Effect of dietary synbiotics on growth, immune response and body composition of Caspian roach (*Rutilus rutilus*)

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Abstract

Effects of dietary synbiotics on growth performance, survival, stress resistance, body composition and immune response in the Caspian roach (*Rutilus rutilus*) were evaluated. Fish with an initial average weight of 4.14±0.25 g were randomly distributed into tanks (50 fish per tank) and triplicate groups were fed a control diet or diets containing 1 g kg^{-1} and 2 g kg^{-1} synbiotics. After an 8-week feeding period, a general enhanced growth performance and feed efficiency were observed in fish fed on the diet containing 2 g kg^{-1}synbiotics (p<0.05). Subsequently, immune responses (Ig levels, lysozyme activity and ACH50) were significantly higher in 2 g kg^{-1} synbiotics fed fish (p<0.05). Although all levels of dietary synbiotics significantly increased resistance to a salinity stress challenge (p<0.05), the highest survival rate was observed in this group. The intestinal tract of the fish with synbiotic diet supplementation had higher concentrations of lactic acid bacteria (7.13±0.32 log CFU g^{-1}). The protein and lipid contents in the whole body increased in the 2 g kg^{-1} synbiotics fed group. At the end of experiment the fish fed synbiotics had the highest survival index after 40 hours exposure to salinity stress (13.8 ppt). Results showed that the addition of synbiotics to the diet of Roach (*Rutilus rutilus*) stimulates the beneficial intestinal microbiota and alters their immune defense system.

Keywords: Synbiotics, Growth, Survival, Body composition, Salinity stress, Immune response, *Rutilus rutilus*

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Introduction

The Caspian roach (*Rutilus rutilus*) is a commercially important species in the Caspian Sea (Abdoli, 2000) and is also a major food source for wild Beluga sturgeon populations (Keyvanshokooh and Kalbassi, 2006). Like other Caspian Sea fishes (e.g. sturgeons) the species is considered threatened due to over fishing, water pollution, and loss of natural habitat and spawning grounds (Kiabi *et al*., 1999). However, the Iranian fisheries organization has developed culture methodologies to rear the Caspian roach up to market size to reduce pressure on natural Caspian Sea populations (Keyvanshokooh *et al.*, 2009). Global demand for safe food has prompted the search for natural and alternative growth promoters to use in fish feeds. A novel approach to these goals is the application of probiotics and prebiotics in the fish farming industry (Irianto and Austin, 2002; Wang and Xu, 2006; Wang *et al.*, 2008). Probiotics are defined as organisms and substances which contribute to intestinal microbial balance. In a practical sense, probiotics are defined as live microorganisms that are used as dietary supplementations in aquaculture and could enhance the growth and health of the host (Gatesoupe, 1999; Kesarcodi-Watson *et al*., 2008). Prebiotics are defined as non-digestible dietary ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of health-promoting bacteria in the gastrointestinal tract (Manning and Gibson, 2004). Gibson and Roberfroid (1995) have defined the mixture of pre- and probiotics as synbiotics that exert synergistic effects in promoting beneficial bacteria and the health of the gastrointestinal tract of the host, thus their potential applications have spurred attention. Although benefits associated with prebiotics and probiotics are desirable, researchers are concerned about a conclusive result, depending on type and amount of pre- and probiotics consumed. Therefore, more studies need to be conducted to provide a better understanding of their direct effects on health. The use of probiotics and prebiotics in aquaculture is now widely accepted but limited data is available regarding the application of synbiotics in aquaculture (Li *et al.*, 2009; Rodriguez-Estrada *et al.*, 2009; Daniels *et al.*, 2010; Zhang *et al.*, 2010; Ai *et al.*, 2011; Ye *et al.*, 2011; Mehrabi *et al.*, 2012; Nekoubin *et al.*, 2012). The aim of the present study was to study the effects of synbiotics (Biomin IMBO) on growth performance, survival rate, lactic acid bacteria (LAB) levels in the intestine, body composition and salinity resistance in roach (*R. rutilus*) fry via supplementation with experimental roach food.

