wang et al., 2017). Among different kinds of immunostimulants, lactoferrin (LF) as a biological factor, xylooligosaccharides (XOS) as a prebiotic and probiotics such as *Lactobacillus plantarum* as functional feed ingredients have been proved to efficiently induce specific and non-specific cellular and humoral immune responses in different farmed aquatic species (Akhter et al., 2015; Vallejos-Vidal et al., 2016; Wang et al., 2017). Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein, which is a part of the transferrin protein family and has several biological functions such as bactericidal, antifungal, antiviral, antiparasitic, enzymatic, anti-inflammatory and anticarcinogenic activities (Giansanti et al., 2016). In addition, xylooligosaccharides are sugar oligomers made up of xylose residues linked through β -(1 $\rightarrow$ 4)-linkages, which are produced by chemical or enzymatic hydrolysis of xylan (an important component of hemicellulose). This oligosaccharide as feed additive could increase the population of beneficial micro-organisms (especially *Bifidobacteria*) and may promote the maintenance of lactic acid-producing bacteria as well as inhibit the adhesion of pathogenic bacteria in the intestine through competitive expulsion (Mussatto and Mancilha, 2007; Aachary and Prapulla, 2011). Furthermore, lactic acid bacteria such as *L. plantarum* play an important role in the host intestine by producing antimicrobial substances (i.e. lactic acid, acetic acid and bacteriocin) and modulating the bacterial community that prevent opportunistic pathogens (Akhter et al., 2015; Liu et al., 2016). Based on the studies of Son et al. (2009), Mohammadian et al. (2017) and Pageh et al. (2017), the above-mentioned immunostimulants have positive effects on growth performance, immunological responses (Son et al., 2009; Van Doan et al., 2016; Pagheh et al., 2018), digestive enzyme activities (Xu et al., 2009; Hoseinifar et al., 2014; Dawood et al., 2015; Mohammadian et al., 2017; Pagheh et al., 2018) in different fish species.

Silvery-black porgy, *Sparidentex hasta* is recognized as one of the most promising candidates for aquaculture diversification in the Persian Gulf and the Oman Sea regions (Basurco et al., 2011). Due to its great aquaculture potential (i.e. spawning in captivity, high growth rate, adaptation to a wide range of culture conditions, high market price and high quality of meat), the propagation and farming techniques of this species have been rapidly developed in the region (Mozanzadeh et al., 2017). Moreover, the nutritional requirements of *S. hasta* for optimizing diet formulation have been established during recent years (Mozanzadeh et al., 2017). Regarding dietary immunostimulants supplementation, Morshedi et al. (2015; 2016 a, b, c) did not observe positive effects of dietary lactoferrin (400 and 800 mg kg⁻¹) and XOS (5 and 10 g kg⁻¹) on growth performance, feed utilization, digestive enzyme activities (i.e. α -amylase, lipase and total protease), and humoral immune responses (i.e. lysozyme, complement and bactericidal activities) in *S. hasta* fingerling (initial body weight (BW_i) = 7.6 $\pm$ 0.3 g) for 6 weeks. In contrast, Khademi et al. (2013) reported that dietary inclusion of 2 g Protexin kg⁻¹ (a multi-species probiotic, consisting mainly of a variety of lactic acid bacteria and also yeast and fungi species) increased growth and feeding performance and stress resistance in *S. hasta* juveniles. In this regard, the aim of the current study was to evaluate combined effects of dietary LF, XOS and Pro on growth performance, hemato-immunological, digestive enzymes activities

and expression of immune-related and growth-related genes of *S. hasta* fingerlings for 8 weeks.

