

Effects of dietary fructo-oligosaccharide supplementation on the growth performance, haemato-immunological parameters, gut microbiota and stress resistance of common carp (*Cyprinus carpio*) fry

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Abstract

The present study was conducted to investigate the effects of dietary fructo-oligosaccharide (FOS) (0, 1, 2 and 3%) supplementation on the growth performance, haemato-immunological parameters, cultivable autochthonous (non-adherent) intestinal microbiota and stress resistance of common carp (*Cyprinus carpio*) fry (3–23 (SEM 0.14) g). These parameters were measured after feeding the carp fry with the experimental diets for 7 weeks. Dietary FOS supplementation had no significant effects on the growth performance and food intake of carp fry compared with the control treatment. It also had no significant effects on the following haematological parameters: erythrocyte count; leucocyte counts (WBC); haematocrit; Hb; mean corpuscular volume; mean corpuscular Hb content; mean corpuscular Hb concentration. However, WBC and respiratory burst activity were significantly affected by dietary FOS supplementation. Evaluation of the cultivable autochthonous intestinal microbiota revealed a significant increase in the levels of total viable heterotrophic aerobic bacteria and lactic acid bacteria in fish fed diets supplemented with 2 and 3% FOS. Furthermore, dietary FOS supplementation significantly increased the survival rate and stress resistance of carp fry compared with the control treatment. These results encourage conducting further research on the administration of FOS and other prebiotics in carp fry studies.

Key words: *Cyprinus carpio*: Fructo-oligosaccharide: Growth performance: Haemato-immunological parameters: Gut microbiota: Stress resistance

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of health-promoting bacteria in the intestinal tract⁽¹⁾. Fructo-oligosaccharide (FOS or oligofructose) is a fructan with a degree of polymerisation (2–20) that is obtained by enzymatic hydrolysis of inulin⁽²⁾. FOS is present in a number of common foods such as garlic, onion, artichoke and asparagus⁽³⁾, and dietary FOS has received great attention as a prebiotic for aquatic animals^(4,5). Despite some contradictory results^(5–7), beneficial effects of dietary FOS supplementation on growth performance and survival^(8–14), gut microbiota^(14,15), immune response^(12,16,17) and digestive enzyme activity^(12,13) have been reported in several fish species.

Common carp (*Cyprinus carpio*) is a widespread freshwater fish and is the main aquaculture species in many European

and Asian countries⁽¹⁸⁾. Considering the progress in culture methodologies, specifically elevation of the production by intensification of culture, which causes deterioration of water quality, stress to cultured organisms and outbreaks of infectious diseases, elevation of fish resistance and improvement of fish health status through dietary supplements are of importance in commercial carp aquaculture, especially in sensitive periods (i.e. larval and fry culture)⁽¹⁹⁾. However, despite the well-documented beneficial effects of prebiotics in finfish^(4,5), to our knowledge, there is no available information on the effects of dietary FOS supplementation in carp fry.

Therefore, the aim of the present study was to investigate the effects of dietary FOS supplementation on the growth performance, haemato-immunological parameters, culturable gut microbiota and stress resistance of common carp fry.

Abbreviations: FOS, fructo-oligosaccharide; Ht, haematocrit; LAB, lactic acid bacteria; WBC, leucocyte counts.

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Experimental methods

Prebiotic

FOS used in the present study was kindly provided by Orafiti (Raffinerie Tirléfontoise). According to the manufacturer, the chemical composition of the product was 98.2% DM and 1.8% crude ash.

Experimental diets

Experimental diets were prepared by supplementing a basal formulated diet with different levels (0% (control), 1%, 2% and 3%) of FOS (Table 1). The ingredients were blended thoroughly in a mixer and pelleted using a meat grinder equipped with a 2 mm die. The pelleted diets were air-dried and stored in plastic bags at 4°C until further use.

Fish culture and feeding trial

A total of 480 common carp fry (3.23 (SEM 0.14) g) obtained from the Siowal Caspian sea teleost fish propagation and cultivation centre (Golestan province, Iran) were acclimatised to the experimental conditions for 1 week. Thereafter, fish were randomly allocated to twelve 500-litre tanks (forty fish per tank and triplicate tanks per treatment). Water temperature, dissolved oxygen content and pH were monitored daily and maintained at 25.74 ± 1.79°C, 7.13 (SEM 0.23) mg/l and 7.11 (SEM 0.18), respectively. Each tank was aerated continuously through an airstone connected to a central air compressor.

