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Effect of dietary prebiotic fructooligosaccharide on histology, digestive enzyme activity, biochemical and immunological parameters of Asian seabass (*Lates calcarifer*) fingerlings

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ABSTRACT

The aim of the study was to evaluate the effect of fructooligosaccharide (FOS) on histology, digestive enzyme activity,biochemical and immunological parameters of Asian seabass fingerlings with an average weight of 11.6±0.05 g. Experimental diets containing 0 (control), 0.25, 0.5, 0.75 and 1% FOS were prepared and the feeding experiment was carried out for a duration of 45 days. Analysis of biochemical parameters in the serum of post-fed animals revealed that glucose, urea, cholesterol, triglyceride, albumin, albumin globulin ratio (A/G ratio), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) showed significant differences ($p<0.05$) among the treatment diets. Similarly, serum immunological parameters such as alternative complement pathway and nitrobluetetrazolium assay showed significantly ($p<0.05$) higher activities in fishes fed with diets containing 0.75 and 1% FOS. Histological observation of post-fed experimental animals revealed increase in length and density of microvilli in the intestine and glycogen deposition in liver in FOS supplemented diets. The digestive enzyme activities were significantly ($p<0.05$) elevated with increasing levels of dietary FOS. The results indicated that supplementation of FOS at 1% modulates the intestinal and liver histology, digestive enzyme activities, biochemical and immunological parameters in Asian seabass.

Keywords: Asian seabass, Biochemical parameters, Fructooligosaccharide, Histology, Intestinal enzyme activities

Introduction

Asian seabass *Lates calcarifer* (Bloch, 1790) popularly known as bhetki or barramundi is a commercially valuable, carnivorous and euryhaline fish species being widely cultured in Australia and South-east Asia under intensive system in fresh, brackish and marine water resources (Ali *et al*., 2018). In India seabass is considered as a potential alternate candidate species because of its high market demand and excellent growth potential for coastal aquaculture (FAO, 2016). Aquacultured fishes reared in intensive systems are exposed to stressful conditions and are easily susceptible to diseases leading to huge economic losses (Hoseinifar *et al*., 2016). Conventionally, antibiotics and chemicals are used as therapeutic agents to control disease in fish (Cabello, 2006). Recent studies illustrated that various feed additives used in aquaculture enhance the innate immunity, disease resistance, gut microbial community, growth performance and survival of various aquacultured organisms (Wang*et al*., 2017). Among the various feed additives used in aquaculture, prebiotics like fructooligosaccharides, mannanoligosaccahrides, xylooligosaccharides and inulin have recently attracted extensive attention (Ali *et al*., 2017a).

Prebiotics are a group of functional, non-digestible dietary supplements which beneficially affect the host by selectively stimulating and activating the metabolism of health promoting intestinal commensal microflora (Wang *et al*., 2017). As an alternative strategy to antibiotics, prebiotics are often used as prophylactic agents rather than curative (Talpur *et al*.,2014). Fructooligosaccharide is a short chain oligosaccharide that is composed of a glucose molecule liked to 2-4 fructose molecules. FOS are not significantly digested in small intestine but fermented in the large intestine to selectively stimulate growth performance and survival of some health promoting bacteria such as lactobacilli and bifidobacteiral species (Wu *et al*., 2013). Supplementation of dietary prebiotics has been shown to improve the health and growth performance of various aquatic species (Safari *et al*., 2014; Eshaghzadeh *et al*., 2015; Guerreiro *et al*., 2015; Ali *et al*., 2016, 2017a, b, c, 2018; Morshedi *et al*., 2018). Therefore the current study was carried out to investigate the effect of dietary fructooligosaccharide supplementation on intestinal and liver histology, digestive enzyme activity, biochemical and immunological parameters of *L. calcarifer* fingerlings.

