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Effects of dietary fenugreek seed extracts on growth performance, plasma biochemical parameters, lipid metabolism, Nrf2 antioxidant capacity and immune response of juvenile blunt snout bream (*Megalobrama amblycephala*)

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A R T I C L E I N F O

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A B S T R A C T

Dietary administration of some plant-derived substances have been proved of great economic value in aquaculture. In order to investigate the effects of dietary fenugreek seed extracts (FSE) on juvenile blunt snout bream (*Megalobrama amblycephala*), a feeding trial was conducted for 8 weeks. The results showed that final weight (FW), weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR) were not significantly affected by dietary FSE levels. The whole body lipid contents of fish fed with 0.04%, 0.08% and 0.16% FSE diets were significantly lowered compared to the control group. Dietary FSE diets significantly affected plasma complement component 3 (C3), immunoglobulin M (IgM), albumin (ALB) and total protein (TP). The relative expressions of acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element binding protein-1 (SREBP1) mRNA in the liver of fish decreased significantly with increasing dietary FSE levels from 0% up to 0.04%. FSE supplementation diets lowered the liver pro-inflammatory genes expressions by regulating tumor necrosis factor-α (TNF-α) and interleukin 8 (IL-8) mRNA levels and increased anti-inflammatory genes expression by regulating transforming growth factor (TGF-β) and interleukin 10 (IL-10). FSE diets increased growth factor-1 (IGF-1) and target of rapamycin (TOR) mRNA levels from 0% up to 0.04%, 0.04% FSE diets significantly increased growth factor-1 (IGF-1) mRNA levels and S6 kinase-polypeptide 1 (S6K1) mRNA levels compared to the control group. 0.04% FSE diets significantly increased superoxide dismutase (SOD) activities and 0.08% FSE diets significantly increased catalase (CAT) and glutathione peroxidase (GPx) activities, 0.16% FSE diets significantly increased total antioxidant capacity (T-AOC) activities compared to the control group. Additionally, compared to the control group, 0.04% dietary FSE significantly up-regulated nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA levels and glutathione peroxidase-1 (GPx1) mRNA levels, at the same time, 0.02%, 0.04%, 0.08%, 0.16% FSE diets significantly down-regulated kelch-like ECH-associated protein 1 (Keap1) mRNA levels. However, no significant effects were observed on copper zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD). Our study indicated that dietary FSE could improve plasma biochemical parameters, regulate lipid metabolism related genes, promote Nrf2 antioxidant capacity and enhance immune response of juvenile blunt snout bream.

1. Introduction

The dietary administration of some plant-derived substances have been proved effective in enhancing immune responses, reducing drug resistance and restricting disease progression in fish [1]. Certain substances in plants have natural antioxidant properties that can be applied to some kinds of fish [2–7]. Fenugreek (*Trigonella foenum graecum L.*), an annually traditional herb, comes from the Leguminosae family [8] contains active substances like alkaloids trigonelline, trigocoumarin, diosgenin, galactomannan and saponin [9,10]. Previous study showed that trigonelline, one of the fenugreek seed extracts (FSE), can be used to prevent oxidative stress [11]. The galactomannan is extracted from

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Canadian fenugreek seeds has the potential in reducing blood glucose [12]. Furthermore, polysaccharide (FWEP) extracted from fenugreek (*Trigonella foenumgraecum*) seeds is able to heal its wounds through its antioxidant properties [13]. In Sea Bass (*Dicentrarchus labrax*), dietary administration of fenugreek supplementation has been shown to improve a series of biochemical parameters [14]. In the Middle East and South Asia, fenugreek seed is considered as a kind of spice, researches have shown that fenugreek seeds have anti-cancer, restrain inflammatory response and antioxidant properties [15–17]. In addition, the fenugreek seeds are also proved to have immunological function [18].

In rats, Fenugreek extract was reported to have the potential to improve lipid metabolism [12]. In aquatic animals, however, studies on lipid metabolism in relation to FSE is still scarce. Evaluation of the immune and antioxidant capacity of fish is very crucial in preventing fish diseases. Complement component 3 (C3) and immunoglobulin M (IgM) are two important parameters that provide information regarding to the immune state in fish [19,20]. Dietary fenugreek was shown to enhance immune response of gilthead seabream [21]. Inflammatory cytokines occupied an important position in the immune system of fish [22]. Tumour necrosis factor-α (TNF-α) and interleukin 8 (IL-8) are vital pro-inflammatory factors in fish [23], while transforming growth factor (TGF-β) and gaiinterleukin 10 (IL-10) are important anti-inflammatory factors [24]. The target of rapamycin (TOR) signaling pathway occupied an important position in regulating some inflammatory responses in mammals [25]. Some related studies had shown that fenugreek seeds can improve antioxidant capacity [26]. In fish, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were considered as the primary antioxidant enzymes [27]. However, as far as we know, no studies had been undertaken to evaluate the effects of FSE on antioxidant enzyme activity and expression in juvenile blunt snout bream. The nuclear factor erythroid 2-related factor 2 (Nrf2) was shown to be able to regulate some antioxidant genes in European eel (*Anguilla anguilla*) [28] and grass carp (*Ctenopharyngodon idella*) [29]. Growing attention has been paid on Nrf2 signaling pathway in regulating antioxidant capacity of aquatic animals in recent years [30,31].