Material and methods

Fish maintenance and feeding

This study was carried out at the Mariculture Research Station of the South Iranian Aquaculture Research Center (SIARC), Sarbandar, Iran. A total of 410 fish (35.64 ± 0.30 g) were randomly distributed into 21 cylindrical polyethylene 250 l tanks (seven treatments with three replicates). Fish were acclimated for 2 weeks before the onset of the nutritional trial. Tanks were supplied with filtered running seawater (1 l min^{-1}); salinity and temperature were $48.0 \pm 0.5\text{‰}$ and $27.1 \pm 0.9^\circ\text{C}$, respectively during the experimental period. Average values for dissolved oxygen and pH were $6.3 \pm 0.6 \text{ mg l}^{-1}$ and 7.5 ± 0.2 , respectively, and the photoperiod was 14L:10D (light: darkness). Selection of the dosages and preparation of the experimental diets was done based on suggestions of previously published works (Esteban et al., 2005; Ren et al., 2007; Xu et al., 2009; Mohammadian et al., 2017). To prepare Lf-supplemented feeds 400 and 800 mg of Lf (90% purity, Biopole, Belgium) and XOS-supplemented feeds 5000 and 10000 mg of XOS (98% purity, Longlive Bio-Technology, China) were dissolved in 100 mL distilled water and sprayed over 1000 g of commercial dry feed (Beyza Feed Mill 21, Shiraz, Iran; 47% crude protein, 17% crude fat, crude fiber 2% and 14% ash). The feeds were then oven-dried with a continuous 35°C air current for 60 min. The dried feeds with supplements and the control feed (commercial dry feed only) were sprayed with 50 mL gelatin and stored at 4°C until used (Chang and Liu, 2002; Chitsaz et al., 2016). *L. plantarum* (PTCC 1058; Persian Type Culture Collection, Tehran, Iran, $1 \times 10^6 \text{ CFU g}^{-1}$) supplemented diets were prepared according to the method described by Irianto and Austin (2002). The above-mentioned immunostimulants were dissolved in sterile saline (0.9%), then sprayed on the basal diet and coated with 3% gelatin in order to avoid their leaching in water. Finally, the pellets were dried at room temperature and stored in plastic bags at -20°C until their use. Feeds were tested by triplicate; fish were fed by hand to apparent satiation (visual observation of first feed refusal) 2 times per day (1000 and 1700 hrs) for 56 days.

Sample collection

At the end of the trial, fish were fasted for 24 h before being anaesthetized (2-phenoxyethanol at 0.5 ml l^{-1} ; Merck, Schuchardt, Germany) and individually weighed (BW_p). Three ($n = 9$ fish per dietary treatment, $n = 3$ fish per replicate) specimens from each replicate were anaesthetized with 2-phenoxyethanol and then sacrificed with an overdose of this anesthetic for the assessment of digestive enzyme activities, and immediately eviscerated on an ice surface ($0\text{--}4^\circ\text{C}$). The alimentary tract was dissected, adherent adipose and connective tissues carefully removed, and placed in individually marked plastic test tubes and stored at -80°C until their analysis. Blood was collected from the caudal vein ($n = 15$ fish per diet treatment, $n = 5$ fish

per replicate) with heparinized syringes (300–700 μl per specimen) and pooled together (each pool contained the blood from two fish), and it was aliquoted into three parts of 1 ml each. An aliquot of blood was used for hematological factors and the other aliquots centrifuged (4000 g, 10 min at room temperature) and plasma separated and stored at -80°C until their analysis (Ashouri et al., 2013; Azodi et al., 2015).

Chemical analysis

After 8 weeks, all the fish were fasted for 24 h. Three fish from each replicate tank were randomly collected and taken for whole body proximate analysis. The analyses of proximate composition of fish were performed using the standard methods of AOAC methods (AOAC, 1995). Briefly, dry matter was measured gravimetrically after oven drying of homogenized samples for 24 h at 105°C (AMB50; ADAM, Milton Keynes, UK). Crude protein ($N \times 6.25$) was determined by the Kjeldahl procedure using an automatic Kjeldahl system (BÜCHI, Auto-Kjeldahl K-370; Switzerland). Crude lipid was determined by ether extraction using Soxhlet (Barnstead/Electrothermal, UK).

Hemato-immunological status

Hematocrit (Hct; %), hemoglobin concentration (Hb; g dl^{-1}), the number of red blood cells (RBC) and white blood cells (WBC) counts, as well as differential WBC percentage (lymphocyte portions as WBC%) were assessed according to methods described by Blaxhall and Daisley (1973).

The alternative complement pathway hemolytic activity (ACH50) was estimated following the procedure of Tort et al. (1996) and the volume yielding 50% hemolysis of rabbit red blood cells (RaRBC) was determined and used for calculating the complement activity of the sample.

The levels of lysozyme in plasma were determined using a turbidimetric assay according to Ellis (1990) by measuring the lytic activity of plasma against lyophilized *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA). A volume of 135 μl of *M. lysodeikticus* at a concentration of 0.2 mg ml^{-1} (w/v) in 0.02 M sodium citrate buffer (SCB), pH 5.8 was added to 15 μl of plasma sample. As a negative control, SCB was replaced instead of plasma. Results were expressed in mg of lysozyme ml^{-1} of plasma. Hen egg white lysozyme (Sigma) in phosphate-buffered saline was used as a standard. A unit of lysozyme activity was defined as the amount of plasma causing a reduction of absorbance of 0.001 per min at 450 nm at 22°C .