Materials and methods

Fish culture and feeding trial

Caspian roach (average weight 4.14±0.25 g), obtained from the Sijowal Caspian Sea Teleost Fish Propagation

and Cultivation Center (Golestan Province, Iran) was randomly stocked into 9 tanks (300 L) at a density of 50 fish per tank (3 tanks per treatment). Water temperature, dissolved oxygen, pH and salinity were monitored daily and maintained at 25.2±0.9°C, 5.9±0.6 mg L^{-1}, 7.53±0.12 and 0.4±0.12 ppt, respectively. Continuous aeration was provided to each tank through an air stone connected to a central air compressor.

Feeding and synbiotic supplement preparation

The type of synbiotics applied in this study was Biomin IMBO (Biomin, Herzogenburg, Austria) which was comprised of probiotic (*Entercoccus faecium* 5×10^{11} CFU/kg) and Fructooligosaccharide (FOS) as the prebiotic. A basal diet was formulated for Caspian roach (Table 1); this basal diet served as the control diet and the experimental diets were produced by supplementation of the basal formulation with varying levels of synbiotics (1 and 2 g kg^{-1}). The ingredients were blended thoroughly in a mixer and pelleted using a meat grinder. The pelleted diets were air-dried, ground and sieved to produce a suitable crumble (1 mm). Then the feed was stored at 4 °C until feeding trials began. The experimental fish were weighted every 15 days in order to adjust the daily feed rate which was 3–5 g kg-1 of the total biomass. The fish were fed twice daily to apparent satiation for 60 days (Akrami *et al.*, 2010). The chemical composition of formulated diets was determined according to standard AOAC (AOAC, 1990) methodology.

Growth and feeding performance

In order to measure the growth parameters, weight and length of all fish were measured at every 15 day interval. After an 8-week feeding period, Weight Gain (g kg^{-1}), Specific Growth Rate (SGR g kg^{-1} /day), Feed Conversion Ratio (FCR), Condition Factor (CF g/cm^3) and Survival Rate (g kg-1) were calculated according to the following equations (Bekcan *et al.*, 2006): WG (g kg^{-1})=$(Wt-W_0)\times 100/W_0$, SGR= $(\text{Ln } Wt-\text{Ln } W_0)\times100/t$, FCR=dry feed fed in g/Wet weight gain in g, CF=$100\times Wt/Lt^3$, Survival rate = $(Nt/N_0)\times 100$. Here Wt and W_0 are final and initial body weights (g) respectively, t is duration of experimental days, N_0 is the initial number of fish and Nt is the final number of fish.

Chemical analysis of diets and fish carcasses

The chemical composition of formulated diets and fish carcasses were determined according to standard AOAC methodology (AOAC, 1990). At the end of the experiment, 15 randomly sampled fish from each treatment (5 fish from each tank) were collected for carcass analysis. Crude protein content was determined by kjeldahl method using Auto Kjeldahl System, crude lipid content by soxhlet extraction method, ash content in a furnace muffler (550 °C for 4 h), moisture content in a dry oven

(105 °C for 24 h) and crude fiber content using an automatic analyzer (Fibertec, Sweden) (AOAC, 1990).

Salinity stress challenge

At the end of the feeding trial, 10 fish were sampled from each tank and subjected to a salinity stress challenge. The fish were exposed to 13.8 g L^{-1} salinity according to Akrami *et al.* (2010). The survival rate of Caspian roach was calculated at 40 h post challenge (Akrami *et al.*, 2010).

Intestinal microbiota

The analysis of intestinal microbiota was conducted at the end of the nutrition trial. Three fish were sampled in each treatment and starved for 24 h prior to microbiological sampling. The fish were killed by physical destruction of the brain and the skin washed in a solution of 0.1g kg^{-1} benzalkonium chloride before opening the ventral surface with sterile scissors. Intestinal tract of sampled fish were removed, weighed, and suspended in sterile saline [0.85g kg^{-1} (w/v) NaCl]. The suspension, serially diluted to 10^{-6} and 0.1 mL of the solution, was spread in triplicate on to nutrient agar (NA). DeMan, Rogosa and Sharpe (MRS) was also used to detect Lactic Acid Bacteria (LAB). All of the plates were incubated at room temperature (25°C) and examined for 5 days (Rengpipat *et al*., 1998; Mahious and Ollevier, 2005), and the number of colonies were counted. Identification of the samples was carried out according to Bergy's method (Peter and Sneath, 1986).