Table 1. Formulation (%) and proximate composition of the experimental diets

	Control	1%	2%	3%
Ingredients				
Fishmeal	40.0	39.8	39.7	39.6
Wheat flour	21.0	20.8	20.5	20.2
Soyabean meal	13.5	13.4	13.2	13.1
Gluten	5.5	5.4	5.2	5.1
Soyabean oil	6.0	5.9	5.8	5.6
Fish oil	6.0	5.9	5.8	5.6
Mineral premix*	3.0	3.0	3.0	3.0
Vitamin premix*	2.0	2.0	2.0	2.0
Binder†	2.0	2.0	2.0	2.0
Antifungal agent‡	0.5	0.5	0.5	0.5
Antioxidant§	0.5	0.5	0.5	0.5
FOS	0.0	1.0	2.0	3.0
Proximate analysis (% DM basis)				
DM	89.50	89.41	89.64	89.60
Crude protein	38.22	38.30	38.21	38.29
Crude lipid	10.24	10.20	10.28	10.21
Ash	3.45	3.50	3.40	3.60
Fibre	11.20	11.30	11.20	11.40
NFE¶	26.39	26.11	26.55	26.10
Energy (MJ/kg)**	17.55	17.50	17.59	17.50

FOS, fructo-oligosaccharide; NFE, nitrogen-free extracts.

* Premix detailed by Hoseinifar *et al.*⁽¹⁵⁾.

† Amet binder™; Mehr Taban-e-Yazd.

‡ ToxiBan antifungal agent (Vet-A-Mix; Shenandoah).

§ Butylated hydroxytoluene (Merck).

|| FOS; Raffinerie Tirléfontoise (FOS content > 93%).

¶ NFE = DM - (crude protein + crude lipid + ash + fibre).

** Gross energy (MJ/kg) calculated according to 23.6 kJ/g for protein, 39.5 kJ/g for lipid and 17.0 kJ/g for NFE.

During the rearing experiment (7 weeks), fish were hand-fed to apparent satiation twice a day (09.00 and 15.00 hours). Uneaten feed was collected 1 h after feeding and dried at 60°C⁽²⁰⁾.

Determination of growth performance and survival rate

The growth performance parameters including weight gain (%), specific growth rates (SGR), condition factor (CF), feed conversion ratio (FCR) and survival rate were calculated according to the following formulae:

$$\text{Weight gain (\%)} = W_2 - W_1,$$

$$\text{SGR} = 100 (\ln W_2 - \ln W_1) / T,$$

$$\text{CF} = 100 \times (\text{body weight (g)}) / (\text{body length (cm)})^3,$$

$$\text{FCR} = \text{FO} / \text{WG},$$

where W_1 is the initial weight (g), W_2 is the final weight (g), T is time (d), FO is the feed provided (g) and WG is the weight gain (g),

$$\text{Survival rate} = (N_f / N_0) \times 100,$$

where N_0 is the initial number of fish and N_f is the final number of fish.

Haemato-immunological analysis

At the end of the feeding trial, after 7 weeks, blood was collected from the caudal vein of carp fry (three fish per tank) for haemato-immunological analysis. Whole blood was suspended in the Natt & Herrick diluent⁽²¹⁾ for performing erythrocyte counts and total leucocyte counts (WBC) using a haemocytometer. Haematocrit (Ht) was determined using the micro-Ht method as described by Brown⁽²²⁾, and Ht values are reported as packed cell volume percentage. Hb levels were estimated using Sahli's method⁽²³⁾. Differential WBC (neutrophils, lymphocytes and monocytes) were performed using May-Grunwald-Giemsa-stained blood smears.

The respiratory burst activity was measured in triplicate by chemiluminescent assay (measurement of light emission) based on the modified protocol of Mathews *et al.*⁽²⁴⁾ as described by Khoshbavar-Rostami *et al.*⁽²⁵⁾ using an automated system for chemiluminescent analysis (Luminoskan Ascent T392; Thermo Fisher Scientific, Inc.).