Materials and methods

Diet preparation

The experimental diets were formulated based on the nutrient requirements of Asian seabass fingerlings (Table 1). Diets were prepared to contain 40% protein and 9% lipid (Table 2) by supplementing FOS at different levels (0, 0.25, 0.5, 0.75 and 1.0%) following Ali *et al.* (2018). The proximate composition of formulated diets was analysed as per the standard procedures of AOAC (2012).

Experimental design and feeding trial

Healthy Asian seabass fingerlings (average body weight 11.6±0.03 g) procured from a farm near Kuluthumedu, Chennai, were randomly stocked in 15 oval fiberglass reinforced plastic (FRP) tanks (1000 l) at the rate of 20 fishes per tank (3 tanks per treatment). During the experimental period, the water quality parameters such as salinity (30-36‰), temperature (27-29ºC), dissolved oxygen (6.2-7.5 mg l^{-1}) and pH (7.0-8.2) in the experimental tanks were recorded following conventional methods (APHA, 1998). Continious aeration was provided with air diffuser stones. During expermiental period the fishes were hand fed twice a day. (09.00 and 15.00 hrs). The faecal matter was removed daily before feeding.

Blood sampling

At the end of the feeding experiment, fishes were starved for 24 h before sampling. Four fish from each replicate were randomly collected and anaesthetised (2-phenoxyethanol at 0.5 ml l^{-1}) for blood sample collection (Ali *et al*., 2018).

Serum biochemical parameters

Glucose, urea, cholesterol, triglycerides, total protein, albumin and globulin were analysed according to Ali *et al*. (2018) and the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were analysed

Table 1. Formulation of experimental diets supplemented with different levels of FOS

Experimental diets	FOS 0%	FOS 0.25%	FOS 0.5%	FOS 0.75%	FOS 1%
Ingredients (%)					
Fish meal[1]	39	39	39	39	39
Soybean meal	27	27	27	27	27
Wheat	13	13	13	13	13
Rice	6	6	6	6	6
Maize	4	4	4	4	4
Fish oil[1]	4	4	4	4	4
Lecithin	2	2	2	2	2
Vitamin and mineral mix[2]	3	3	3	3	3
Binder[3]	1	1	1	1	1
Cellulose	1	0.75	0.5	0.25	0
FOS[4]	0	0.25	0.5	0.75	1

[1]Sardine fishmeal and fish oil. (Bismi fisheries, Mayiladuthurai, Tamil Nadu, India)
[2]Commercially sourced premix (Sarabhai Zydus Animal Health Ltd., Vadodara, Gujarat, India) which contains (per kg): Vitamin A - 2000000 IU, Vitamin D - 40000 IU, Vitamin E - 300 U, Vitamin K - 450 mg, Riboflavin - 800 mg, Panthothenic acid - 1 g, Nicotinamide - 4 g, Vitamin B12 - 2.4 mg, Choline chloride - 60 g, Ca - 300 g, Mg - 11 g, I - 400 mg, Fe - 3 g, Zn - 6g, Cu - 800 mg, Co - 180 mg
[3]Pegabind, Bentoli Agri nutrition Asia pvt Ltd, Singapore.
[4]FOS (Himedia, Mumbai, India).

Table 2. Proximate composition (% dry matter basis) of experimental diets containing varying levels of FOS (n=3)

Parameters	FOS 0%	FOS 0.25%	FOS 0.5%	FOS 0.75%	FOS 1%
Moisture	8.75	8.26	8.44	8.20	8.24
Crude protein	40.32	40.37	40.36	40.32	40.36
Crude lipid	8.83	8.82	8.85	8.81	8.82
Crude fiber	2.06	2.12	2.27	2.12	2.12
Total ash	14.18	14.01	14.47	14.12	14.0
NFE	25.86	26.42	25.61	26.43	26.46

following Reitman and Frankel (1957) and Borges *et al*. (2004) respectively. Lactate dehydrogenase (LDH) was assayed as per Anon. (1984).

Serum immunological parameters

Alternative complement pathway activity was performed as described by Sunyer and Tort (1995). Nitrobluetetrazolium assay (NBT) was performed following the standard procedures of Anderson and Siwicki (1995).