Immunological assays

Serum total immunoglobulin (Ig) levels were determined according to the method described by Siwicki and Anderson Siwicki and Anderson (1993). Briefly, serum total protein content was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the immunoglobulin molecules, using a 12g kg^{-1} solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content. Serum lysozyme activity was determined according to Demers and Bayne (Demers and Bayne, 1997) and based on lysis of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma). Alternative complement activity was assayed according to the procedure of Yano (Yano, 1992) by using rabbit red blood cells (RaRBC). The volume of serum yielding 50g kg^{-1} haemolysis was determined and used to calculate the complement activity of the sample (value of ACH50 is in units per millilitre).

Statistical analysis

Data were analyzed by one-way analysis of variance using the statistical software SPSS version 18.0. Subsequently, significant differences between the groups were determined using Duncan's new multiple range test. Data are presented as treatment

means±standard deviation (SD). Differences were considered significant when $p<0.05$.

Results

The growth performance of roach fed diets supplemented with varying levels of dietary synbiotics is presented in Table 2. Compared to the control treatment, roach fed 2.0 g synbiotics kg^{-1} diet displayed improved ($p<0.05$) growth performance, including weight gain (g kg^{-1}), SGR, FCR and CF. Furthermore, roach fed 1.0 and 2.0 g synbiotics kg^{-1} diet had significantly higher survival compared to the control ($p<0.05$) (Table 2).

According to the body analysis composition data (Table 3) at the end of the experiment, the percentages of body protein and lipid in fish fed with synbiotics was significantly ($p<0.05$) higher than that from the control fish whereas the percentage of ash content was not ($p>0.05$).

Intestinal microbiota analyses are shown in Table 4. There were significant differences in intestinal lactic acid bacteria count in fish fed with 2.0 g synbiotics kg^{-1} diet (7.13±0.32 CFU g^{-1}) ($p<0.05$) although the concentration of total heterotrophic bacteria did not differ ($p>0.05$). The effects of the different dietary levels of synbiotics on the immune responses of roach juveniles are shown in Table 5. All immune responses measured (i.e. total Ig, lysozyme activity and ACH50) were significantly higher ($p<0.05$) in 2.0 g synbiotics kg^{-1} diets fed fish compared to the control group. Fish fed 2.0 g synbiotics kg^{-1} diets displayed significantly elevated lysozyme activity (51.6±3.8 µg mL^{-1}) compared to the control (33.6±3.8 µg mL^{-1}). The concentration of total immunoglobulin (7.2±0.8 mg ml^{-1}) and ACH50 (54.23±6.92 U mL^{-1}) were significantly higher in fish fed with 2.0 g synbiotics kg^{-1} diets compared to the control group (Table 5).

Results from the salinity challenge are presented in Fig. 1. The dietary synbiotics significantly increased the resistance of roach to the salinity stress challenge ($p<0.05$). Survival of fish fed diets containing 2.0 g synbiotics kg^{-1} supplementation was significantly higher than fish fed the basal diet after the same period (Fig. 1).

Table 1: Formulation (g kg^{-1}) and proximate composition of diets.

Ingredient	Diets		
	Control	1 g kg^{-1} synbiotic	2 g kg^{-1} synbiotic
Fish meal	40	40	40
Wheat flour	25	24	23
Soybean meal	15	15	15
Corn gluten	5	5	5
Soybean oil	6	6	6
Fish oil	6	6	6
Synbiotic[a]	0	1	2
Vitamin/ Mineral premixa[b]	3	3	3
Proximate composition (g kg^{-1})[c]			
Crude protein	35.1	35.3	35.2
Crude lipid	12.1	11.9	12.1
Ash	7.9	8.1	7.8
Moisture	10	9.8	9.7
Crude fiber	5	5.1	4.8
NFE[d]	19.9	20	20.7
Gross energy (MJ/kg)[e]	16.51	16.48	16.66

[a] The type of synbiotics applied in this study was Biomin IMBO (Biomin, Herzogenburg, Austria) which was comprised of probiotic (Entercoccus faecium 5×1011 CFU/kg) and Fructo-oligosaccharide (FOS) as prebiotic.

