Original Research Article

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Modulation of growth performance, haemato-immunological parameters, gut microbiota and stress resistance upon feeding juvenile *Schizothorax zarudnyi* (Nikolskii, 1897) by fructo-oligosaccharid

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Keywords

Schizothorax zarudnyi, Fructo-oligosaccharide, Growth, Haemato-immunological parameters, Gut microbiota

Abstract

A 63-day experiment was carried out under controlled conditions to compare the effects of fructo-oligosaccharide (FOS) at four levels (5, 10, 20 and 30 g/kg) on growth performance, nutritional efficiency indices, haemato-immunological parameters, stress resistance, digestive enzymes and cultivable autochthonous intestinal microbiota of juvenile (68.52 ± 1.52 g) Khaju fish *Schizothorax zarudnyi*. Fish fed the diet containing 20 g/kg FOS had significantly ($p < 0.05$) higher weight. Dietary FOS supplementation (5-20 g/kg) showed significant effects on SGR compared with control treatment. Hb, Haematocrit, MCV, MCH and lymphocytes in fish fed with the diet containing 20-30 g/kg FOS were significantly higher than those in fish fed with control treatment. After 63-day feeding period and also, 5-min air exposure challenge test, the activities of IG, LYZ and ACP in serum of fish fed with the diet containing 10-30 g/kg FOS showed a significantly higher trend than other treatments. The ratio of lactobacillus count to total autochthonous intestinal microbiota in fish fed with 10-30 g/kg FOS was significantly higher than that in other treatment groups. Furthermore, dietary FOS supplementation significantly increased survival rate of juvenile Khaju fish. Polynomial regression of SGR, FCR, PPV and PER suggested that the optimum dietary FOS level could be higher than 18.2 and < 23.8 g/kg in fish reared in culture conditions. These results indicate the beneficial effects of FOS, and emphasizes the need for further research to analyze the use of prebiotics on growth performance of fish.

Abbreviations

FOS: Fructo-Oligosaccharide
DP: Degree of Polymerization
SGR: Specific Growth Rate
VFI: Voluntary Feed Intake

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FCR: Feed Conversion Ratio
PER: Protein Efficiency Ratio
PPV: Protein Productive Value
NEI: Nutritional Efficiency Index
IG: Total immunoglobulin
LYZ: Lysozyme
ACP: alternative complement
Ht: Haematocrit
LAB: Lactic Acid Bacteria
WBC: White Blood Cell
ADC: Apparent Digestibility Coefficient
RBC: Red Blood Cell
NFE: Nitrogen Free Extract
DGGE: Denaturing Gradient Gel Electrophoresis

Introduction

Khaju fish, snow trout, *Schizothorax zarudnyi*, is an endangered endemic species in the southeast of Iran [1]. Successful propagation and high efficiency hatching techniques in freshwaters have led to domestication process of this species during the last decade [2,3]. To determine nutrient requirements, suitable ingredients and inclusion levels in the diet of aquatic species are critically regarded as key indices to acclimatize aquatic species in captive areas [4–8]. Using feed additives (nucleotides, probiotics, prebiotics and synbiotics) in the aquaculture industry is an important option to obtain sustainable production due to increase in non-specific defense mechanisms as well as improvement of fish health status [6,8–10].

Fructo-oligosaccharide (FOS or oligofructose), a fructan existing in a number of common foods (garlic, onion, artichoke and asparagus), is a hydrolyzed product of inulin [11,12]. To achieve an integrated approach in order to opt a profitable prebiotic in the aquafeeds, it is crucial to consider main parameters including prebiotic origin (fungi or plant), chemical structure, degree of polymerization (DP), initial weight, feeding period, supplement dose, basal diet formulation, intestinal flora, type of tested biological responses including growth performance, survival rate, carcass quality, enzymatic and immunological indices and knowledge of setting up a challenge test (e.g. physical, chemical or biological stressors) [6,8,13]. In spite of conflicting results [14–16], profitable effects of dietary FOS supplementation on growth performance and survival [11,17–20], gut microbiota [19,21], immune response [Buentello et al., 2010; Hoseinifar et al., 2011] and digestive enzyme ac-