Plasma total immunoglobulin (Ig) was measured using the method described by Siwicki et al. (1994). Primary separation of immunoglobulins from the plasma was achieved by precipitation with polyethylene glycol (PEG) and the resulting supernatant analyzed. To perform the assay, 100 μl of plasma were combined with 100 μl 12% PEG and incubated at room temperature for 2 h in continuous agitation. Following the incubation time, the mixture was centrifuged (400 g, 10 min at room temperature) and total protein concentration in the supernatant determined by the biuret method. The total Ig levels were calculated considering total protein values less the quantity of protein in the supernatant.

Digestive enzymes

The digestive tracts were pooled and homogenized (1–2 min at 0–4°C; 3 volumes v/w of 50 mM 2 mM Tris–HCl buffer, pH 7.0) (Chong et al., 2002). Samples were centrifuged (10,000 g, 20 min at 4°C) and the supernatant was collected, aliquoted and frozen at –80°C for the determination of selected pancreatic enzymes. Total alkaline proteases were assayed by the azo-casein method described by Garcia-Carreño et al. (1993) using 50 mM Tris–HCl buffer (pH 9.0). Total alkaline protease activity (U) was defined as the mg of substrate hydrolyzed during 10 min ml⁻¹ of the extract at 25°C at 440 nm. Bile salt-activated lipase (E.C. 3.1.1) activity was assayed for 30 min at 30°C using p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris–HCl (pH 9.0), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract centrifuged (6080 g, 2 min at 4°C) and the absorbance of the supernatant read at 405 nm. Lipase activity (U) was defined as the 1 µmol of p-nitrophenyl myristate hydrolyzed min⁻¹ ml⁻¹ of extract (Iijima et al., 1998). Alpha-amylase (E.C.3.2.1.1) activity was determined according to Worthington (1991) using starch as substrate. In brief, starch (1%) was diluted in a 0.02 M Na₂HPO₄ and 0.006 M NaCl buffer (pH = 6.9) and incubated with the enzyme crude extract for 4 min at 25°C. Then, 0.5 ml of 1% dinitrosalicylic acid solution was added and boiled for 5 min. After boiling, 5 ml of distilled water was added to the mixture and the absorbance of the solution measured at 540 nm. Blanks were similarly prepared, but without the crude enzyme extracts. Maltose (0.3–5 µmol ml⁻¹) was used for the preparation of the standard curve. The α-amylase specific activity was defined by the µmol of maltose produced per min per mg protein at 25°C. The soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (1976) using bovine serum albumin as standard. All the assays were made in triplicate (methodological replicates). All immunological and digestive enzymes activities were measured by a microplate scanning spectrophotometer (PowerWave HT, BioTek®, USA).

Gene expression studies

Primers used in the identification of the *S. hasta* interleukin-1β (*IL-1β*) and insulin-like growth factor I (*IGF-I*) sequences were designed with Primer 3. Primers for *IL-1β* and *IGF-I* genes were designed based on nucleotide sequences with the gene bank numbers AY669059.1, AJ277166.2, JQ973887.1, and AJ269472.1: AY608674.1, AF030573.1, AY996779.2, and AB902571. Primer sequences are shown in Table 1.

Real-time PCR primers were designed based to *S. hasta* *IL-1β* and *IGF-I* sequences, and to the beta actin gene (based on alignments from AY491380.1, KY388508.1, and AY510710.2) (primer sequences are shown in Table 1) using Primer 3 online software. Head kidney and liver tissues were homogenized by liquid nitrogen, and total RNA was extracted using RNA extraction kit (Sinagen) according to the manufacturer. RNA samples quality was checked using a NanoDrop ND-1000 spectrophotometer. cDNA was synthesized from 1 µg of total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, K1622). The genes determined in this study included *IGF-I* and *IL-1β*. The cDNAs were used to perform real-time PCR with

specific primers previously reported as shown in Table 1. β -actin gene was used as a housekeeping gene in all experiments. The real-time PCR was performed with the SYBR Green Real-time PCR Master Mix (Sinagene, Iran) in a Rotor-Gen3000 real-time PCR detection system (Corbett Research). Each 20- μ L amplification contained 10 μ L SYBR Green Real-time PCR Master Mix (Sinagene), 10 pmol of each primer and 1 μ L cDNA. The mixtures were run with the following thermal cycling program: an initial activation step at 95°C for 3 min and then 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30s. All real-time PCRs were performed at least three times. Melt curve analysis was performed on the PCR products at the end of each run to ensure that a single product was amplified. Relative targeted gene expression was calculated for each reaction by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