Quantification of autochthonous intestinal microbiota

Samples of the entire intestine of fifteen fish were used for quantifying total viable autochthonous heterotrophic aerobic bacteria and lactic acid bacteria (LAB) at the start and end of the experiment as described previously⁽¹⁵⁾. Samples were processed on an individual basis and were not pooled. Dilutions (100 µl) were spread in triplicate onto Plate Count Agar medium (Merck) and de Man, Rogosa and Sharpe medium (Merck) for quantifying total viable heterotrophic aerobic bacteria and LAB, respectively. The plates were incubated at room temperature (25°C) for 5 d and colony-forming units (CFU)/g

Table 2. Growth performance parameters and survival rate of common carp fry fed diets supplemented with varying levels of fructo-oligosaccharide for 7 weeks

(Mean values with their standard errors)

	Control		1%		2%		3%	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial weight (g)	3.35	0.15	3.30	0.11	3.20	0.20	3.10	0.18
Final weight (g)*	9.79	0.29	9.35	0.62	9.57	0.25	10.10	0.41
Weight gain (%)†	192.20	6.03	178.68	14.46	199.61	13.65	225.22	18.57
SGR	1.91	0.11	1.85	0.16	1.95	0.12	2.05	0.15
CF	1.29	0.08	1.30	0.04	1.30	0.07	1.35	0.09
FCR‡	3.48	0.11	3.71	0.45	3.51	0.26	3.21	0.31
Survival (%)§	61.60 ^a	7.07	83.30 ^{a,b}	9.37	78.40 ^{a,b}	11.73	98.30 ^b	2.40

SGR, specific growth rates; CF, condition factor; FCR, feed conversion ratio.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P > 0.05$).

* $\alpha = 0.05$; $\beta = 0.17$.

† $\alpha = 0.05$; $\beta = 0.15$.

‡ $\alpha = 0.05$; $\beta = 0.11$.

§ $\alpha = 0.05$; $\beta = 0.16$.

were calculated from statistically viable plates (i.e. plates containing thirty to 300 colonies)⁽²⁶⁾.

Salinity stress challenge test

Salinity stress challenge test was carried out at the end of the feeding trial according to the method of Hoseinifar *et al.*⁽²⁰⁾. A total of fifteen fish were sampled from each tank and subjected to salinity stress challenge. The fry were exposed to 15 g/l salinity in triplicate (fifteen fish per tank). The survival rates of fish were calculated 72 h after the challenge.

Statistical analyses

Statistical analyses were conducted using SPSS statistical package version 17.0 (SPSS, Inc.). Each tank served as the unit of analysis. All data were subjected to a one-way ANOVA to test the effects of dietary FOS supplementation on the haemato-immunological parameters, growth performance, culturable gut microbiota and stress resistance of carp fry (*C. carpio*). Duncan's multiple range test was carried out when the differences were significant⁽²⁷⁾. P values < 0.05 were considered to be statistically significant. Data are presented as mean values with their standard errors.

Results

The growth performance parameters of carp fry fed experimental diets containing different levels of FOS are summarised in Table 2. There were no significant differences in the initial weight of the treatment groups ($P > 0.05$). Similarly, at the end of the feeding trial (7 weeks), no significant differences were observed in the final weights, WG, SGR, CF and FCR of common carp fry fed the FOS-supplemented diets and the control diet ($P > 0.05$; Table 2).

Calculation of survival rates at the end of the experiment revealed that dietary FOS supplementation significantly increased the survival rate of carp fry ($P < 0.05$; Table 2). Carp fry fed the 3% FOS diet exhibited the highest survival rate (98.3 (SEM 2.4)%).

The effects of dietary FOS supplementation on the haemato-immunological parameters of carp fry are summarised in Tables 3 and 4. Statistical analyses revealed that erythrocyte count, mean corpuscular volume, mean corpuscular Hb content, mean corpuscular Hb concentration, Hb, Ht and differential leucocyte counts were not significantly affected by dietary FOS supplementation ($P > 0.05$; Table 3). WBC were significantly affected by 3% FOS supplementation ($P < 0.05$; Table 4), while no effect of FOS supplementation was observed on lymphocyte, neutrophil and monocyte counts.

Table 3. Effects of dietary fructo-oligosaccharide (FOS) supplementation on the haematological parameters of common carp fry (Mean values with their standard errors)

Groups	Erythrocyte count ($\times 10^6$ per μ l)*		Hb (mmol/l)†		Haematocrit (%)‡		MCV (fl)		MCH (pg)		MCHC (mmol/l)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	1.49	0.26	3.41	0.25	23.00	3.60	155.07	19.60	34.16	4.39	13.69	0.92
1% FOS	1.62	0.20	3.08	0.31	23.66	3.51	145.63	13.90	30.54	6.09	13.02	2.44
2% FOS	1.55	0.11	3.28	0.25	23.60	3.78	152.30	19.87	34.15	4.20	14.08	1.98
3% FOS	1.65	0.17	3.22	0.12	25.00	2.64	151.29	13.66	31.88	4.19	13.08	1.57

MCV, mean corpuscular volume; MCH, mean corpuscular Hb content; MCHC, mean corpuscular Hb concentration.