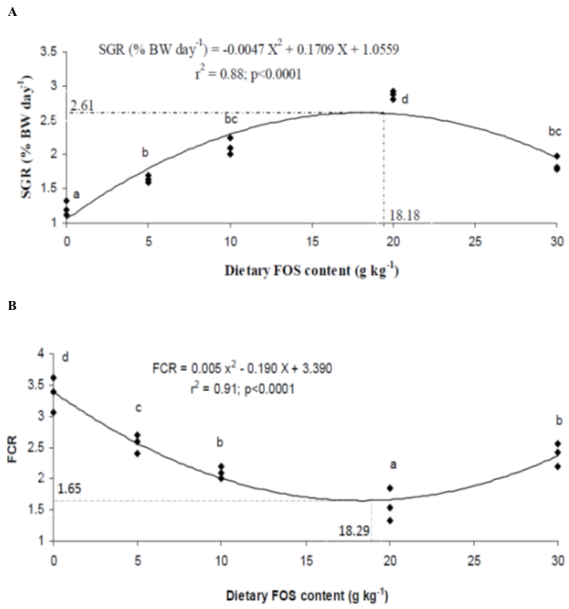

Figure 1
Polynomial model fitting for growth rate and conversion ratio
(a) specific growth rate (SGR; % BW/day) and (b) feed conversion ratio (FCR) to dietary fructo-oligosaccharide (FOS; g/kg) in juvenile Khaju fish at three replicates. Different letters indicate significant differences ($p < 0.05$).

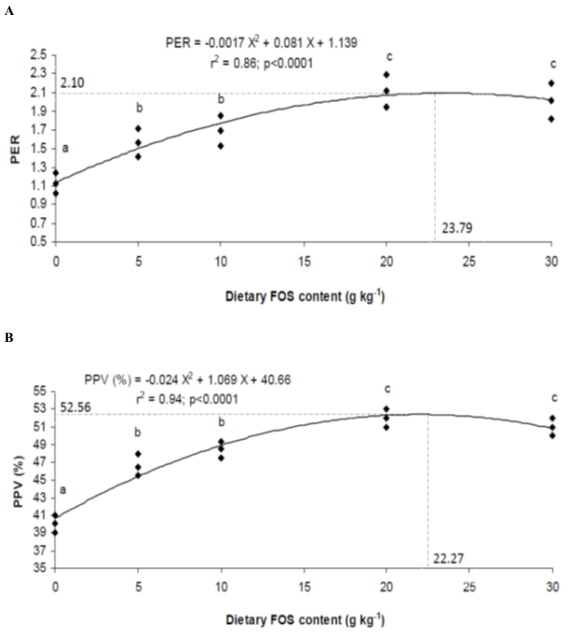

Figure 2
Polynomial model fitting for protein efficiency and protein productive value
(a) protein efficiency ratio (PER) and (b) protein productive value (PPV; %) to dietary fructo-oligosaccharide (FOS; g kg-1) in juvenile Khaju fish at three replicates. Different letters indicate significant differences ($p < 0.05$).

Table 1
Composition of the experimental diets (g/kg dry matter)

Ingredient	Control	Dietary fructo-oligosaccharide (g/kg)			
		5	10	20	30
Menhaden fish meala	393	393	393	393	393
Soybean meala	146	146	146	146	146
Corn glutena	75	75	75	75	75
Wheat floura	167	167	167	167	167
Fish oila	54	54	54	54	54
Canola oila	54	54	54	54	54
Choline chloride (70%)[b]	20	20	20	20	20
Vitamin C (stay)c	11	11	11	11	11
Vitamin premixb[f]	20	20	20	20	20
Mineral premixb[f]	20	20	20	20	20
Carboxymethyl cellulosed	40	35	30	20	10
FOS [e]	0	5	10	20	30
Chemical composition					
Dry matter	904				
Crude protein	350				
Crude fat	120				
Crude fiber	94				
Nitrogen free extract	382				
Ash	54				
Gross energy (Mj/kg)	18.90				

a Behparvar Aquafeed Co, Iran.

b Kimia Roshd Co. Iran.

c Sigma, Germany.

d Scharloo Chemical Co, Spain.

e Fructo-oligosaccharide (FOS, Raftilose® P95, Orafti Co., Belgium)

f Mineral premix contains (mg kg-1) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant (BHT), 100. Vitamin premix contains (mg kg-1) E, 30; K, 3; Thiamine, 2; Riboflavin, 7; Pyridoxine, 3; Pantothenic acid, 18; Niacin, 40; Folacin, 1.5; Choline, 600; Biotin, 0.7 and Cyanocobalamin, 0.02

tivity [18,20] have been reported in several fish species. Since culture intensification of Khaju fish, can led to inappropriate water quality, and to increased levels of physical, chemical and biological stressors in cultured organisms, it is important to decrease outbreaks of infectious diseases, to increase fish resistance, and to keep the high health status of fish using dietary supplements. To the best of our knowledge, there is no available information on the effects of dietary FOS supplemen-