In aquaculture, the balance between fish and water is affected by some environmental factors such as the temperature, water quality, bacteria and viruses, leading to stress responses in fish [32]. Too much stress would suppress the fish's immune system [33–35] and eventually lethal [36,37]. As a kind of freshwater fish, blunt snout bream (*Megalobrama amblycephala*) has delicious meat and high economic value [38]. The annual output of blunt snout bream was about 820,000 tons in 2016 [39]. But disease outbreaks have led to high mortality rates for blunt snout bream in recent years, especially in summer [40]. Therefore, in order to promote the quality of blunt snout bream, it is necessary to improve its feeding. So far, no studies regarding the effects of dietary FSE in blunt snout bream have been conducted. Hence, this study was aimed at evaluating the effects of FSE on growth performance, plasma biochemical parameters, lipid metabolism, Nrf2 antioxidant capacity and immune response of juvenile blunt snout bream.

2. Materials and methods

2.1. Experimental diet

Ingredient and nutrient composition of the feeds were showed in Table 1. The crude protein content in the basal diet was 32.64% and the lipid content was 6.82%. Diets were developed according to feed formulations into six dietary FSE grading levels (0.00, 0.01%, 0.02%, 0.04%, 0.08% and 0.16%). Fish meal, soybean meal, rapeseed meal and cotton meal were used as protein sources, and soybean lecithin and soybean oil were used as sources of lipid. Ground all ingredients into powder, mixed well with soybean oil and water, and then pelleted (die diameter 1.5 mm and 2.0 mm) using Lab extruder (Science and Technology Industrial Factory of South China University of Technology, China). After drying, the pellets were packed into airtight plastic bags and stored at −20 °C.

Table 1
Ingredient and nutrient composition of the feeds.

Ingredients	Diet1	Diet2	Diet3	Diet4	Diet5	Diet6
Fish meal[a]	3	3	3	3	3	3
Soybean meal[a]	30	30	30	30	30	30
Rapeseed meal[a]	22	22	22	22	22	22
Cotton meal[a]	5	5	5	5	5	5
Wheat meal[a]	29.72	29.71	29.70	29.68	29.64	29.56
Soybean lecithin	1	1	1	1	1	1
Soybean oil	2	2	2	2	2	2
monocalcium phosphate	2	2	2	2	2	2
Mineral premix[b]	1	1	1	1	1	1
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Bentonite	3.00	3.00	3.00	3.00	3.00	3.00
Lysine	0.45	0.45	0.45	0.45	0.45	0.45
Methionine	0.32	0.32	0.32	0.32	0.32	0.32
Threonine	0.31	0.31	0.31	0.31	0.31	0.31
Fenugreek seed extracts	0	0.01	0.02	0.04	0.08	0.16
Proximate analysis by dry diet						
Crude protein	32.59	32.59	32.71	32.82	32.63	32.47
Crude lipid	6.74	6.75	6.83	6.86	6.93	6.83
Gross Energy (KJ g^{-1})	18.51	18.52	18.50	18.48	18.51	18.46

[a] The ingredients used to formulate the diets were obtained from Wuxi Tongwei feedstuffs Co., Ltd, Wuxi, China.
[b] Mineral premix (g kg^{-1} of diet): calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulphate, 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02 g; manganese sulphate, 0.03 g; sodium selenate, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

2.2. Experimental procedures

Experimental juvenile blunt snout bream were obtained from Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences. Fish rearing methods were similar to those described in previous studies [41,42]. Before feeding, the healthy fish with similar size (4.75 ± 0.04 g) were randomly distributed into 18 cages (1 m × 1 m × 1 m, triplicate cages) with 20 fish in each cage in a farm pond culture and fed with commercial diet containing 330 g/kg protein and 70 g/kg lipid (Wuxi Tongwei feedstuffs Co. Ltd., Wuxi China). Fish were fed three times a day (8:00, 12:00, 16:00) for 8 weeks. During the experimental period, water temperature (24–26 °C), dissolved oxygen (≥6.0 mg/l), total ammonia nitrogen (0.005–0.009 mg/L) and pH (7.0–7.5) were recorded by a data logger weekly.

2.3. Sample collection

At the end of the experiment, sampling was conducted after 24 h of the last feeding. Five fish from each cage were obtained after the fasting period (24 h from the last feeding day). Two fish were used to measure composition of whole body and three fish were anesthetized by MS-222 (100 mg/L). Blood samples were collected from the caudal vein and then centrifuged (3000 rpm, 10 min, 4 °C) to prepare the plasma. Plasma and liver samples were stored at −80 °C until analysis. The moisture, crude protein, fat and ash contents of the feed and whole fish body were determined according to the method described by AOAC (2003), dry matter by atmospheric pressure drying method at 105 °C oven, drying to constant weight; crude protein content (N) after the acid solution using automatic Kjeldahl nitrogen determination apparatus (FOSS KT260, Switzerland); crude fat content using soxhlet extraction method; ash content after subjecting the samples on the carbide in electric stove to 560 °C burning in the muffle furnace for 5 h.