Intestine digestive enzyme activity

On termination of the feeding trail, 3 fish from each treatment group were taken for enzymatic analysis (Yanbo and Zirong, 2006). Total protease activity was assayed by the casein hydrolysis method of Hidalgo *et al*. (1999). Amylase activity was determined according to Bernfeld (1955) using soluble starch as substrate, and the maltose released by hydrolysis of starch was measured by reaction with dinitrosalicylic acid reagent. Lipase activity was assayed according to the method of Borlongan (1990) following the release of *p*-nitrophenol from *p*-nitrophenylpalmitate (*p*NPP) and the specific activity was expressed.

Intestine and liver histology

On termination of the feeding experiment, intestine and liver samples from 3 fish per tank were taken for histological analysis. Samples were fixed in phosphate buffered formalin, dehydrated in graded levels of alcohol, cleared in xylene, embeded in paraffin wax, sectioned and stained with Haematoxylin and Eosin (H&E) following the standard procedure of Roberts (2001).

Statistical analysis

Data were subjected to statistical analysis using one-way ANOVA and the means were compared using Duncan's multiple range test. Mean values were considered significantly different at $p<0.05$. Statistical analysis was performed with SPSS statistical package ver. 17.0 (SPSS, Chicago, USA)

Results

Serum biochemical parameters of Asian seabass fingerlings fed with different levels of FOS supplemented diets is shown in Table 3. Results showed that glucose, urea, cholesterol, triglyceride, albumin, globulin, albumin globulin ratio (A/G ratio), AST, ALT, ALP and LDH showed significant differences ($p<0.05$) between control and FOS supplemented diets. No significant differences ($p>0.05$) were observed for total protein and globulin among different treatments.

Serum immunological parameters of Asian seabass fingerlings fed with different levels of FOS supplemented diets are shown in Fig. 1 and 2. Alternate complement activity and nitroblue tetrazolium assay showed significant ($p<0.05$) difference between control and FOS supplemented diets. Fish fed with 1% FOS supplemented diet showed highest ACP and neutrophil oxidative radical production activity compared to rest of the treatments.

Digestive enzyme activities of seabass fed with FOS supplemented diets (protease, amylase and lipase) are shown in Fig. 3a, b and c. Results showed that significantly higher ($p<0.05$) activity of protease and lipase was observed in 1% FOS supplemented group compared to rest of the treatments. Fish fed diet supplemented with 0.75% FOS exhibited the highest amylase activity compared to other treatments.

Histological analysis showed that the intestine of seabss fingerlings fed with control diet appeared normal (Fig. 4a). The intestine showed normal features with the villi appearing straight without any side branches. In the FOS supplemented groups, histological changes were evident by the increase in the intestinal villi side

Table 3. Biochemical parameters of Asian seabass fingerlings fed different levels of fructooligosaccharide (FOS)