Table 2
The mean (± SD[1]) of initial weight (g), final weight (g), voluntary feed intake (% BW/day) and survival rate (%) of juvenile Khaju fish fed the experimental diets after 63 days (n=3)[2]

	Control	Dietary fructo-oligosaccharide (g/kg)				*p* value
		5	10	20	30	
Initial weight (g)	69.06 ± 0.85	67.29 ± 1.12	68.05 ± 1.15	69.36 ± 1.23	68.85 ± 1.21	0.466
Final weight (g)	86.72 ± 1.12[a]	96.85 ± 1.25[b]	110.45 ± 1.32[cd]	132.28 ± 2.35[e]	106.31 ± 2.30[c]	0.042
VFI (% BW/day)	1.82 ± 0.12[a]	2.12 ± 0.08[b]	2.58 ± 0.05[c]	3.12 ± 0.07[d]	2.06 ± 0.25[b]	0.0001
Survival rate (%)	96.00 ± 0.25[a]	97.00 ± 0.52[b]	98.00 ± 0.75[c]	99.00 ± 0.23[d]	99.00 ± 0.21[d]	0.0001

1 Standard Deviation; 2 Different superscripts within a row indicate significant differences at $p < 0.05$

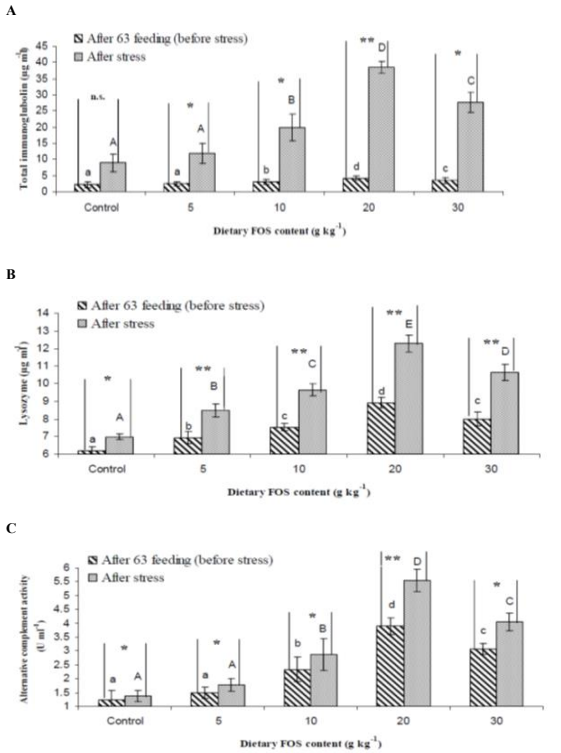

Figure 3
The mean (± SD) of (a) total immunoglubolin (µg/ml), (b) lysozyme (U/ml) and (c) alternative complement (U/ml) activities of juvenile Khaju fish fed the experimental diets after 63 days (before stress) and air exposure challenge (after stress). Values in lower case indicate significant changes after 63 days feeding within the control group. Values in capital letters indicate significant changes after 6 hr air exposure stress. Values with * and ** mean significant changes between groups at a specific sampling time with $p < 0.05$ and $p < 0.001$, respectively (n=3).

tation in juvenile Khaju fish. Therefore, the aim of the present study was to evaluate the effects of dietary FOS supplementation on growth performance, haemato-immunological parameters, nutritional efficiency indices, cultivable gut microbiota, digestive enzymes and stress resistance of juvenile Khaju fish.

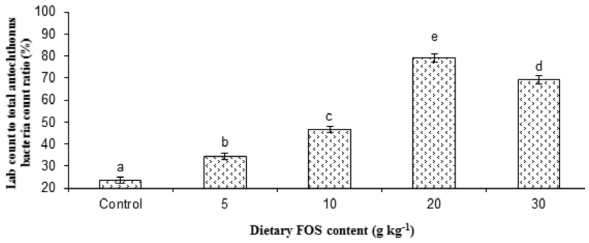

Figure 4
The mean (± SD) of lactic acid bacteria (lab) count (CFU/g) to total autochthonous bacteria count (CFU/g) ratio (%) of intestine extracted from juvenile Khaju fish fed the experimental diets after 63 days at three replicates.
Different letters indicate significant differences ($p < 0.05$).