2.4. Chemical analysis

Activities of plasma immunoglobulin M (IgM) levels, complement component 3 (C3) content, albumin (ALB), total protein (TP), total triglyceride (TG), glucose (GLU), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested by Mindray BS-400 automatic biochemical analyser (Mindray Medical International Ltd., Shenzhen, China) as described before (Habte-Tsion et al., 2015). Activities of L-Glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and total antioxidant capacity (T-AOC) were assayed by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Real-time PCR analysis

The relative genes expression of lipid metabolism, TOR and Nrf2 signaling pathway were determined using Real-time PCR analysis as described by previous studies [42]. In brief, total RNA of liver was extracted by using a Takara RNAiso Plus Kit. The mRNA levels of acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element binding protein-1 (SREBP1), tumour necrosis factor-α (TNF-α), interleukin 8 (IL-8), transforming growth factor-β (TGF-β), interleukin 10 (IL-10), growth factor-1 (IGF-1), target of rapamycin (TOR) and ribosomal protein S6 kinase-polypeptide 1 (S6K1), nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione peroxi-dase 1 (GPx1), Kelch-like ECH-associated protein 1 (Keap1), copper-zinc superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD) were tested using a Takara PrimeScript™ Reagent Kit and a 7500 Real Time PCR System (Applied Biosystems, USA). On the basis of the partial cDNA sequences, the blunt snout bream transcriptome analysis was used to design specific primers (Table 2) [43]. β-actin was used as a nonregulated reference gene in previous studies [44,45]. In our investigation, no changes in the expression of the β-actin gene were observed. The Pfaffl's mathematical model was used to relatively quantify the amount of target gene expression [46].

2.6. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using the software SPSS version 17.0. Significant differences between means were evaluated by Turkey's Multiple Range Test. Probabilities of $P < 0.05$ were considered significant. Data were represented as mean ± SEM.

3. Results

3.1. Growth performance

Results of growth performance of the juvenile blunt snout bream were shown in Table 3. Final weight (FW), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were not significantly affected ($P > 0.05$) by dietary FSE levels.

3.2. Whole body composition

Whole body compositions were presented in Table 4. Dietary FSE showed no significant effects on moisture, protein, and ash contents of whole body in blunt snout bream ($P > 0.05$). Compared to control group, 0.04%, 0.08% and 0.16% FSE diets significantly lowered the whole body lipid contents of fish ($P < 0.05$).

Table 2
Real-time PCR primer sequences.

Primer	Sequence Information	
	Forward primer (5′–3′)	Reverse primer (5′–3′)
ACC[a]	TAGCAGTGAGCATTGGCACA	CATCGCTGGCGTATGAGGAT
FAS[b]	GTTTGCCAACCGCTTGTCTT	GGCCATGGCGAATAGCATTG
SREBP1[c]	ACAACAGTAGCGACACCCTG	AGGAGCGGTAGCGTTTTTCA
TNF-α[d]	TGGAGAGTGAACCAGGACCA	AGAGACCTGGCTGTAGACGA
IL-8[e]	CAGAGAGTCGACGCATTGGT	ATTCACGGTGCTTTGTTGGC
TGF-β[f]	ACTGGACAAACAGAGAGGCG	CAGGGGAGTTGCCGTTAGAG
IL-10[g]	GTGTTTTCGGGTGCAAGTGG	ATGAACGAGATCCTGCGCTT
IGF-1[h]	CTCACTGGTGCTGTTCGTCCTC	TGAAAGCAGCATTCGTCCACA
TOR[i]	TTTACACGAGCAAGTCTACGGA	CTTCATCTTGGCTCAGCTCTCT
S6K1[j]	GGTGCATGTCACCTTATGGG	AGCTGGCAGCACTTCTAGTC
Nrf2[k]	GGGGAAGTCCTTGAACGGAG	AACCAGCGGGAATATCTCGG
GPX1[l]	GAACGCCCACCCTCTGTTTG	CGATGTCATTCCGGTTCACG
Keap1[m]	AATATCCGCCGGCTGTGTAG	TGAGTCCGAGGTGTTTCGTG
Cu/Zn-SOD[n]	AGTTGCCATGTGCACTTTTCT	AGGTGCTAGTCGAGTGTTAGG
Mn-SOD[o]	AGCTGCACCACAGCAAGCAC	TCCTCCACCATTCGGTGACA
β-actin	TCGTCCACCGCAAATGCTTCTA	CCGTCACCTTCACCGTTCCAGT

[a] ACC, acetyl CoA carboxylase.
[b] FAS, fatty acid synthase.
[c] SREBP1, sterol regulatory element binding protein-1.
[d] TNF-α, tumour necrosis factor-α.
[e] IL-8, interleukin 8.
[f] TGF-β, transforming growth factor.
[g] IL-10, interleukin 10.
[h] IGF-1, growth factor-1.
[i] TOR, target of rapamycin.
[j] S6K1, ribosomal protein S6 kinase-polypeptide 1.
[k] Nrf2, nuclear factor erythroid 2-related factor 2.
[l] Keap1, kelch-like ECH-associated protein 1.
[m] GPx1, glutathione peroxidase-1.
[n] Cu/Zn-SOD, copper zinc superoxide dismutase.
[o] Mn-SOD, manganese superoxide dismutase.

Table 3
Effect of fenugreek seed extracts on growth performance and feed utilisation of juvenile blunt snout bream (*Megalobrama amblycephala*)(means ± S.E.M.)[a].