* $\alpha = 0.05$; $\beta = 0.10$.

† $\alpha = 0.05$; $\beta = 0.14$.

‡ $\alpha = 0.05$; $\beta = 0.13$.

Table 4. Differential leucocyte counts of common carp fry fed diets supplemented with different levels of fructo-oligosaccharide (FOS) for 7 weeks

(Mean values with their standard errors)

Groups	Leucocyte count ($\times 10^3$ per μl) [*]		Lymphocytes (%)		Neutrophils (%)		Monocytes (%)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	32.66 ^a	2.08	92.33 ^a	2.08	5.66 ^a	0.57	2.01 ^a	0.32
1% FOS	36.26 ^{a,b}	3.55	93.03 ^a	1.25	5.36 ^a	1.52	1.61 ^a	0.70
2% FOS	37.33 ^{a,b}	2.81	92.96 ^a	1.15	5.33 ^a	1.15	1.71 ^a	0.41
3% FOS	39.30 ^b	1.15	92.60 ^a	2.64	5.60 ^a	1.63	1.80 ^a	0.90

^{a,b} Mean values within a column with unlike superscript letters were significantly different ($P > 0.05$).

^{*} $\alpha = 0.05$; $\beta = 0.12$.

Dietary FOS supplementation significantly increased the respiratory burst activity compared with the control treatment ($P < 0.05$; Fig. 1).

The levels of total heterotrophic autochthonous gut bacteria in the control group (4.05 (SEM 0.07) CFU/g) were significantly lower than those in carp fry fed the FOS-supplemented diets ($P < 0.05$; Fig. 2). Total autochthonous intestinal heterotrophic bacteria levels were significantly higher in the 2 and 3% FOS groups (5.68 (SEM 0.10) and 5.57 (SEM 0.24) CFU/g, respectively). However, no cultivable LAB were detected in the control and 1% FOS groups; the 2 and 3% FOS groups had 1.50 (SEM 0.04) and 2.42 (SEM 0.06) CFU/g of autochthonous LAB, respectively (Fig. 2).

The results of the salinity challenge test are shown in Fig. 3. Low survival rates were observed 72 h after the challenge in the control group, while carp fry fed the FOS-supplemented diets had significantly improved the survival rate ($P < 0.05$; Fig. 3). The survival rates of the 1, 2 and 3% FOS groups were 53.4 (SEM 3.9), 57.7 (SEM 4.52) and 59.6 (SEM 8.16)%, respectively.

Discussion

To our knowledge, this is the first study to investigate the effects of dietary FOS supplementation on the growth performance, haemato-immunological parameters, cultivable gut microbiota and stress resistance of common carp fry. The results of the present study showed that 1, 2 or 3% FOS supplementation had no significant effects on the growth performance of carp fry. These results are not in accordance with those of previous studies in blunt snout bream (*Megalobrama amblycephala*) fingerlings⁽¹³⁾, stellate sturgeon (*Acipenser stellatus*) juveniles⁽¹⁴⁾, farmed rainbow trout (*Oncorhynchus mykiss*)⁽²⁸⁾ and red drum (*Sciaenops ocellatus*)⁽¹⁷⁾. However, Hoseinifar *et al.*⁽¹⁵⁾ and Grisdale-Helland *et al.*⁽²⁹⁾ reported that dietary FOS supplementation had no significant effects on the growth performance of beluga (*Huso huso*) juveniles and Atlantic salmon (*Salmo salar*). The differences observed in the results of these studies might be due to the different methods of prebiotic administration, dosage levels, different intestinal morphology, gut microbiota and life stages⁽¹¹⁾.

Dietary FOS supplementation significantly increased the survival rate of common carp fry compared with the

control treatment. Similarly, improved survival of cobia (*Rachycentron canadum*) larvae⁽³⁰⁾, rainbow trout⁽³¹⁾ and beluga juveniles⁽¹⁵⁾ has been observed upon prebiotic administration. Akrami *et al.*⁽¹⁴⁾ reported that the survival rate of stellate sturgeon juveniles was not affected by 1 or 2% dietary FOS supplementation. The improved survival rate of prebiotic-fed cultured organisms might be due to improved general health or immune status, as reported previously⁽¹²⁾, but further investigations are needed to clarify the underlying mechanisms.