Table 1. Nucleotide sequences of the primers used for cloning and assay gene expression

Primer Name	Nucleotide sequences (5'-3')
<i>IGF-I</i> Forward	ATGTCTAGCGCTCTTTCCTTTC
<i>IGF-I</i> Reverse	CTACATTCGGTAATTTCGCCCC
<i>IL-1β</i> Forward	TGGAATCCGAGATGACATGCAAC
<i>IL-1β</i> Reverse	CACTCGCCATCCCCACTG
<i>IGF-I</i> Forward (Real-Time PCR)	TGTAGCCACACCCTCTCACT
<i>IGF-I</i> Reverse (Real-Time PCR)	AGCCTCTCTCTCCACACACA
<i>IL-1β</i> (Real-Time PCR)	CTGGACTTGAGATTGCCCA
<i>IL-1β</i> Reverse (Real-Time PCR)	CTTCCACTGCGCTCTCCAG
Beta actin F (Real-Time PCR)	AGGGAAATCGTGCGTGACAT
Beta actin R (Real-Time PCR)	CGAGGAAGGATGGCTGGAAG

Interleukin-1 β (*IL-1 β*), Insulin-like growth factor I (*IGF-I*).

Statistical analyses

Data were analyzed using SPSS ver. 19.0 (Chicago, Illinois, USA). All data are presented as mean $\pm$ standard error of the mean calculated from three biological replicates. Arcsine transformations were conducted on data expressed as percentage. One way ANOVA was performed at a significance level of 0.05 following confirmation of normality and homogeneity of the variance. Tukey's procedure was used for multiple comparisons when statistical differences were found among groups by the one-way ANOVA.

Results

There was no mortality in the trial. The growth performance of juvenile sobaity seabream fed the diets containing LF, XOS and probiotic (*Lactobacillus plantarum*) over a period of 8 weeks is reported in Table 2. No significant differences were observed for final weight, percent weight gain, specific growth rate, protein efficiency ratio of juvenile sobaity seabream between dietary treatments and control treatment ($P>0.05$).

Table 2. Growth and feeding performance of *S. hasta* juvenile fed experimental diets supplemented with LF, XOS and *L. plantarum* for 8 weeks (mean $\pm$ SEM, n = 3)

Parameters	Control	LF ₄₀₀ + XOS ₅₀₀₀	LF ₄₀₀ + XOS ₁₀₀₀₀	LF ₄₀₀ + Pro	LF ₈₀₀ + XOS ₅₀₀₀	LF ₈₀₀ + XOS ₁₀₀₀₀	LF ₈₀₀ + Pro
Initial body weight (g)	35.98 $\pm$ 0.03	36.07 $\pm$ 0.13	35.94 $\pm$ 0.13	35.95 $\pm$ 0.00	36.18 $\pm$ 0.23	35.94 $\pm$ 0.06	36.21 $\pm$ 0.06
Final body weight (g)	76.09 $\pm$ 1.75	77.46 $\pm$ 0.87	78.16 $\pm$ 1.01	76.74 $\pm$ 0.74	74.43 $\pm$ 0.63	79.05 $\pm$ 4.49	76.84 $\pm$ 0.75
SGR (% day ⁻¹)	1.34 $\pm$ 0.04	1.36 $\pm$ 0.01	1.38 $\pm$ 0.12	1.35 $\pm$ 0.01	1.28 $\pm$ 0.00	1.40 $\pm$ 0.10	1.34 $\pm$ 0.02
WG (%)	111.46 $\pm$ 4.67	114.69 $\pm$ 1.60	117.48 $\pm$ 4.75	113.48 $\pm$ 2.06	105.72 $\pm$ 0.44	119.92 $\pm$ 2.91	112.18 $\pm$ 2.45
FCR	1.57 $\pm$ 0.03	1.62 $\pm$ 0.00	1.59 $\pm$ 0.14	1.62 $\pm$ 0.04	1.74 $\pm$ 0.01	1.58 $\pm$ 0.11	1.64 $\pm$ 0.04
PER	1.23 $\pm$ 0.03	1.19 $\pm$ 0.00	1.22 $\pm$ 0.11	1.19 $\pm$ 0.03	1.11 $\pm$ 0.00	1.23 $\pm$ 0.08	1.18 $\pm$ 0.03

SGR = $100 \times [\ln(\text{Mean final body weight}) - \ln(\text{Mean initial body weight})] / \text{time (days)}$, WG (%) = $100[(\text{Mean final body weight} - \text{Mean initial body weight}) / \text{body weight}]$, FCR = $\text{dry feed intake (g)} / \text{wet weight gain (g)}$, PER = $\text{wet weight gain (g)} / \text{protein intake (g)}$. No significant changes were observed ($P > 0.05$).