Fenugreek seed extracts(%)	Initial weight(g)	Final weight(g)	WG (%)[b]	FCR[c]	SGR (% day $^{-1}$)[d]	HSI (%)[e]	CF (%)[f]
0	4.77 ± 0.03	36.67 ± 2.53	669.41 ± 32.65	0.95 ± 0.04	2.75 ± 0.06	8.44 ± 0.19	2.13 ± 0.05
0.01	4.75 ± 0.02	35.03 ± 1.17	638.49 ± 15.45	0.99 ± 0.03	2.70 ± 0.03	8.00 ± 0.19	2.23 ± 0.03
0.02	4.77 ± 0.02	35.59 ± 0.39	646.61 ± 3.03	1.02 ± 0.03	2.72 ± 0.00	7.73 ± 0.26	2.29 ± 0.06
0.04	4.77 ± 0.01	36.37 ± 2.31	663.07 ± 28.65	1.01 ± 0.01	2.74 ± 0.05	7.60 ± 0.23	2.22 ± 0.03
0.08	4.76 ± 0.01	37.68 ± 2.44	691.64 ± 29.17	0.88 ± 0.03	2.79 ± 0.05	7.86 ± 0.25	2.17 ± 0.04
0.16	4.76 ± 0.02	34.21 ± 0.83	618.71 ± 11.37	1.02 ± 0.01	2.65 ± 0.00	7.74 ± 0.21	2.26 ± 0.04

[a] Data are means of triplicate, value in the same column with different superscripts are significantly different (*P < 0.05*).
[b] Weight gain (WG) (%) = 100 × (final weight (g) − initial weight (g))/initial weight (g).
[c] Feed conversion ratio (FCR) = dry feed fed (g)/wet weight gain (g).
[d] Specific growth rate (SGR) (%/d) = 100 × [(ln(final body weight (g)) − ln(initial body weight (g)))/days].
[e] Hepatosomatic index (HSI) (%) = 100 × (liver weight (g)/body weight (g)).
[f] Condition factor (CF) (%) = 100 × fish weight (g)/[body length (cm)]^3.

Table 4
Effect of fenugreek seed extracts on whole body composition of juvenile blunt snout bream (*Megalobrama amblycephala*)(means ± S.E.M.)[1].

Fenugreek seed extracts (%)	Moisture (%)	Protein (%)	Ash (%)	Lipid (%)
0	73.59 ± 0.45	16.20 ± 0.11	3.14 ± 0.06	7.36 ± 0.04^{c}
0.01	73.30 ± 0.22	16.58 ± 0.09	2.82 ± 0.04	7.29 ± 0.05^{bc}
0.02	73.22 ± 0.46	16.52 ± 0.17	2.95 ± 0.12	7.16 ± 0.04^{abc}
0.04	73.32 ± 0.08	16.39 ± 0.18	3.02 ± 0.04	7.12 ± 0.03^{ab}
0.08	72.44 ± 0.15	16.46 ± 0.26	2.90 ± 0.12	7.06 ± 0.05^{a}
0.16	72.79 ± 0.32	16.49 ± 0.02	2.89 ± 0.11	7.08 ± 0.03^{ab}

[1]Data are means of triplicate, value in the same column with different superscripts are significantly different (*P < 0.05*).

3.3. Plasma biochemical parameters

Plasma biochemical parameters data were shown in Table 5. Immunoglobulin M (IgM) and complement component 3 (C3) contents increased significantly with increasing dietary FSE levels from 0.01% up to 0.16% FSE diets. Compared to the control group, at dietary FSE levels of 0.08% and 0.16%, IgM levels were significantly affected ($P < 0.05$), at the same time, some significant differences on C3 levels were observed in fish fed with 0.04%, 0.08% and 0.16% dietary FSE levels ($P < 0.05$). Albumin (ALB) contents and total protein (TP) levels of the fish fed with dietary FSE levels of 0.02%, 0.04% and 0.08% were significant higher than those fed with 0.01% FSE diets and the control group, but total triglyceride (TG) was not significantly affected by the graded FSE levels ($P > 0.05$). No significant differences were observed on glucose (GLU) levels between the control and treatment groups ($P > 0.05$). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the fish fed with 0.08% FSE were lower than those fed with other diets, but the difference was not significant ($P > 0.05$).

3.4. Gene expressions of lipid metabolism

The relative expressions of acetyl CoA carboxylase (ACC) (Fig. 1. A), fatty acid synthase (FAS) (Fig. 1. B) and sterol regulatory element binding protein-1 (SREBP1) (Fig. 1. C) mRNA in the liver of fish decreased significantly with increasing dietary FSE levels from 0% up to 0.04%. There were significant differences observed on ACC and SREBP1 mRNA levels between the control and treatment groups ($P < 0.05$). Furthermore, 0.04% and 0.16% dietary FSE levels significantly decreased the relative expressions of FAS mRNA compared to the control group ($P < 0.05$).

3.5. Relative genes expressions of pro-inflammatory and anti-inflammatory factor

The relative expressions of tumor necrosis factor-α (TNF-α) mRNA (Fig. 2. A) in the liver of fish were decreased significantly with increasing dietary FSE levels up to 0.16% diets ($P < 0.05$) and interleukin 8 (IL-8) (Fig. 2. B) mRNA decreased significantly with increasing dietary FSE levels up to 0.08% diets ($P < 0.05$). Transforming growth factor (TGF-β) mRNA levels (Fig. 2. C) increased with increasing dietary FSE levels from 0% up to 0.02% and then decreased. However, TGF-β mRNA levels with 0.02% dietary FSE levels were significantly higher than the control group ($P < 0.05$). Similarly, interleukin 10 (IL-10) mRNA levels (Fig. 2. D) increased with increasing dietary FSE levels

Table 5
Effect of fenugreek seed extracts on plasma biochemical parameters of juvenile blunt snout bream (*Megalobrama amblycephala*)(means ± S.E.M.)[1].