Haemato-immunological parameters are valuable tools for the determination of cultured organism health status⁽³²⁾. These parameters have been widely used in prebiotic studies for the evaluation of changes in health status as a result of prebiotic administration^(4,6). The results of the present study showed that dietary FOS supplementation had no significant effects on haematological parameters including erythrocyte count, mean corpuscular volume, mean corpuscular Hb content, mean corpuscular Hb concentration, Hb, Ht and differential leucocyte counts, but the significantly higher WBC values in the prebiotic-fed fish are in accordance with the results of the study carried out by Akrami *et al.*⁽¹⁴⁾. Similar elevation of WBC has been observed in beluga (*H. huso*) juveniles fed FOS⁽¹⁶⁾. In the present study, dietary FOS supplementation increased the respiratory burst activity compared with the control treatment.

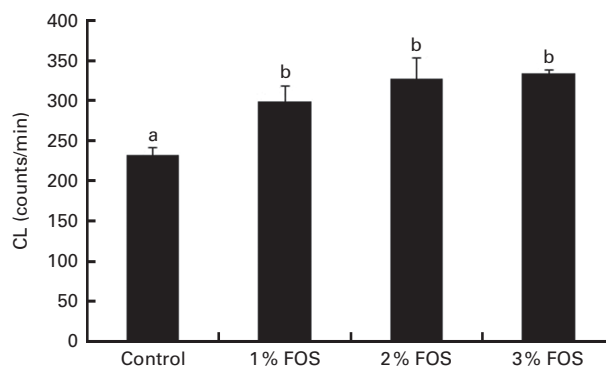


Fig. 1. Respiratory burst activity (chemiluminescence (CL) response; light emission count/min) ($\alpha = 0.05$; $\beta = 0.17$) of carp fry fed diets supplemented with different levels (0% (control), 1%, 2% and 3%) of fructo-oligosaccharide (FOS) for 7 weeks. Values are means, with their standard errors represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

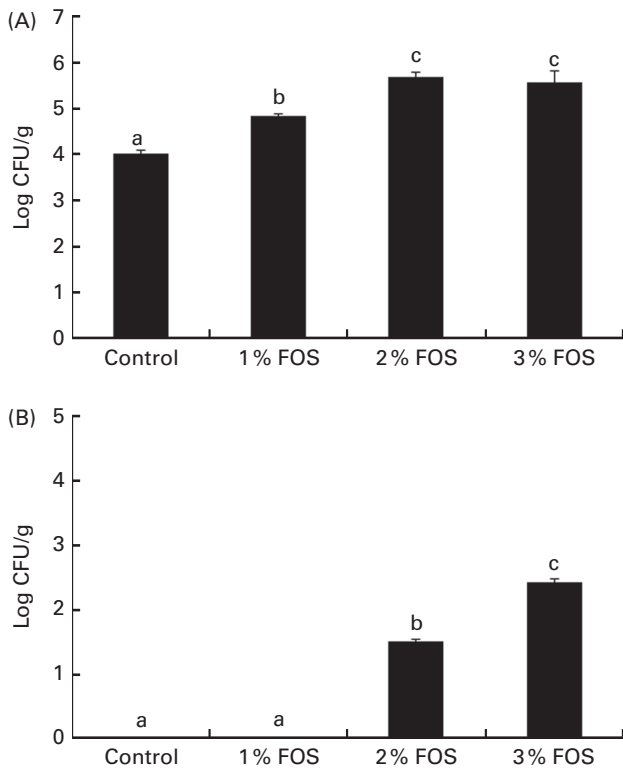


Fig. 2. Levels of total culturable autochthonous bacteria (A) ($\alpha = 0.05$; $\beta = 0.14$) and autochthonous lactic acid bacteria (B) ($\alpha = 0.05$; $\beta = 0.12$) (log colony-forming units (CFU)/g intestine) of carp fry fed diets supplemented with different levels (0% (control), 1%, 2% and 3%) of fructo-oligosaccharide (FOS) for 7 weeks. Values are means, with their standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$).

Similarly, previous studies on the effects of synbiotic administration in large yellow croaker (*Larimichthys crocea*)⁽³³⁾, gilthead sea bream (*Sparus aurata* L.)⁽³⁴⁾ and koi (*C. carpio koi*)⁽³⁵⁾ revealed a significantly higher respiratory burst activity. In contrast to these results, Akrami *et al.*⁽¹⁴⁾ reported that FOS supplementation had no effect on the respiratory burst activity of stellate sturgeon. Respiratory bursts, produced by phagocytes to attack invasive pathogens during phagocytosis, have been widely used for the evaluation of defence ability against pathogens⁽³⁶⁾. Elevation of WBC and respiratory burst activity observed in the present study is probably caused by the stimulation of carp immune response due to FOS supplementation.