Table 3. Body composition of *S. hasta* juveniles fed control diet and diets supplemented with LF, XOS and *L. plantarum* for 8 weeks (mean $\pm$ SEM, n = 3)

Parameters	Control	LF ₄₀₀ + XOS ₅₀₀₀	LF ₈₀₀ + XOS ₁₀₀₀₀	LF ₄₀₀ + XOS ₁₀₀₀₀	LF ₈₀₀ + XOS ₁₀₀₀₀	LF ₄₀₀ + Pro	LF ₈₀₀ + Pro
Protein (%)	23.20 $\pm$ 0.66	21.00 $\pm$ 0.16	23.03 $\pm$ 0.60	21.91 $\pm$ 0.27	21.67 $\pm$ 0.07	22.97 $\pm$ 0.25	23.27 $\pm$ 0.11
Lipid (%)	7.91 $\pm$ 0.47	8.26 $\pm$ 0.08	8.74 $\pm$ 0.07	8.36 $\pm$ 0.20	7.96 $\pm$ 0.35	8.62 $\pm$ 0.60	8.20 $\pm$ 0.26
Dry matter (%)	39.96 $\pm$ 1.21	36.66 $\pm$ 0.40	40.63 $\pm$ 0.72	37.69 $\pm$ 0.07	37.08 $\pm$ 0.35	39.65 $\pm$ 0.03	40.32 $\pm$ 0.57

No significant changes were observed (P>0.05).

Table 4. Hematological factors of *S. hasta* juveniles fed experimental diets supplemented with LF, XOS and *L. plantarum* for 8 weeks (mean $\pm$ SEM, n = 3)

Parameters	Control	LF ₄₀₀ + XOS ₅₀₀₀	LF ₄₀₀ + XOS ₁₀₀₀₀	LF ₄₀₀ + Pro	LF ₈₀₀ + XOS ₅₀₀₀	LF ₈₀₀ + XOS ₁₀₀₀₀	LF ₈₀₀ + Pro
RBC ($\times 10^6 \mu\text{l}^{-1}$)	2.54 $\pm$ 0.06 a	2.29 $\pm$ 0.04 a	2.57 $\pm$ 0.08 a	2.27 $\pm$ 0.02 a	2.50 $\pm$ 0.02 a	2.15 $\pm$ 0.06 a	2.48 $\pm$ 0.04 a
Hb (g dl ⁻¹)	8.32 $\pm$ 0.18 a	7.50 $\pm$ 0.10 a	8.40 $\pm$ 0.24 a	7.52 $\pm$ 0.04 a	8.15 $\pm$ 0.71 a	7.02 $\pm$ 0.17 a	8.18 $\pm$ 0.14 a
Hct (%)	59.00 $\pm$ 1.58 a	53.25 $\pm$ 1.10 a	59.25 $\pm$ 1.79 a	52.75 $\pm$ 0.25 a	57.75 $\pm$ 2.58 a	49.00 $\pm$ 1.29 a	57.00 $\pm$ 1.29 a
WBC (μl^{-1})	4950 $\pm$ 306.8 a	4075 $\pm$ 213.6 ab	3975 $\pm$ 597.7 ab	4125 $\pm$ 394.4 ab	3650 $\pm$ 155.4 b	4350 $\pm$ 184.8 ab	4500 $\pm$ 496.6 ab
Lymphocytes (%)	72.75 $\pm$ 0.47 a	71.75 $\pm$ 0.47 a	75.50 $\pm$ 1.7 a	74.25 $\pm$ 0.85 a	75.25 $\pm$ 0.47 a	74.50 $\pm$ 1.04 a	71.75 $\pm$ 1.03 a
Neutrophils (%)	24.25 $\pm$ 0.25 a	22.75 $\pm$ 0.47 a	21.00 $\pm$ 1.47 a	21.50 $\pm$ 0.28 a	21.25 $\pm$ 0.47 a	22.75 $\pm$ 6.3 a	23.25 $\pm$ 1.10 a
Monocytes (%)	2.75 $\pm$ 0.47 a	4.75 $\pm$ 0.25 a	2.75 $\pm$ 0.47 a	3.25 $\pm$ 0.62 a	3.25 $\pm$ 0.47 a	2.50 $\pm$ 0.28 a	4.50 $\pm$ 0.28 a

A different letters in the same row denotes statistically significant differences (P<0.05).