Fenugreek seed extracts(%)	IgM(mg L^{-1})[2]	C3(mg L^{-1})[3]	ALB(g L^{-1})[4]	TP(g L^{-1})[5]	TG(mmol L^{-1})[6]	GLU(mmol L^{-1})[7]	ALT(U L^{-1})[8]	AST(U L^{-1})[9]
0	1.77 ± 0.07^{a}	5.28 ± 0.05^{a}	10.61 ± 0.32^{a}	31.09 ± 0.41^{a}	3.41 ± 0.13	9.42 ± 0.18	4.32 ± 0.42	124.41 ± 6.92
0.01	1.79 ± 0.04^{ab}	5.27 ± 0.08^{a}	10.58 ± 0.40^{a}	31.29 ± 0.49^{a}	3.42 ± 0.12	9.57 ± 0.39	4.29 ± 0.64	125.42 ± 8.10
0.02	1.84 ± 0.07^{ab}	5.48 ± 0.04^{ab}	12.31 ± 0.35^{b}	33.92 ± 0.71^{b}	3.83 ± 0.17	9.61 ± 0.09	5.70 ± 0.38	145.85 ± 7.83
0.04	1.96 ± 0.02^{abc}	5.56 ± 0.02^{b}	12.33 ± 0.41^{b}	34.31 ± 0.63^{b}	3.89 ± 0.26	10.23 ± 0.16	4.35 ± 0.57	144.20 ± 6.67
0.08	2.01 ± 0.05^{bc}	5.69 ± 0.08^{bc}	12.33 ± 0.36^{b}	34.42 ± 0.61^{b}	3.43 ± 0.26	9.65 ± 0.13	4.21 ± 0.62	122.43 ± 6.98
0.16	2.06 ± 0.04^{c}	5.81 ± 0.06^{c}	10.86 ± 0.31^{ab}	32.16 ± 0.63^{ab}	3.40 ± 0.15	10.32 ± 0.49	5.50 ± 0.52	137.10 ± 6.99

[1]Data are means of triplicate, value in the same column with different superscripts are significantly different (*P < 0.05*).
[2]IgM, immunoglobulin M.
[3]C3, complement component 3.
[4]ALB, albumin.
[5]TP, total protein.
[6]TG, total triglyceride.
[7]GLU, glucose.
[8]ALT, alanine aminotransferase.
[9]AST, aspartate aminotransferase.

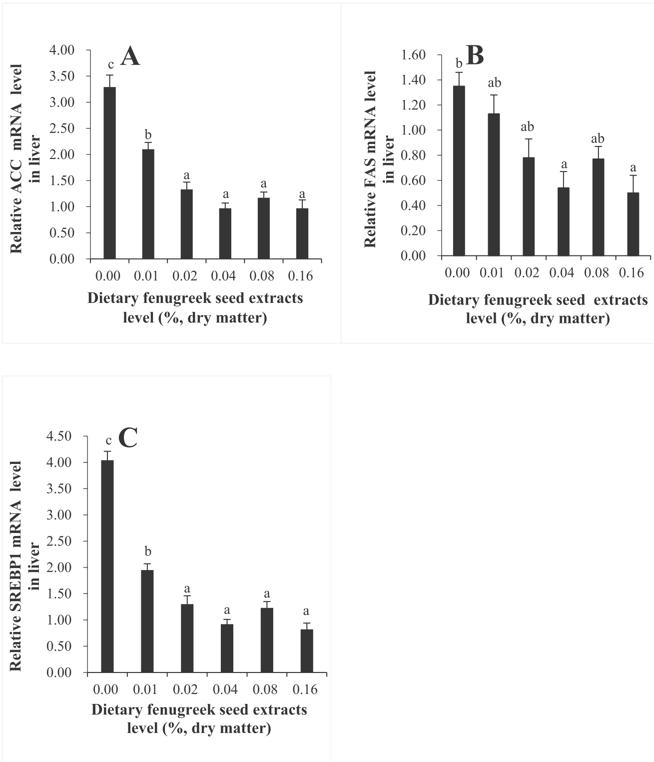

Fig. 1. Relative expressions of lipid metabolism signaling pathway. Such as ACC (A), FAS (B), SREBP1 (C) genes in the liver of blunt snout bream fed diets with diferent fenugreek seed extracts levels. Data are expressed as means with SEM. Value with diferent superscripts are signifcantly diferent ($P < 0.05$).