Results

Growth performance and survival rate

There were no significant differences in the initial weight of treatment groups ($p > 0.05$). Increasing the levels of dietary FOS from 5 to 30 g/kg had significant ($p < 0.05$) effects on the final weight and specific growth rate (SGR) of juvenile Khaju fish compared with control diet (Table 2; Figure 1). VFI (2.06-3.12 % BW/day) and survival rate (97-99%) of juvenile Khaju fish fed the diets containing 5-30 g FOS kg-1 were significantly (p<0.05) higher than those of fed the control diet (1.12 % BW/day and 96%; respectively) (Table 2). Based on the broken line regression model, the dietary FOS re-

Table 3
The mean (± SD[1]) of the haematological parameters and differential leucocyte counts of juvenile Khaju fish fed the experimental diets after 63 days (n=3)[2]

	Control	Dietary fructo-oligosaccharide (g/kg)				P-value
		5	10	20	30	
Erythrocyte count (×10⁶/µl)	1.87 ± 0.07	1.89 ± 0.80	2.00 ± 0.09	2.15 ± 0.11	2.05 ± 0.13	0.875
Hb (mmol/l)	3.01 ± 0.11 [a]	3.05 ± 0.13 [ab]	3.28 ± 0.12 [bc]	3.52 ± 0.15 [c]	3.30 ± 0.14 [c]	0.005
Haematocrit (%)	24.10 ± 0.13 [a]	24.17 ± 0.14 [a]	24.63 ± 0.15 [b]	25.82 ± 0.12 [c]	25.71 ± 0.13 [c]	0.0001
MCV (fl)	151.28 ± 1.23 [a]	152.00 ± 1.33 [a]	153.71 ± 1.32 [a]	158.92 ± 1.30 [b]	158.89 ± 1.27 [b]	0.0001
MCH (pg)	32.16 ± 1.11 [a]	33.25 ± 1.13 [a]	36.72 ± 1.13 [b]	38.21 ± 1.14 [b]	37.02 ± 1.12 [b]	0.0001
MCHC (mmol/l)	13.68 ± 1.34	13.72 ± 1.34	14.09 ± 1.36	14.38 ± 1.39	14.11 ± 1.37	0.572
Leucocyte count (×10³/µl)	34.45 ± 1.39	35.15 ± 1.27	36.00 ± 1.39	36.98 ± 1.76	37.00 ± 1.87	0.252
Lymphocytes (%)	87.13 ± 1.30 [a]	88.27 ± 1.45 [ab]	89.37 ± 1.32 [ab]	90.27 ± 1.43 [bc]	92.30 ± 1.53 [c]	0.011
Neutrophils (%)	5.21 ± 1.23	5.89 ± 1.56	6.17 ± 1.49	6.48 ± 1.42	5.75 ± 0.76	0.812
Monocytes (%)	7.66 ± 1.53 [b]	5.84 ± 1.01[ab]	4.46 ± 1.81[ab]	3.25 ± 1.85 [ab]	1.95 ± 0.92 [a]	0.041

1 Standard Deviation; 2 Different superscripts within a row indicate significant differences at $p < 0.05$;

quirement for maximum growth (SGR) and minimum FCR of juvenile Khaju fish were estimated to be 18.18 and 18.29 g/kg, respectively (Figure 1).

Nutritional efficiency indices (NEIs)

The juvenile Khaju fish fed the control diet showed significantly ($p < 0.05$) lower values for NEIs (Figure 2). Increasing the levels of dietary FOS from 5 to 30 g/kg had significant ($p < 0.05$) effects on PER (1.56-2.12) and PPV (48.76-52.92%) compared with fed control diet (1.13 and 40.23%, respectively). Based on the broken line regression model, the dietary FOS requirement for maximum PER and PPV of juvenile Khaju fish were estimated to be 23.79 and 22.27 %, respectively (Figure 2).

Haemato-immunological parameters

The effects of dietary FOS supplementation on the haemato-immunological parameters of juvenile Khaju fish are summarized in Table 3 and Figure 3. Statistical analyses revealed that erythrocyte count, MCHC, leucocyte count and neutrophils were not significantly affected by dietary FOS supplementation ($p > 0.05$; Table 3). Hb, haematocrit, MCV, MCH and lymphocyte of the juvenile Khaju fish fed the diets containing 20 and 30 g/kg FOS were significantly ($p < 0.05$) higher than those of control diet (Table 3). The significantly ($p < 0.05$) lower number of monocytes (1.95%) were measured in fish fed the 30 g/kg FOS in diet (Table 3). After a 63-day feeding trial, dietary FOS supplementation (10-30 g/kg) significantly ($p < 0.05$) increased the activities of IG and ACP compared with those fed with 10 g/kg FOS and control diet (Figures 3a, 3c). Feeding 5-30 g/kg FOS in Khaju fish significantly ($p < 0.05$) increased the LYZ activity compared with control group (Figure 3b). After 5 min air exposure challenge, the significantly ($p < 0.05$) higher activities of IG, LYZ and ACP were observed in fish fed the diet containing 20 g/kg FOS (Figures. 3a, 3b and 3c).