In the present study, whole body dry matter, protein and lipid did not change among dietary treatments ($P>0.05$; Table 3).

The changes in some hemato-immunological parameters of sobaity seabream fed different levels of dietary LF are shown in Table 4. At the end of the experiment, the effects of different levels of dietary LF, xylooligosaccharide and probiotic had no significant effects on RBC, HCT content, Hb concentration and differential WBC counts ($P>0.05$). However, fish fed the LF800 + XOS5000 showed lower WBC counts than other groups ($P<0.05$).

The amount of Total Ig and lysozyme activity in sobaity seabream did not markedly vary between the control and the supplemented diet with LF, XOS and probiotic ($P>0.05$; Figure 1, 2).

Figure 1. Total immunoglobulin of sobaity juvenile fed the different levels of synbiotic for 8 weeks (mean $\pm$ SE). Bars assigned with different superscripts are significantly different ($P>0.05$)

Figure 2. Lysozyme activity of sobaity juvenile fed the different levels of synbiotic for 8 weeks (mean $\pm$ SE). Bars assigned with different superscripts are significantly different ($P<0.05$)

Table 5. Digestive enzyme activity of *S. hasta* juveniles fed experimental diets supplemented with LF, XOS and *L. plantarum* for 8 weeks (mean $\pm$ SEM, n = 3)

Parameters	Control	LF ₄₀₀	LF ₄₀₀ + XOS ₅₀₀₀	LF ₄₀₀ + XOS ₁₀₀₀₀	LF ₄₀₀ + Pro	LF ₈₀₀ + XOS ₅₀₀₀	LF ₈₀₀ + XOS ₁₀₀₀₀	LF ₈₀₀ + Pro
Total protease (U mg ⁻¹ protein)	0.15 $\pm$ 0.01 b	0.17 $\pm$ 0.02 ab	0.32 $\pm$ 0.02 a	0.37 $\pm$ 0.02 a	0.09 $\pm$ 0.01 ab	0.06 $\pm$ 0.01 ab	0.28 $\pm$ 0.02 a	0.07 $\pm$ 0.02 ab
Lipase (μ mol g ⁻¹ h ⁻¹)	0.33 $\pm$ 0.02 a	0.28 $\pm$ 0.02 a	0.37 $\pm$ 0.02 a	0.30 $\pm$ 0.02 a	0.30 $\pm$ 0.02 a	0.24 $\pm$ 0.02 a	0.35 $\pm$ 0.01 a	0.25 $\pm$ 0.01 a
α -Amylase (μ mol mg ⁻¹ protein)	4.82 $\pm$ 0.12 b	4.54 $\pm$ 0.04 ab	5.73 $\pm$ 0.15 a	4.62 $\pm$ 0.08 ab	4.91 $\pm$ 0.33 ab	5.19 $\pm$ 0.21 ab	4.49 $\pm$ 0.28 ab	

A different letters in the same row denotes statistically significant differences (P<0.05).

As presented in Table 5, the supplementation of diet with LF, XOS and probiotic did not coincide with the activity of lipase ($P>0.05$), but the activities of total protease and amylase improved by different experimental diets ($P<0.05$).

Figure 3. Expression of *IGF-I* gene of sobaity juveniles fed the different levels of synbiotic for 8 weeks (mean ± SE). Bars assigned with different superscripts are significantly different ($P<0.05$)

Figure 4. Expression of *IL-1β* gene of sobaity juveniles fed the different levels of synbiotic for 8 weeks (mean ± SE). Bars assigned with different superscripts are significantly different ($P>0.05$)

Expression of immune-related and growth-related genes of sobaity fed diets with different levels of LF, XOS and probiotic is presented in Figures 3 and 4. Fish fed the LF800+XOS10000 and LF800+Pro showed higher *IGF-I* gene expression than the control group ($P<0.05$). Nonetheless, *IL1β* gene expression did not affect different experimental groups ($P>0.05$).