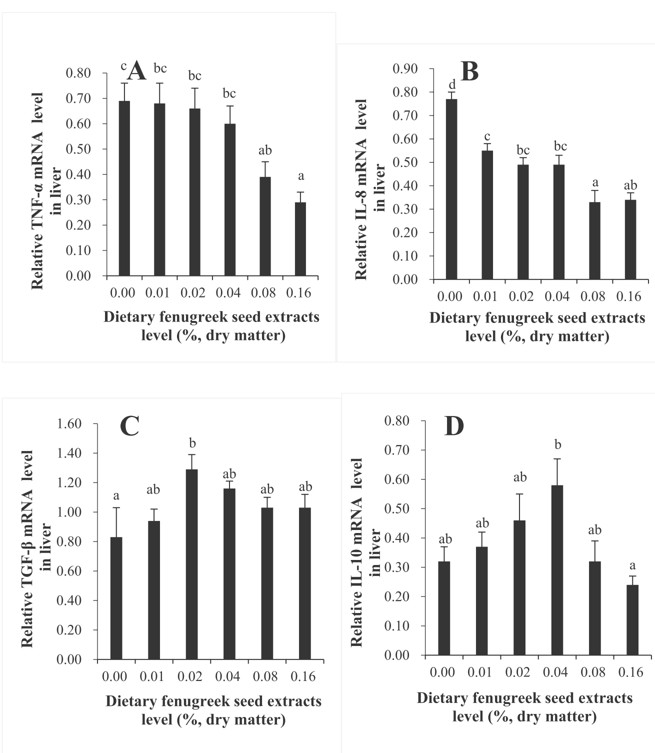

Fig. 2. Relative expressions of TNF-α (A), IL-8 (B), TGF-β (C) IL-10 (D) genes with different fenugreek seed extracts levels. Data are expressed as means ± S.E.M., value with different superscripts are significantly different ($P < 0.05$).

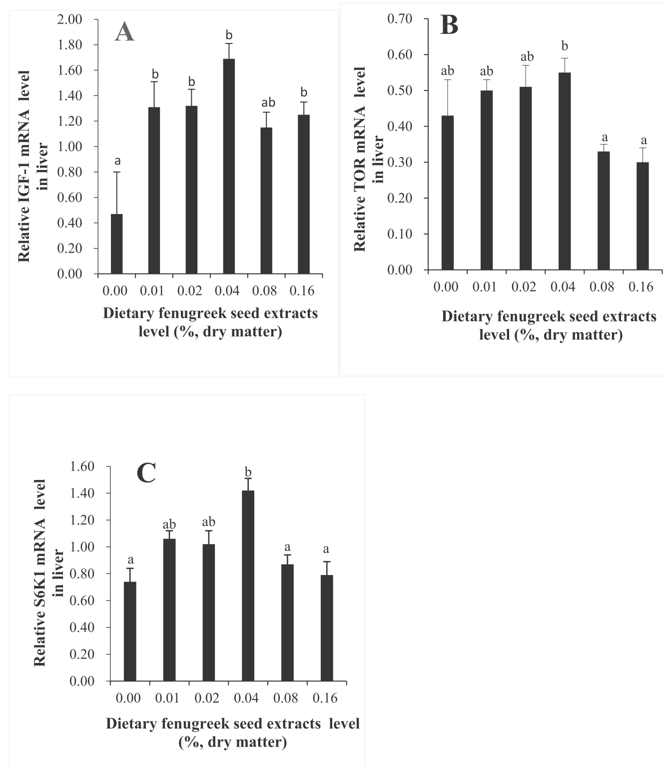

Fig. 3. Relative expressions of TOR signaling pathway with different fenugreek seed extracts levels in liver. Such as IGF-1 (A); TOR (B); S6K1 (C). Data are expressed as means ± S.E.M., value with different superscripts are significantly different ($P < 0.05$).

from 0% up to 0.04% and then decreased significantly ($P < 0.05$) towards 0.16%.

3.6. Relative expressions of TOR in the liver

Growth factor-1 (IGF-1) (Fig. 3. A) and target of rapamycin (TOR) (Fig. 3. B) mRNA levels increased with increasing dietary FSE levels from 0% up to 0.04%. Compared to the control group, 0.01%, 0.02%, 0.04% and 0.16% dietary FSE levels significantly increased the relative expressions of IGF-1 mRNA levels ($P < 0.05$). Furthermore, TOR mRNA levels of fish fed with 0.04% dietary FSE levels were significantly higher than those fed with 0.08% and 0.16% dietary FSE ($P < 0.05$). Moreover, S6 kinase-polypeptide 1 (S6K1) mRNA levels in the fish fed with 0.04% dietary FSE levels were significantly higher than the 0.08%, 0.16% diets and the control groups ($P < 0.05$) (Fig. 3. C).

3.7. Nrf2 antioxidant signaling pathway

Plasma biochemical parameters Antioxidant enzyme activities results were shown in Table 6. No significant differences on L-Glutathione (GSH) and malondialdehyde (MDA) activities were observed in fish fed with dietary FSE levels ($P > 0.05$). Superoxide dismutase (SOD) activities increased significantly with increasing dietary FSE levels from 0% up to 0.04% FSE diets ($P < 0.05$). Catalase (CAT) and glutathione peroxidase (GPx) activities increased significantly with increasing dietary FSE levels from 0% up to 0.08% FSE diets ($P < 0.05$). In the fish fed with 0.08% FSE levels, CAT and GPx activities were significantly higher than the control group ($P < 0.05$). Total antioxidant capacity (T-AOC) activities of the fish fed with 0.16% FSE were increased significantly compared to the control group ($P < 0.05$).

Gene expressions parameters Nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA levels increased with increasing dietary FSE

Table 6
Effect of fenugreek seed extracts on enzymes activities of juvenile blunt snout bream (*Megalobrama amblycephala*)(means ± S.E.M.)[1].