Parameters	FOS 0%	FOS 0.25%	FOS 0.5%	FOS 0.75%	FOS 1%
Glucose (mg dl^{-1})	$53.66^{a}\pm2.83$	$74.93^{b}\pm4.50$	$78.70^{b}\pm4.56$	$77.60^{b}\pm8.81$	$92.53^{c}\pm4.96$
Urea (mg dl^{-1})	$37.43^{c}\pm5.21$	$22.76^{b}\pm4.06$	$18.50^{ab}\pm6.30$	$11.90^{a}\pm2.21$	$21.26^{b}\pm5.70$
Cholesterol (mg dl^{-1})	$186.6^{cd}\pm9.45$	$195.91^{d}\pm7.12$	$178.20^{c}\pm9.80$	$122.23^{a}\pm10.1$	$154.16^{b}\pm3.18$
Triglyceride (mg dl^{-1})	$264.09^{c}\pm9.67$	$290.43^{d}\pm7.72$	$252.90^{c}\pm9.85$	$172.06^{a}\pm9.42$	$194.40^{b}\pm4.91$
Total Protein (g dl^{-1})	2.94 ± 0.84	3.92 ± 0.80	3.42 ± 0.29	3.35 ± 0.76	3.26 ± 0.60
Albumin (g dl^{-1})	$0.81^{a}\pm0.07$	$1.15^{d}\pm0.03$	$1.53^{e}\pm0.02$	$1.03^{c}\pm0.06$	$0.96^{b}\pm0.02$
Globulin (g dl^{-1})*	2.04 ± 0.03	2.67 ± 0.80	1.86 ± 0.30	2.32 ± 0.76	1.99 ± 0.02
A/G Ratio	$0.39^{a}\pm0.03$	$0.40^{a}\pm0.10$	$0.82^{b}\pm0.14$	$0.47^{a}\pm0.15$	$0.48^{a}\pm0.06$
AST (U l^{-1})	$154.83^{b}\pm5.90$	$165.46^{b}\pm7.98$	$106.20^{a}\pm6.26$	$93.60^{a}\pm7.52$	$104.23^{a}\pm5.11$
ALT (U l^{-1})	$27.03^{ab}\pm5.95$	$29.76^{c}\pm1.92$	$43.56^{d}\pm7.10$	$25.73^{ab}\pm7.03$	$18.5^{a}\pm4.60$
ALP (U l^{-1})*	20.40 ± 5.81	19.86 ± 3.74	22.80 ± 3.86	23.43 ± 4.42	27.53 ± 1.50
LDH (U l^{-1})	$1431.93^{b}\pm35.22$	$1572.13^{b}\pm30.65$	$1237.86^{a}\pm24.88$	$1261.03^{a}\pm15.56$	$1762.83^{d}\pm15.67$

Values are mean±SE of three replicates

Means in the same row bearing different superscripts are significantly different ($p<0.05$).

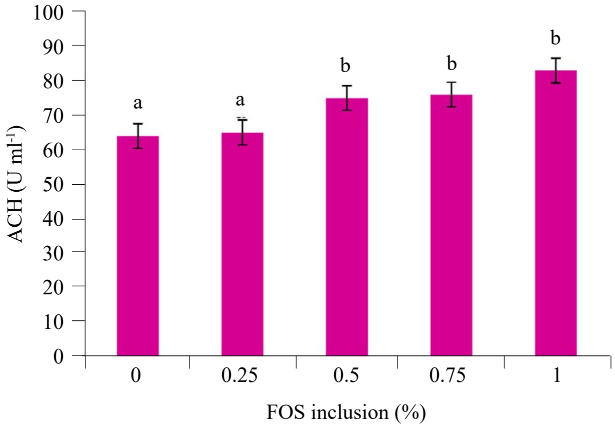


Fig. 1. Serum alternative complement activity of Asian seabass fingerlings fed different levels of dietary fructooligosaccharides. Superscript denotes significant differences between control and treatment groups ($p<0.05$)

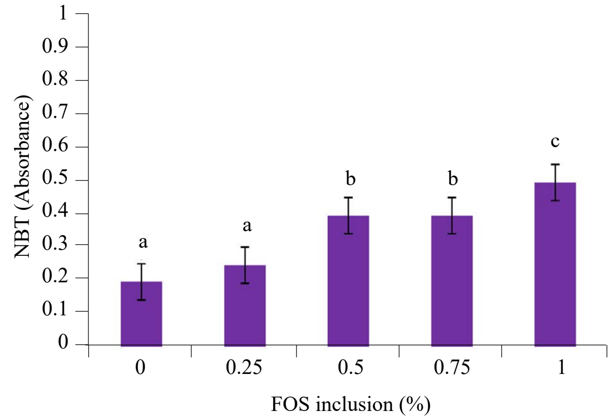


Fig. 2. NBT assay of Asian seabass fingerlings fed different levels of dietary fructooligosaccharides (FOS). Superscript denotes significant differences between control and treatment groups ($p<0.05$)