Digestive enzyme activities

Amylase activity (0.37-0.45 U/mg protein/min) was not significantly affected by dietary FOS supplementation (Table 4). Trypsin activity of fish fed 5-30 g/kg FOS were significantly ($p < 0.05$) higher than that of control treatment (2.30-4.03 vs. 1.87 U/mg protein/min) (Table 4). The significantly ($p < 0.05$) higher trypsin activity (4.03 U/mg protein/min) was observed in fish fed with 20 g/kg FOS (Table 4). Lipase activity in fish fed 10-30 g/kg FOS (2.25-2.95 U/mg protein/min) showed significantly higher activity than those fed the diet 5 g/kg FOS and control group (Table 4).

Microbiological analysis

The lab count/total autochthonous bacteria count ratio in the extracted intestine in the juvenile Khaju fish fed the diets containing 5-30 g/kg FOS (34.5-79%) showed significantly ($p < 0.05$) higher values than those fed control diet (23.3%) (Figure 4). The significantly higher ($p < 0.05$) lab count/total autochthonous bacteria count ratio was observed in Khaju fish fed the diet containing 20 g/kg FOS (Figure 4).

Discussion

Growth performance, survival rate and digestive enzymes

The main challenge facing Khaju fish culture as a domesticated species in the northeast of Iran is the improvement of feed formulation in or-

Table 4
The mean (± SD[1]) of initial weight (g), final weight (g), voluntary feed intake (% BW/day) and survival rate (%) of juvenile Khaju fish fed the experimental diets after 63 days (n=3)[2]

	Control	Dietary fructo-oligosaccharide (g/kg)				*p* value
		5	10	20	30	
Trypsin (U/mg protein/min)	1.87 ± 0.15^{a}	2.30 ± 0.11^{b}	3.11 ± 0.13^{c}	4.03 ± 0.21^{d}	3.20 ± 0.20^{c}	0.0001
Lipase (U/mg protein/min)	1.62 ± 0.21^{a}	2.12 ± 0.31^{ab}	2.25 ± 0.92^{bc}	2.95 ± 0.18^{b}	2.73 ± 0.31^{b}	0.001
Amylase (U/mg protein/min)	0.38 ± 0.01	0.37 ± 0.02	0.41 ± 0.07	0.45 ± 0.09	0.43 ± 0.08	0.452

1 Standard Deviation; 2 Different superscripts within a row indicate significant differences at $p < 0.05$

der to optimize growth performance and disease resistance through the development of health-promoting diets [23,24]. Critically evaluation of key parameters such as feeding regime (feedstuffs, inclusion levels and supplements) and preference in cultivable aquatic species (behavioral aspects and palatability) helps aqua-feed industry to formulate suitable diets according to biological requirements [6,8]. To our knowledge, this is the first study to investigate the effects of dietary FOS supplementation on growth performance, haemato-immunological parameters, nutritional efficiency indices, cultivable gut microbiota, digestive enzymes and stress resistance of juvenile Khaju fish. The results of present study showed that 5-30 g/kg FOS had significant effects on growth performance of Khaju fish. These results are according to findings in blunt snout bream (Megalobrama amblycephala) [20], stellate sturgeon (Acipenser stellatus) [19], rainbow trout (Oncorhynchus mykiss) [25] and red drum (Sciaenops ocellatus) [22]. However, FOS supplementation in the diets of carp (Cyprinus carpio) fry [10], beluga (Huso huso) juvenile [21] and Atlantic salmon (Salmo salar) [26] had no significant effects on growth performance. Using prebiotics as a feed additive is considered as a way to maximize the potential of modulating the mucosa-associated microbiota and luminal bifidobacteria to efficiently discriminate and eliminate pathogenic organisms [7,27]. Consequently, the developed gastrointestinal microflora balance leads to boost In vivo digestion and absorption processes and finally, improvement of host health and growth performance [7,28,29]. Overall improvement of FCR may be regarded to produce some metabolites such as butyrate in the gastrointestinal tract [22]. A general increase in propionate and total fatty acid production was recorded through In vitro incubation of red drum, Sciaenops ocellatus chyme with Grobiotic® in the culture media [30]. The differences observed in the results of these studies might be due to the prebiotic chemical properties (origin and DP), feeding trial design (initial weight, feeding period, food formulation etc) and tested biological indices [7,8,13]. Improvements in the nutrient retention efficiency in the aquatic species are needed to reduce the environmental impacts of aquaculture, also, to make more efficient use of dietary nutrients. Perhaps the greatest potential for improving nutrient retention lies in the selection of broodstock having higher nutrient retention rates, but opportunities to improve protein retention through the formulation of ideal proteins and by increasing dietary energy levels or supplements influencing metabolism pathways also needed to be explored [7,31].