Fenugreek seed extracts(%)	GSH(umol L^{-1})[2]	MDA(nmol ml^{-1})[3]	SOD(U ml^{-1})[4]	CAT(U ml^{-1})[5]	GPx(U ml^{-1})[6]	T-AOC(U ml^{-1})[7]
0	244.02 ± 20.79	2.50 ± 0.15	35.56 ± 7.54^{a}	6.62 ± 0.67^{a}	83.65 ± 6.99^{a}	0.09 ± 0.02^{a}
0.01	289.81 ± 26.80	2.00 ± 0.38	62.45 ± 3.47^{a}	6.85 ± 0.84^{a}	92.53 ± 5.96^{a}	0.11 ± 0.01^{ab}
0.02	301.09 ± 23.63	1.88 ± 0.13	67.30 ± 8.68^{ab}	8.13 ± 0.73^{ab}	102.07 ± 6.68^{ab}	0.12 ± 0.01^{ab}
0.04	307.61 ± 26.28	1.85 ± 0.23	99.91 ± 6.62^{b}	9.64 ± 0.27^{ab}	110.96 ± 10.48^{ab}	0.12 ± 0.01^{ab}
0.08	321.43 ± 25.53	1.79 ± 0.15	35.85 ± 6.44^{a}	10.84 ± 0.23^{b}	133.49 ± 4.60^{b}	0.12 ± 0.01^{ab}
0.16	275.00 ± 27.79	1.92 ± 0.26	50.30 ± 6.41^{a}	10.01 ± 0.87^{ab}	104.82 ± 6.51^{ab}	0.17 ± 0.01^{b}

[1]Data are means of triplicate, value in the same column with different superscripts are significantly different (*P* < *0.05*).
[2]GSH, L-Glutathione.
[3]MDA, malondialdehyde.
[4]SOD, superoxide dismutase.
[5]CAT, catalase.
[6]GPx, glutathione peroxidase.
[7]T-AOC, total antioxidant capacity.

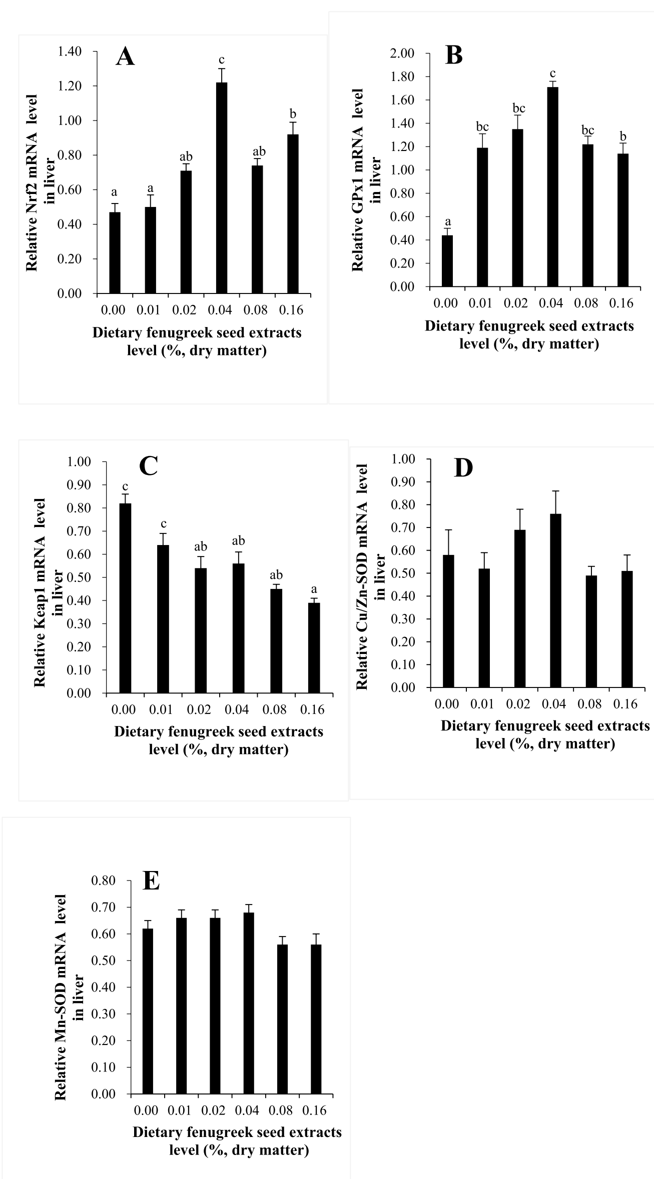

Fig. 4. Relative expressions of Nrf2 signaling pathway with different fenugreek seed extracts levels in liver. Such as Nrf2 (A); GPx1 (B); Keap1 (C); Cu/Zn-SOD (D); Mn-SOD (E). Data are expressed as means ± S.E.M., value with different superscripts are significantly different ($P < 0.05$).

levels up to 0.04%, and the levels in 0.04% group was significantly higher than the other groups ($P < 0.05$) (Fig. 4. A). The relative expressions of glutathione peroxidase-1 (GPx1) mRNA levels (Fig. 4. B) increased with increasing dietary FSE levels up to 0.04% and then decreased. In the fish fed with 0.04% FSE levels, GPx1 mRNA levels were significantly higher than that in fish fed the 0.16% and the control diets ($P < 0.05$). Kelch-like ECH-associated protein 1 (Keap1) mRNA levels of fish fed with 0.02%, 0.04%, 0.08%, 0.16% diets were significantly lower than that in 0.01% dietary FSE levels and the control group ($P < 0.05$) (Fig. 4. C). There were not significant differences were observed on copper zinc superoxide dismutase (Cu/Zn-SOD) (Fig. 4. D) and manganese superoxide dismutase (Mn-SOD) (Fig. 4. E) mRNA levels between the control and treatment groups ($P > 0.05$).