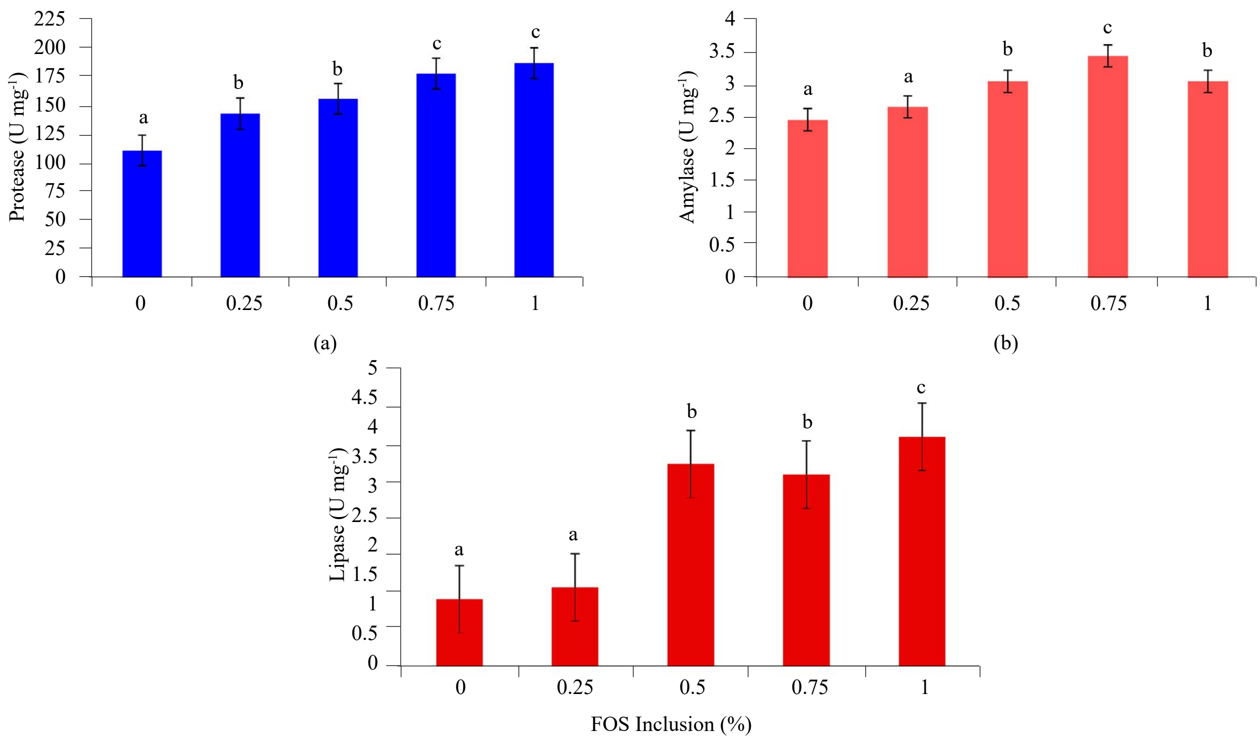
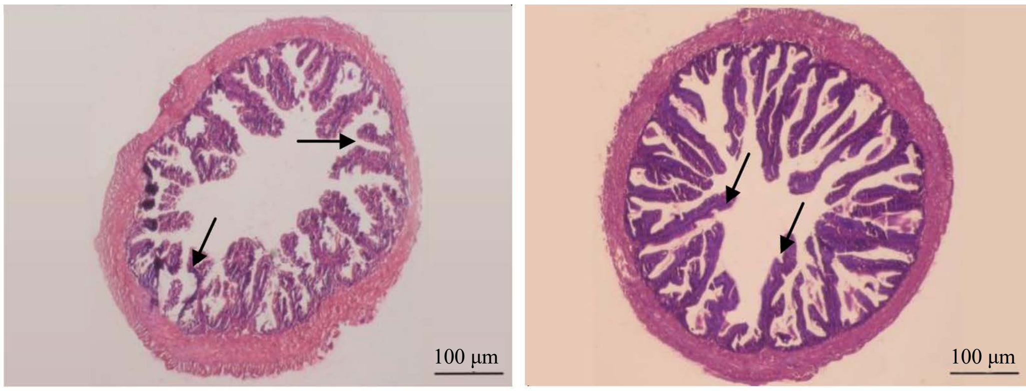