Discussion

The results of the current study showed that supplementation of diet with combination of LF, XOS and *L. plantarum* did not improve growth performance in *S. hasta*, which might be a consequence of low levels of dietary immunostimulants supplementation. Similar to our results, in most of the consulted studies, LF administration did not have positive effects on growth performance in other fish species such as Atlantic salmon (*Salmo salar*) (Lygren et al., 1999), gilthead seabream (*Sparus aurata*) (Esteban et al., 2005), Japanese flounder (*Paralichthys olivaceus*) (Yokoyama et al., 2005), orange spotted grouper (*Epinephelus coioides*) (Yokoyama et al., 2006), Nile tilapia (*Oreochromis niloticus*) (Welker et al., 2007), Siberian sturgeon (*Acipenser baerii*) (Falahatkar et al., 2014), and sobaity seabream (Morshedi et al., 2015). In contrast, dietary LF supplementation led to an increase in growth performance in goldfish (*Carassius auratus*; effective LF dose = up to 1000 mg kg⁻¹; Kakuta et al., 1996) and *S. hasta* (effective dose = 800 mg kg⁻¹; Pagheh et al., 2018) compared with the LF-free group. Multiple factors such as the synergistic effects between LF and some other unknown feed ingredients (Yokoyama et al., 2006), feed processing (Lonnerdal, 2009), species-specific ability to dietary LF absorption (Eslamloo et al., 2012), and/or experimental design (i.e. dietary LF dose, feeding duration and culture conditions) (Moradian et al., 2018) might be differentially involved in promoting growth performance in fish and be accountable for such differences between above mentioned studies. Moreover, several other studies have revealed that growth parameters have remained unaffected with prebiotic (Grisdale-Helland et al., 2008; Dimitroglou et al., 2010; Hoseinifar et al., 2011; Geraylou et al., 2012) or synbiotic (Nekoubin and Sudagar, 2012) applications in different fish species, which is in line with the results of this study. In contrast, a plethora of researches reported growth performance and feed utilization improved by dietary prebiotics, probiotics and synbiotics in different fish species (Guzmán-Villanueva et al., 2014; Talpur et al., 2014; Van Doan et al., 2014; Faggio et al., 2015; Azimirad et al., 2016; Lee et al., 2016; Ringo et al., 2018).

In the present study, whole body composition did not change among dietary treatments. There is no available data to study body composition in fish supplemented with Lf, XOS and Pro. However, from previous studies, it has been suggested that changes in body composition especially protein and lipid are likely to be attributed to changes in deposition rate in muscle and different growth rates (Abdel-Tawwab et al., 2006, 2008). The results of the present study showed that effects of the supplementation of diet with different synbiotics on body composition were not efficient.

The present study revealed that RBC, Hb and Hct were not significantly affected by levels of dietary immunostimulants, indicating that mixture of above mentioned immunostimulants could not play a role in enhancing general health status of the fish. In this context, previous studies have reported that neither dietary LF (Welker et al., 2007; Eslamloo et al., 2012; Rahimnejad et al., 2012; Moradian et al., 2018; Pagheh et al., 2018) nor synbiotic (Welker et al., 2007; Sado et al., 2008) improved hematological parameters in different fish species. In contrast, in our previous studies single inclusion of dietary LF (400 and 800 mg kg⁻¹) or XOS (5000 and

10000 mg kg⁻¹) increased RBC, Hb and Hct in *S. hasta* juveniles (Morshedi et al., 2015, 2016 c). Welker et al. (2010) reported that dietary LF increased in blood Hct of channel catfish, *Ictalurus punctatus* fed diets supplemented with 800 and 1600 mg kg⁻¹ LF. In addition, supplementation of diet with synbiotic led to an increase in RBC, Hb and Hct in *Labeo rohita* (Andrews et al., 2009), rainbow trout (*Oncorhynchus mykiss*; Rodriguez-Estrada et al., 2009) and red seabream (*Pagrus major*; Dawood et al., 2015). These differences may be attributed to the fact that animal health condition and iron concentration in the diet may be responsible for improving hematological parameters (Kawakami et al., 1988).