Dietary FOS supplementation significantly increased the survival rate of juvenile Khaju fish compared with the control group. Similarly, improved survival of common carp fry [10], cobia [Rachycentron canadum] larvae [32], rainbow trout [33] and beluga juveniles [21] has been reported upon FOS prebiotic administration. This can be related to improved general health or immune status [7,18].

Data from the present study can confirm that FOS as a non-digestible feed ingredient via selective fermentation affected the composition of intestinal microflora by stimulating Bifidobacteria and Lactobacilli, which are present in the intestine bacterial flora. Burr et al. reported the supplementation of soybean meal-based diets of red drum with mannanoligosaccharide, trans-galactooligosaccharide and Grobiotic (a mixture of partially autolysed brewer's yeast, dairy components and dried fermentation products) increased apparent digestibility coefficient (ADC) of nutrients than those of control diet [30]. Improvement of ADC values could be potentially related to up-regulation of the activities of specific digestive enzymes [22]. In the present study, the activities of digestive enzymes including trypsin and lipase in juvenile Khaju fish fed the diets containing FOS were higher than those of control. In this regard, supplemented-diets containing FOS significantly increased the activities of amylase and total protease in male broilers [34]. The effect of dietary-supplementation of different prebiotics was obvious after 49 days in chicken and 7 weeks or more in fish [22] because the development of microbial ecology and synchronous changes in the morphology and function of GIT are very complex processes that depends on several factors including the enteric environment, host physiology, microbial interactions, nutrition history and genetics [6,35,36].

Haemato-immunological parameters and stress resistance

Haemato-immunological parameters are considered as valuable tools in order to evaluate the potential of prebiotics in aquafeeds [14,37,38]. The results of the present study showed that dietary FOS supplementation had significant effects

on Hb, haematocrit, MCV, MCH, lymphocytes and monocytes. Similar results were observed on stellate sturgeon [19] and beluga [21]. In the present study, dietary FOS supplementation (10-30 g/kg) increased the IG, LYZ and ACP activities compared with the control treatment. Similarly, symbiotic administration in large yellow croaker (Larimichthys crocea) [39], gilthead sea bream (Sparus aurata L.) [40], koi (Cyprinus carpio koi) [41] and common carp fry [10] revealed a significantly higher respiratory burst activity. Elevation of lymphocytes, IG, LYZ and ACP activities is probably caused by the stimulation of Khaju fish immune responses due to FOS supplementation.

The intestinal flora of fish as a dynamic environment containing a diverse community of micro-organism [42,43] was affected by genetic, nutritional and environmental factors [44]. In spite of considering minor ratio of LAB count in intestinal microbiota, they have been regarded as beneficial components of the fish intestine [45]. Dietary manipulations using probiotics and prebiotics can increase the resistance of animals through mechanism of pathogen inhibition in GIT [46]. Competition for territory in GIT, reduction in pH and release of natural antibiotics from beneficial microbial populations can cause the pathogen inhibition [47]. Enhanced resistance also might be attributable to the beneficial effect of GIT microbes on host innate and adaptive immunity [48]. In this regard, lactic acid bacteria-containing yogurt could inhibit the growth of intestinal carcinoma through increased activity of immunoglobulin A, T cells and macrophages in mice [49]. In the present study, dietary FOS of 10-30 g/kg supplementation significantly increased the ratio of total authochthonous intestinal heterotrophic bacteria count to LAB count. The changes in the ratios of lacrobacillus count/total count noted in the present study may have benefited the Khaju fish, possibly by increasing nonspecific immune responses and by increasing concentrations and/or production of volatile fatty acids and butyrate as by-products of fermentation process in GIT. Both of these benefits have been demonstrated in chicken [50] and swine [51]. It was confirmed that butyrate as an increasing factor of disease resistance could down-regulate the expression of invasion genes in Salmonella sp. [52], although, the values of volatile fatty acids and butyrate were not measured in this study. However, further evaluations with use of denaturing gradient gel electrophoresis [DGGE] and 16S rRNA sequencing need to identify the LAB species in order to confirm their beneficial effects in juvenile Khaju fish. Similar to the present results, FOS has been reported to increase the levels of total cultivable autochthonous intestinal heterotrophic bacteria and LAB in common carp fry [10], stellate sturgeon [19] and beluga juveniles [21]. Although, the results of the present study confirmed that modulation of the intestinal microbiota in Khaju fish can be achieved through dietary FOS supplementation, but further considerations are needed.