Fig. 3. Specific activities of digestive enzymes (U mg^{-1}) of Asian seabass fingerlings fed different levels of dietary fructooligosaccharides (FOS). (a) Protease; (b) Amylase and (c) Lipase. Superscript denotes significant differences between control and treatment groups. ($p<0.05$).

branches (Fig. 4b). Moreover, increase in secretory cells were noticed in comparison to control fishes. The group fed with 1% FOS supplemented diet showed increased villi growth compared to other treatments. Similarly the hepatic tissue appeared normal with normal hepatocytes in fish fed with control diet (Fig. 5a). Supplementation of FOS at varying levels showed vacuolations and glycogen storage in hepatocytes and some of the hepatocytes appeared swollen with presence of irregular vacuolations (Fig. 5b).

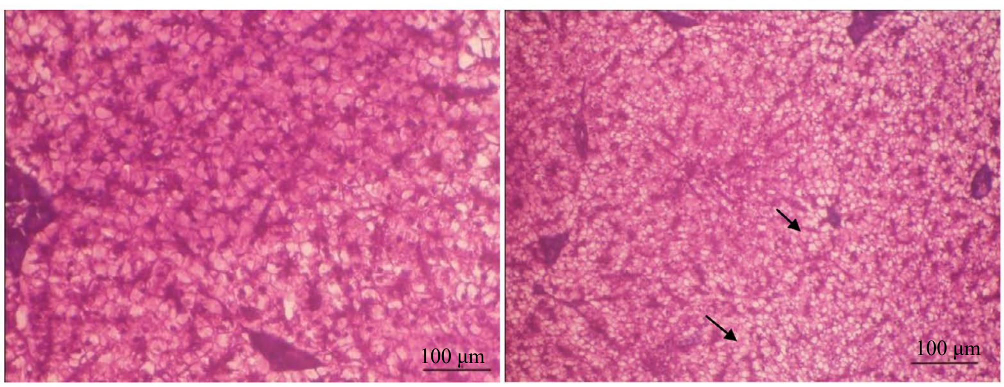
Discussion

This forms the first study to investigate the effect of dietary supplementation of fructooligosaccahrride on



(a) (b)

Fig. 4. Photomicrographs of intestinal sections of Asian seabass fingerlings fed (a) Control diet, without any changes in the intestinal folding; (b) 1% FOS supplemented diet - Arrows denotes increase in the length of intestinal folding (H&E)



(a) (b)

Fig. 5. Photomicrographs of Liver sections of Asian seabass fingerlings fed (a) Control diet; (b) 1% FOS supplemented diet - Arrows denotes glycogen deposition (H&E)

intestine and liver histology, digestive enzyme activities, biochemical and immunological parameters in Asian seabass fingerlings. The current study revealed that there was a significant increase in various serum biochemical parameters like glucose, urea, cholesterol, triglyceride, albumin, globulin, A/G ratio, AST, ALT, ALP and LDH in fish fed with the diets supplemented with FOS. Whereas no significant differences were observed for total protein and globulin among the various treatments. On the contrary to our results Akrami *et al.* (2009) in juvenile beluga reported that in an 8 weeks feeding trial, various biochemical parameters were not significantly affected by dietary prebiotic inulin. The enzyme activities LDH, ALP, ALT and AST levles were not affected by supplemetation of mannanoligosaccahrides at 0, 0.5, 1, 1.5 and 2% in Asian seabass fingerlings (Ali *et al,* 2017). The total amount of blood glucose content in fish varies from one species to another and the value ranges from 20-360 mg dl^{-1} (Shakoori*et al*., 1996). In the current study, the total blood glucose content ranged from 53-92 mg dl^{-1}. Albumin and globulin are the two components of total proteins. Globulin is made up of fractions of β and globulins (Alexander *et al*., 2011). Increase in total serum protein and globulin contents plays an important role in defense mechanism and innate immunity (Magnadottir, 2006). However, the decreased albumin and total protein levels in treatment groups would be indicative of an abnormality in liver or kidney functions. It appears that changes in biochemical parameters may vary from one species to another species and may be affected by various factors namely, supplementation rate of prebiotics in diet, type of feed ingredients and the experimental conditions (Taati *et al*., 2011). The elevated levels in serum AST and ALT may be related to stressed condition.