[b] Vitamin/mineral premix contains (multivitamin and trace minerals per 500 g mixture): vitamin A 1,000 IU, vitamin D_3 3,000 IU, vitamin E_3 mg, vitamin B_1 2 mg, vitamin B_2 2 mg, vitamin B_6 1 mg, nicotinamid 15 mg, calcium pentotenate 5 mg, vitamin K_3 2 mg, Cu^{+2} 3 mg, Fe^{+2} 12 mg, Zn^{+2} 15 mg, Mn^{+2} 25 mg

[c] Means of the two replicate analyses sample expressed in dry-matter basis

[d] NFE = 100 - (g kg^{-1} crude protein+g kg^{-1} crude lipid + g kg^{-1} ash + g kg^{-1} fiber + g kg^{-1} moisture)

[e] Gross energy (GE) (MJ/kg) = (g kg^{-1} crude protein × 23.6+g kg^{-1} crude lipid × 39.5+g kg^{-1}NFE × 17)

Table 2: Growth performance of Caspian juvenile fry fed different dietary levels of synbiotics for 8-week.

	Control	1.0 g synbiotics kg^{-1}	2.0 g synbiotics kg^{-1}
WG (g kg^{-1})	105.60± 5.9 [a]	132.73±9.41 [b]	168.83± 7.75 [c]
SGR (g kg^{-1}/day)	1.72±0.06[a]	2.14±0.09 [b]	2.35±0.04 [c]
FCR	4.41±0.18 [a]	3.45± 0.31 [b]	2.79±0.17 [c]
CF	0.84±0.04 [a]	0.96±0.01[b]	1.1±0.02 [c]
Survival (g kg^{-1})	74.5±8.66 [a]	87.3±3.5 [b]	95.6± 2.9 [b]

Values in a row with different superscripts denote a significant difference (p<0.05).

Table 3: Whole body composition of Caspian roach juvenile fed diets containing various levels of synbiotics for 8 weeks.

Composition (% dry matter)	Control	1.0 g synbiotics kg^{-1}	2.0 g synbiotics kg^{-1}
Moisture	78.3 ±0.78 [a]	77.84 ±0.65 [b]	71.3 ±0.56 [c]
Protein	14.38 ±0.39 [a]	15.98 ±0.26 [ab]	18.16 ±1.13 [b]
Lipid	3.38± 0.39 [a]	4.18 ±0.26 [ab]	6.16 ±0.23 [b]
Ash	2.49 ±0.26 [a]	2.5 ±0.31 [a]	2.6 ±0.22 [a]

Values in a row with different superscripts denote a significant difference (p<0.05).

Table 4: Bacteria counts of the intestinal tract of Caspian roach juvenile fed different dietary levels of synbiotics.

Bacteria counts log (CFU g^{-1})	Control	1.0 g synbiotics kg^{-1}	2.0 g synbiotics kg^{-1}
Total bacteria	5.86±1.94 ^{ab}	5.88±1.2 ^{ab}	6.1±0.79 ^{a}
Lactic acid	5.42±0.14 ^{b}	5.73±0.4 ^{b}	7.13±0.32 ^{a}

Values in a row with different superscripts denote a significant difference (p<0.05).

Table 5: Immune responses of Caspian roach juvenile fed different dietary levels of synbiotics.

	Control	1.0 g synbiotics kg^{-1}	2.0 g synbiotics kg^{-1}
Lysozym (μg mL^{-1})	33.6±3.8 ^{a}	42.6± 4.35 ^{ab}	51.6±3.8 ^{b}
ACH50 (U mL^{-1})	48.86 ±2.51 ^{a}	49.83±8.13 ^{ab}	54.23±6.92 ^{b}
Total Ig (mg mL^{-1})	4.9±0.97 ^{a}	5.5±0.71 ^{a}	7.2±0.8 ^{b}

Values in a row with different superscripts denote a significant difference (p<0.05).