Resistance during different stressors (physical, chemical and biological) has been considered as an important indicator to evaluate the efficiency of feed [7,53]. The critical factors to set up a challenge test are type, quantity and exposure time of stressor, genetic and feeding history of target animal, sampling method (plasma or serum) and physicochemical conditions of rearing facilities [7,54]. Regarding the focus of aquaculture industry to increase stocking rate, air exposure challenge is considered as one of limiting factors of production. It has been confirmed that numbers of activated macrophages (innate immunity), T-cell (adaptive immunity) activation and the recruitment of surveillance T cells increased during an acute stress as a primary stress response [55,56]. The results of the present study revealed that dietary FOS supplementation significantly increased fish resistance in the air exposure challenge test compared with the control diet and this is in accordance with previous reports on pacu (Piaractus brachypomus) [57], silver dollar (Metynnis argenteus) [58] and zebra fish (Danio rerio) [59] fed dietary FOS supplementation. Although, it is not clear how to describe the results of previous studies, it can be related to up-regulate immune system and finally, to obtain a higher level of homeostasis via the metabolic pathways of oxygen consumption process by mitochondria [7,24,31].

Conclusion

In the current trial, dietary FOS supplementation had significant effects on the growth performance and haemato-immunological parameters of juvenile Khaju fish and modulated autochthonous gut microbiota levels and stress resistance. The results of this study boost planning further studies on the use of FOS and other prebiotics in juvenile Khaju fish. To distinguish the metabolic pathways

and optimum inclusion level is a topic that guides further research.

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Materials and methods

Experimental diets

A basal diet (350 g/kg, crude protein; 120 g/kg, crude fat; 18.9 Mj/kg, Gross energy) as control diet (Table 1) was formulated [NRC, 2011; Safari, 2016] with WUFFDA (windows-based user-friendly feed formulation, done again; University of Georgia, Georgia, USA) software. The prebiotic, fructooligosaccharide (FOS, Raftilose® P95, Orafti Co., Belgium) was used at four doses 5, 10, 20 and 30 g/kg in the place of carboxymethyl cellulose [14]. The minimum level of fructose in FOS guranteed by manufacturer is 91%. DP of fructose in FOS ranges from 2-8% (Mahious, A. S., Ollevier, 2005). The other components are mainly glucose, fructose and sucrose. All feedstuffs were ground to a particle size < 250 µm [Glencross et al., 2007]. After adding fish oil, supplements and water (320 g/kg) contents, respectively, the mash was transferred from the hand pelletizer (Abzarsazan CO, Iran) with a 3 mm die, dried at 30˚C, packed in three-layer water-proof nylon bags and maintained at -20˚C [Hardy and Barrows, 2003] until feeding trials were started.

Fish culture and feeding trial

Two hundred twenty five healthy juvenile Khaju fish (68.52±1.52 g) were obtained from the Zahak reproduction and restocking center of warmwater and native fishes (Zabol, Iran) and stocked at a density of ten fish per 100 L tank (0.8 × 0.25 × 0.5 m) in a semi-recirculating system with daily water exchange rate of 30% at three replicates for each experimental diet. Most adapted to the feeding regime within three days. Unconsumed feed was collected three hours after feeding and weighed. Water temperature was maintained at 22.9°C throughout the feeding trial. DO (5.3 ± 0.10 mg/l), pH (8.6 ± 0.15), hardness (115 ± 1.3 mg/l as $CaCO_3$), unionized ammonia (< 0.06 mg/l) and nitrite contents (< 0.6 mg/l) were evaluated every week. Animals were held under L:D 12:12 h. Each diet was randomly assigned to three tanks of Khaju fish and they were fed 4% body weight twice daily (6:00 and 14:00) for 63 days. Biometry was done during first and last day of the experiment.

Evaluation of growth performance, survival rate and nutritional efficiency indices

At the end of the feeding trial, each fish was individually weighed (± 0.01) on an electronic scale (AND, Japan). All parameters were corrected based on the ingested feed. Growth parameters, survival rate and nutrient efficiency indices (PER and PPV) were calculated as follows [7]:

Specific Growth Rate (SGR ; %/day) = [(lnWf –lnWi)/ t] × 100

Volountary Feed Intake (VFI; % body weight/day) = [(Feed consumed (DM))/ (Wmean × t)

Survival Rate (%) = (Final Individual Numbers/Initial Individual Numbers) × 100

Feed Conversion Ratio (FCR) = (Feed consumed/W gain)

Protein Efficiency Ratio (PER) = (Wgain/Crude protein consumed)

Protein Productive Value (PPV; %) = 100 × [(Protein retained)/(Protein consumed)

In the above equations, Wi, Wf, Wmean and Wgain, t, and Feedconsumed are initial weight, final weight, mean weight, weight increment (g), time period (day) and consumed feed (g), respectively.