In the current study, the fact that humoral immune responses of fish (except for fish fed the LF800 + XOS5000 that showed higher lysozyme activity than other groups) did not improve by different experimental diets might be due to the same WBC counts and lymphocytes in different groups. In our previous study, Morshedi et al. (2015, 2016 c) reported that single supplementation of dietary LF (400 and 800 mg kg⁻¹), XOS (5000 and 10000 mg kg⁻¹) and *L. plantarum* (1×10^5) for 6 weeks did not affect the same immunological parameters in *S. hasta* fingerlings. On the contrary, Pagheh et al. (2018) reported that dietary inclusion of LF (800 mg kg⁻¹) increased lysozyme activity, but not plasma total Ig content in *S. hasta* in grow-out stage (80 g). The contradictory result between our study and that of Pagheh et al. (2018) may result from different inclusion of LF (spray vs formulation) and/or fish growth stage (fingerling vs juvenile stage). Similar to the results of current study, it has been reported that dietary LF inclusion did not affect lysozyme (Lygren et al., 1999; Welker et al., 2007; Eslamloo et al., 2012) as well as plasma total Ig (Kumari et al., 2003; Eslamloo et al., 2012) in different fish species. Moreover, some studies also reported that dietary prebiotics and probiotics did not have significant effects on immunological responses of fish (Cerezuela et al., 2008; Grisdale-Helland et al., 2008; Geraylou et al., 2012). It seems that in the present study the selected dosages of dietary LF, XOS and *L. plantarum* were not enough for stimulating humoral immune response in *S. hasta*. In contrast, a plethora of studies reported the positive effects of dietary LF (Rahimnejad et al., 2012; Moradian et al., 2018), XOS (Hoseinifar et al., 2014) and *L. plantarum* (Son et al., 2009; Dawood et al., 2015; Van Doan et al., 2016) on immunological responses of different fish species.

In our study, fish fed the LF₈₀₀ + XOS₅₀₀₀ showed higher digestive enzymes activities (protease and amylase) than other groups, which indicated certain content of dietary immunostimulants can improve digestion process in fish. In the previous studies in *S. hasta*, Morshedi et al. (2016 a, b) reported that dietary LF (400 and 800 mg kg⁻¹) or XOS (5000 and 10000 mg kg⁻¹) alone did not improve total alkaline protease, lipase and α -amylase. These results indicated that the synergic effects of combination of dietary LF (800 mg kg⁻¹) and XOS (5000 mg kg⁻¹) could improve digestive enzyme activities in *S. hasta* juveniles. In this context, it has been reported that dietary LF increased intestinal epithelial cell proliferation and protected intestinal crypt and villous structure in neonatal piglets (Nguyen et al., 2014). These authors proposed that dietary LF promoted the integrity of intestinal mucosa integrity, helped in intestinal brush border stabilization and prevented loss of digestive enzymes to the gut lumen by interacting with LF receptors on intestinal brush border membranes.

Moreover, several authors have reported that the dietary administration of different bacterial forms enhanced the secretion and activity of digestive pancreatic and intestinal enzymes, leading to better feed utilization (Ray et al., 2012; Mohapatra et al., 2012; Dawood et al., 2015). Moreover, it has been reported that the positive effects of XOS on digestive enzyme activities might be associated with the manipulation of the gut flora towards a potentially more beneficial microbial community (Xu et al., 2009) or improvement of gut morphology, especially the microvilli structures by enhanced proliferation of epithelial cells (Guan et al., 2011).

It is well known that the expression of immune-related genes can be considered a useful tool for evaluating immune response (Alejo and Tafalla, 2011; Hoseinifar et al., 2018; Ahmadifar et al., 2019). The results of this study showed that the growth-related gene such as IGF-I was not affected by administration of dietary lactoferrin, prebiotic and probiotic, although fish fed $LF_{800} + XOS_{10000}$ and $LF_{800} + Pro$ showed higher expression than control groups. Nonetheless, the immune-related gene such as *IL1 β* did not indicate significant variations induced by diet supplementation. Although earlier studies have widely documented the effects of lactoferrin administration on growth performance and immune responses in several fish species, a limited number of studies have focused on the expression of immune-related and growth-related genes. Carnevali et al. (2006) reported in European sea bass (*Dicentrarchus labrax* L.) fed diet containing probiotic *Lactobacillus delbrueckii delbrueckii*, that an increase of *IGF-I* transcription was observed in comparison with control group. These authors suggested that at molecular level, the expression of genes involved in muscular growth was also positively affected by bacterial integrators confirming a beneficial role of probiotics on the whole metabolism. However, more research is needed to expand knowledge on the variation of gene expression in sobaity fish when fish diet is supplemented with combination of LF, XOS and Pro.

Overall, our results suggest that diets supplemented with levels or mixture of LF, XOS and LP could not improve growth performance, hematological factors, humoral immunity and immune-related gene expression. However, digestive enzyme activities and growth-related gene expression in *S. hasta* fingerlings improved by supplementing diet with these synbiotics, indicating that more research is needed to expand knowledge on mechanisms by which dietary synbiotics exert their effects on these parameters.

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