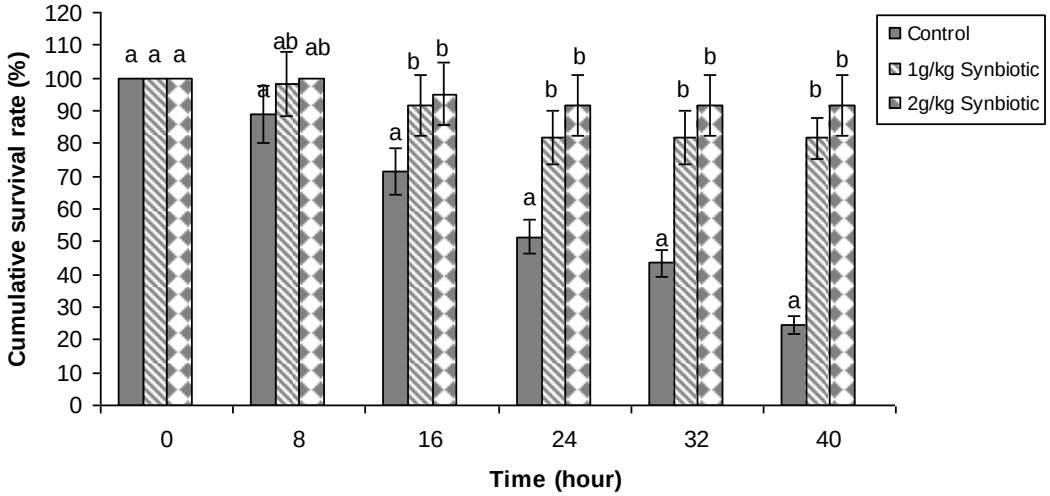

Figure 1: Percent cumulative survival rate of roach fry after salinity stress test; exposure to 13.8 g L^{-1} water salinity for 40 h. Values (means±SD) in bars that do not have same letter are significantly different (p<0.05) (by one way ANOVA).

Discussion

There is some information available to date regarding the interaction between synbiotics and growth performance in animals (Kumprecht and Zobac 1998; Shim 2005; Buteikis *et al.*, 2008; Sahin *et al*., 2008). Shim and his co-workers (2005) reported that a dietary synbiotics fed suckling pig, showed positively improved growth performance parameters. A similar finding was also obtained by Buteikis *et al.* (2008), who presented evidence that dietary synbiotics applied in turkey resulted in reduced mortality. Addition of synbiotics supplement in quail diets improved body weight gain, SGR and reduced FCR (Sahin *et al.*, 2008). In the present experiment, the growth performance, immune response, lactic acid bacteria, salinity resistance and body composition were significantly (p<0.05) improved by supplementing the basal diet with synbiotics. This is in agreement with results of some studies that have revealed the effects of synbiotics in increasing growth performance in fish. For instance, Mehrabi *et al*. (2012), found that in rainbow trout (*Oncorhynchus mykiss*)

fingerlings synbiotics (0.5, 1.0 and 1.5 g kg^{-1} of diet) significantly increased growth performance, survival rate and feeding efficiency parameters compared to the control (Mehrabi *et al.,* 2012). Similarly, application of synbiotics was found to enhance growth performance and survival of Zebrafish (*Danio rerio*) larvae (Nekoubin *et al.,* 2012) and Caspian kutum (*Rutilus frisii*) fry (Talibi Haghighi *et al.,* 2010). Improved growth performance is likely to be brought about by elevated digestive enzyme activities, possible improvements of intestine morphology or via synbiotics fermentation by endogenous gut microbes to produce short chain fatty acids (SCFAs). Moreover, the synbiotics effect might also be potentially influenced by the type of species and the environment. Ye *et al*. (2011) demonstrated that feeding FOS, MOS or *Bacillus clausii* alone, or in various combinations, improved growth performance, feed efficiency and health status of the Japanese flounder (*Paralichthys olivaceus*) which was more pronounced in fish fed the synbiotics than those fed pre- and probiotics alone. Similar synergistic effects were observed in studies with MOS+*Enterococcus faecalis* fed rainbow trout (*Oncorhynchus mykiss*) (Rodriguez- Estrada *et al*., 2009). Synbiotics, the combined application of probiotics and prebiotics, is based on the principle of providing a probiont with a competitive advantage over competing endogenous populations; thus, effectively improving the survival and implantation of the live microbial dietary supplement in the gastrointestinal tract of the host (Gibson and Robefroid, 1995). With the use of synbiotics, it may be possible to produce greater benefits than the application of individual probionts (Merrifield *et al.,* 2010). According to Soleimani *et al*. (2012) Dietary supplementation of FOS improved the innate immune response, stress resistance, digestive enzyme activities and growth performance in Caspian roach (*Rutilus rutilus*) fry. Stimulation of the immune response of fish through dietary supplements is of high interest for commercial aquaculture (Staykov *et al*., 2007). The innate immune system is very important in this regard because aquatic animals are continually vulnerable to numerous opportunistic pathogens and this part of immune response provides the first line of defense for the host (Magnadóttir, 2006). The use of natural immunostimulants is a promising area in aquaculture because they are biodegradable, biocompatible and safe both for the environment and human health (Ortuno *et al.,* 2002). It is clear from the present study that dietary supplementation of synbiotics can modulate the innate immune responses of the Caspian roach. As shown in Table 5, fish fed 2g kg^{-1} synbiotics had significantly greater plasma lysozyme, total immunoglobulin (Ig) and ACH50 compared to those fed the 1g kg^{-1} synbiotics and control diet. Similarly, Ye *et al*. (2011) reported that lysozyme