Haemato-immunological analysis

Six fish from each tank (18 fish per treatment) were anaesthetized by clove solution after 24 h of last feeding time in the 63th day. About 3.5 ml of blood was drawn from the caudal vein. Then, blood samples were introduced to both heparinized and non-heparinized tubes to perform hematological and immunological studies, respectively. For serum isolation, blood samples in nonheparinized tubes were centrifugated at 1000g for 5 min in order to separate the plasma. All assays were done one by one at three replicates. Whole blood was suspended in the diluents described by Natt and Herrick in order to count erythrocyte cells (RBC) and total leucocyte cells (WBC) [60]. Haematocrit (Ht) was determined using the micro-Ht method as described by Brown and Ht values are reported as packed cell volume percentage [61]. Mean corpuscular hemoglobin content (MCV) and hemoglobin (Hb) was estimated using Sahli's method according to method explained by Blaxhall and Daisley [62]. Differential WBC (neutrophils, lymphocytes and monocytes) were done using May-Grunwald-Giemsa blood smears. The non-heparinized tubes were stored at 20°C to measure the activities of total immunoglobulin (IG), lysozyme (LYZ) and alternative complement (ACP) (24).

Digestive enzyme activities

At day 63th, three fish (9 fish per treatment) were starved for 24 h and sampled from each tank for enzymatic analysis. The intestines were isolated and rinsed with cold distilled water at 4°C [24]. The intestinal enzyme extracts were homogenized in phosphate buffered saline (pH 7.5; 30 g/70 ml PBS) using a homogenizer (DI 18 Disperser) and the homogenate was then centrifuged at 15000×g, 4°C, 15 min and the supernatant stored in liquid nitrogen until further analysis. The total protein content of the supernatant was determined as using bovine serum albumin as a standard [24]. Protease activity was measured using casein hydrolysis at pH 8 [24]. Amylase activity was quantified using starch as a substrate at 540 nm [24] and lipase activity was determined via the measurement of fatty acids released following enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil [24]. Digestive enzyme activities (i.e. protease, amylase and lipase) were defined as specific activity (U/mg protein/min).

Chemical analysis

Analysis of dry matter (oven drying, 105°C), crude protein (N × 6.25, Kjeldahl system: Buchi Labortechnik AG, Flawil, Switzerland), crude fat (Soxtec System HT 1043: Foss Tecator, AB), ash (muffle furnace, 550°C), gross energy (Parr bomb calorimetry model 1266, Parr Instrument Co., Moline, IL) and crude fiber (after digestion with H2SO4 and NaOH) analysis of feedstuffs, diets and feces were performed according to standard methods [7]. Nitrogen free extract (NFE) was cal-

culated by subtraction of dry matter from crude protein, crude fat, crude fiber and ash contents.

Microbiological analysis

To quantify total viable autochthonous heterotrophic aerobic bacteria and lactic acid bacteria (LAB), six fish per treatment were transported alive to laboratory, anesthetized with ice, rinsed with benzalkonium chloride (0.1% for 60 min) and dissected with scalpel without pooling samples. Then, entire intestine was removed, homogenized with sodium chloride (0.9 w/v) using a homogenizer (DI 18 Disperser) and the homogenate was then centrifuged at 5000×g, 4°C, for 5 min. Serial dilutions 10^{-1}-10^{-7} were prepared. Nutrient agar (Sigma-Aldrich Co.) and MRS (Merk Co.) were used to determine total viable autochthonous heterotrophic aerobic bacteria and lactic acid bacteria count at room temperature (25°C) for 5 days, respectively. Colony-forming unit (CFU) per gram was calculated from statistically viable plates (i.e. plates containing 30 to 300 colonies) [7, 21].

Air exposure challenge

Three fishes from each tank (9 individuals per a treatment) were exposed to air for 5 min on day 63 [24]. The activities of IG, LYZ and ACP were used to evaluate biological responses of fish fed the experimental diets against air exposure challenge.

Statistical analysis

All percentage data were transformed using arcsine method. After confirming the homogeneity of variance and normality of the data using Leaven and Kolmogorov-Smirnov tests, one-way ANOVA was used to compare the treatments at three replicates. Duncan test was applied to compare significant differences among treatments ($p < 0.05$) with SPSS version 19. All results were given as mean ± SD.

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Author Contributions

Designed the experiment: O.S.; Performed the experiment: F.S.; Analyzed the results: R.V.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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