The intestinal microbiota of fish is a complex and dynamic ecosystem containing a diverse community of micro-organisms^(37,38), and genetic, nutritional and environmental factors are the main factors that modulate the intestinal microbiota⁽³⁹⁾. Although LAB are considered a minor constitute of the intestinal microbiota of several fish species, they have been considered to be beneficial components of the fish intestine⁽⁴⁰⁾. Modulation of the intestinal microbiota of fish towards beneficial communities (i.e. LAB) has been studied through the administration of probiotics, prebiotics and synbiotics^(4,41). The levels of cultivable autochthonous intestinal microbiota observed in the present study revealed that dietary FOS

supplementation significantly increased the levels of total autochthonous intestinal heterotrophic bacteria. In addition, the levels of LAB were significantly elevated in common carp fed the 2 and 3% FOS diets. However, further studies using more sensitive molecular methods such as denaturing gradient gel electrophoresis (DGGE) and 16S rRNA sequencing are required to identify the LAB species to confirm their beneficial effects in common carp fry. Similar to the present results, FOS has been reported to increase the levels of total cultivable autochthonous intestinal heterotrophic bacteria and LAB in stellate sturgeon⁽¹⁴⁾ and beluga juveniles⁽¹⁵⁾. The results of the present study confirmed that modulation of the intestinal microbiota of carp can be achieved through dietary FOS supplementation, but further investigations are needed.

Resistance during exposure to abrupt changes in water salinity (salinity stress test) has been considered an important indicator of fry quality in nutrition trials, especially in prebiotic studies^(20,30,42,43). The results of the present study revealed that dietary FOS supplementation significantly increased fish resistance in the salinity stress challenge test compared with the control treatment. Similarly, Soleimani *et al.*⁽¹²⁾ reported that Caspian roach (*Rutilus rutilus*) fry fed 3% dietary FOS exhibited significantly higher resistance in salinity stress challenge test, and this is in accordance with previous reports on Caspian roach fed galacto-oligosaccharide⁽²⁰⁾ and cobia⁽³⁰⁾ and white sea bream larvae (*Diplodus sargus* L.)⁽⁴⁴⁾ fed mannan oligosaccharide. Although not confirmed in the present study, the main reason for the prebiotic effects on salinity stress resistance has been suggested to be the improved alignment of microvilli⁽⁴⁴⁾, and evaluation of microvillus alignment will be included in further prebiotic studies.

In conclusion, dietary FOS supplementation had no significant effects on the growth performance and haematological parameters of carp fry, but significantly increased WBC and respiratory burst activity and modulated cultivable autochthonous gut microbiota levels and stress resistance. The positive results obtained encourage conducting further research on the

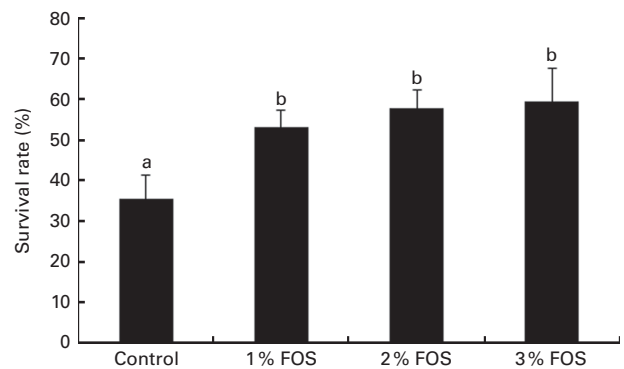


Fig. 3. Survival rate of carp fry fed diets supplemented with different levels (0% (control), 1%, 2% and 3%) of fructo-oligosaccharide (FOS) for 7 weeks after exposure to salinity stress challenge (exposure to 15 g/l water salinity for 72 h) ($\alpha = 0.05$; $\beta = 0.19$). Values are means, with their standard errors represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

administration of FOS and other prebiotics in common carp fry studies. Determination of the mechanisms of action and optimal inclusion levels is a topic that merits further research.

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The authors' contributions are as follows: S. H. H. contributed to the study design, conducted the study and analysed the samples, interpreted the data and wrote a draft of the manuscript; N. S. contributed to the conduction of the study; E. R. contributed to the interpretation of the results and improved the manuscript by critical comments.

None of the authors has any conflicts of interest to declare.

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