activity was significantly higher in Japanese flounder (*Paralichthys olivaceus*) fed a synbiotics diet (FOS + *Bacillus clausii*, MOS+ *Bacillus clausii* or FOS + MOS + *Bacillus clausii*) than in fish fed the control diet. Similar to our results, dietary FOS has been reported to stimulate the innate immune responses, such as serum total immunoglobulin and serum lysozyme activity in roach (Soleimani *et al.,* 2012). The immunostimulatory nature of synbiotics may be attributed to stimulation of the growth of beneficial bacteria such as lactic acid bacteria (Zhang *et al.,* 2010). Supplementation with synbiotics influenced the immune system of fish in this study, evidenced by the increased total lactic acid bacteria in the roach gut. Fish fed a diet containing 2g kg^{-1} synbiotics showed significant difference in lactic acid bacteria in the intestinal tract after 8 weeks. This finding is concordant with several studies on the use of probiotics and prebiotics in fish showing that bacteria can abound in the intestinal tract of freshwater fish and stimulate their immune system (Gatesoupe, 1999). Mourino *et al*. (2012) observed that the administration of synbiotics (inulin and *Weissella cibaria*) to the diet of hybrid surubium (*Pseudoplatystoma* sp.) increased growth of lactic acid bacteria, which is in agreement with the observation of this study. Lactic acid bacteria have been considered beneficial residents of the fish's intestinal ecosystem by producing bacteriocins, which inhibit growth of certain fish pathogens and thus positively affect the host's microflora (Ringø *et al.,* 2010). In the present experiment, higher body protein and lipid content in the fish fed the synbiotics supplemented diet implies this fact that, the ingested food was converted more effectively into the structural protein and lipid subsequently resulted in more muscle as it is a desirable aspect in fish farming. However, application of synbiotics in roach diet did not have any significant effect on ash content. Although supplementation with synbiotics in rainbow trout (Mehrabi *et al.,* 2012) and Caspian kutum fry (Talibi Haghighi *et al.,* 2010), specifically increased the carcass protein, there was no significant difference in lipid and ash content, among experimental treatments. Ye *et al*. (2011) observed higher value of body protein deposition in Japanese flounder (*Paralichthys olivaceus*) supplemented with FOS+*Bacillus clausii* and FOS+MOS+*B.clausii* compared to diets with FOS or FOS + MOS only. The addition of prebiotics to a *B. clausii* supplemented diet did not further decrease body lipid deposition. Salinity stress tests have often been used as a final indicator of fish quality after nutrition trials (Taoka *et al.,* 2006); our results indicated that dietary synbiotics significantly increased Caspian roach resistance to salinity stress. Fish fed dietary synbiotics showed remarkable survival compared to the control group. The improved resistance to salinity stress in the present study was similar to that reported for cobia (*Rachycentron*

canadum, Salze *et al.*, 2008), white sea bream larvae (*Diplodus sargus*, Dimitroglou *et al.*, 2010) and Kutum fry (*Rutilus frisii* ; Akrami *et al.*, 2010). Soleimani *et al*. (2012) reported that dietary FOS significantly increased resistance of roach fry to salinity stress challenges. It has been suggested that greater resistance to salinity stress challenges might be due to improved microvilli alignment, as has been reported in MOS fed fish (Dimitroglou *et al.*, 2010), which may increase the protective function of the mucin barrier and affect ion regulation (Salze *et al.*, 2008); however, future studies are required to test this speculative hypothesis. This study corroborates the functionality of synbiotics in the diet of roach which positively affects growth performance, immune response, beneficial intestinal microbiota and stress resistance.

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