The ACP and NBT activity of seabass fed with FOS supplemented diets showed significant difference between control and treatment groups. The fish fed with 1% FOS supplemented diet showed highest ACP and NBT activity compared to rest of the treatments. Similar to our results Soleimani *et al*. (2012) reported that Caspian roach fry fed 2 and 3% FOS showed improved immunity by increasing serum alternate complement activity. Other studies on allo-gynogenetic silver crucian carp (Wang *et al*., 2008) and large yellow croaker (Ai *et al*., 2011) also confirmed the beneficial effects of FOS on fish innate immune responses. Zhang *et al*. (2013) reported that triangular bream fed 0.3% FOS diet showed relatively higher values of ACP activities. On the contrary in ovate pompano, ACP activity was not affected by dietary FOS levels (Zhang *et al*., 2014). Improved immune capacity of fish can be demonstrated by higher complement activity, triggered through both alternate and classical pathways (Spring, 2002). Data regarding the positive and negative effects of prebiotics FOS on neutral oxidative radical production has been reported by Zhou *et al*. (2010) in red drum (*Sciaenops ocellatus* (Li and Gatlin, 2004) and in hybrid striped bass (*M. Chrysops*×*M. saxatilis*) with dietary yeast inclusion.

Histological analysis revealed that supplementation of FOS at different levels results in increased microvilli density and length in the anterior and posterior intestinal regions. FOS supplementation can produce more microvilli structures which whould provide the potential to improve the nutrient capture. FOS in diets has promoted the health of epithelium as well as the mocrovilli of the gut indicated by a more complicated architecture of the gut with longer villi and hence a larger surface area for nutrient absorption. Similar to our results, Wu *et al*. (2013) reported that dietary supplementation of FOS has increased the microvilli length and improved intestinal folding. Similarly Ali *et al.* (2018) reported that Asian seabass fed 15-20 g kg^{-1} inulin supplementation showed improved length of the microvilli and numerous side branches. Hepatic tissue appeared normal with normal shaped hepatocytes in the fish fed with control diet. Supplementation of FOS at varying levels showed vacuolations and glycogen storage in hepatocytes. Depositon of glycogen in the liver tissue indicates that supplementation of prebiotics significantly increased the amount of glycogen in the liver cells (hepatocytes). Glycogen stored in the liver and the muscles provides the body with a readily available source of energy in case of interrupted feeding which may arise in commercial culture conditions especially during stress conditions.

Results of the present study clearly showed that dietary supplementation of prebiotic FOS at 0.75 and 1% significantly increased the digestive enzyme activities in Asian seabass. Similar to our results, in Caspian roach (*Rutilus rutilus*) fry (Soleimani *et al*., 2012) and in snout bream (*Megalobrama amblycephala*) fingerlings (Wu *et al*., 2013), digestive enzyme activities (protease, amylase and lipase) were found significantly elevated with increasing levels of dietary FOS. Similar results have been reported in Crucian carp (*Carassius auratus gibelio*) fed Xylooligosaccharide (Xu*et al*., 2009) and freshwater crayfish (*Cherax destructor*) fed MOS (Sang *et al*., 2011). The reasons for the elevated levels could be endogenous (host) and/or exogenous microbial activities (potentially modulated by the prebiotic) which needs further elucidation.

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