4. Discussion

Fenugreek seed had been shown to improve the growth performance in common carp [47], nile tilapia fingerlings [48] and gilthead seabream [49]. However, in the present study, FW, WG, FCR and SGR of juvenile blunt snout bream were not significantly affected by dietary FSE levels. The variation in the results from different studies might be due to the difference in the size of the fish, breeding environment and species used [50].

In the present study, whole body lipid contents of the fish were significantly affected by dietary FSE levels. Therefore, in this study, we discussed some genes related to lipid metabolism. ACC and FAS were known to play vital roles in the synthesis of fatty acids [51,52], which had adverse influence on fish liver function because of lipid accumulation [53]. It had also been reported that SREBPs was critical for lipogenesis and cholesterol homeostasis [54–57]. SREBP-1 regulates the biosynthesis of fatty acids through FAS [58]. In our study, ACC, FAS and SREBP1 mRNA levels showed similar trends, and FSE diets resulted in the down-regulation of FAS, ACC and SREBP1 mRNA levels in juvenile blunt snout bream. These results suggest that FSE was responsible for fat reduction, which was exactly consistent with the present result that dietary FSE lowered whole body lipid contents of fish. Our study confirmed that FSE could improve fat metabolism. Similarly, in rats, fenugreek seed was reported to reduce blood glucose and improve lipid metabolism, contributing to the galactomannan in its extract [12]. Therefore, we inferred that FSE had the potential in regulating lipid metabolism in juvenile blunt snout bream, but the mechanism of how FSE affect lipid metabolism was unclear and needed further research.

Blood biochemical parameters could provide information on health conditions in fish [59,60]. 1% dietary fenugreek supplementation could improve blood biochemical indexes of sea bass [61]. The complement system of fish had the ability to dissolve foreign cells and organisms by phagocytes [62]. C3 and C4 played a vital role in the activation of complement system [63]. IgM was considered as one of the most important immunoglobulins in fish [64,65]. Therefore, C3 and IgM occupied an important position in the immune response in fish [66]. In our study, C3 and IgM levels were significantly increased in fish fed with FSE. These results were in line with those of E. Awad et al. who reported that the complement activity increased in gilthead seabream fed with fenugreek diets [49]. Furthermore, Bahi et al. found that fenugreek significantly increased the plasma complement activity of gilthead seabream [67]. Plasma ALB and TP might be considered as indicators of protein metabolism and innate immune function [68–70] and the increase in serum total protein indicated the enhanced immunity of fish [71]. In our study, ALB levels had been increased significantly and reached its maximum at 0.04% and 0.08% FSE diets. Additionally, TP levels at dietary FSE levels of 0.08% reached its maximum, and this was in agreement with the report by Sevdan Yılmaz that TP levels with fenugreek supplementation in the treatment groups were significantly higher than that in the control group in sea bass [61]. Our present study showed that FSE boosted the immunity of juvenile blunt snout bream. These findings were in line with those of previous studies reported that fenugreek supplementation improved immune responses in gilthead seabream [21,49] and also in weanling pig [72].

The serum glucose concentration could be used to evaluate stress levels in fish [32,73]. Yılmaz showed that the GLU levels significantly decreased in treated groups compared to control groups of Sea Bass by dietary Spice supplementations [14]. In our experiment, no significant differences were observed on GLU levels between the control and the treatment group. This observation was similar to the findings of Zahra Roohi that fenugreek seed meal had no obvious influence on glucose levels [47]. On the one hand, no significant effects on glucose were found because fenugreek seeds contain steroidal saponins [74,75]. On the other hand, as Ren et al. and Li et al. reported, the unchanged GLU level might be due to blunt snout bream's strong ability to recover blood sugar since it was herbivorous [76,77]. Blood ALT and AST activities in serum increased when the liver is injured. Therefore, ALT and AST activities could reflect liver function [78]. In the current study, ALT and AST activities were not significantly affected by dietary FSE levels suggesting that dietary FSE did not affect liver function, agreeing with the findings of F.A. Guardiola and E. Awad in gilthead seabream (*Sparus aurata* L) [21,49].

TNF-α and IL-8 are crucial pro-inflammatory cytokines [79,80] while TGF-β and IL-10 are regarded as anti-inflammatory cytokines which occupied an important position in restricting inflammatory response [81,82]. Fenugreek was regarded as anti-allergic and anti-inflammatory because it contained alkaloids and flavonoids [83]. In this study, the relative expressions of TNF-α and IL-8 mRNA in the liver of fish significantly decreased with increasing dietary FSE. 0.02% dietary FSE level significantly increased TGF-β mRNA levels and 0.04% dietary FSE significantly increased IL-10 mRNA levels. This study suggested that dietary FSE could regulate pro-inflammatory and anti-inflammatory genes expression of juvenile blunt snout bream.

Insulin-like growth factor 1 (IGF-1) occupied a vital position in growth and anabolic effects [84]. At the same time, IGF-1 gene was one of the upstream genes regulating the transcription and expression of TOR gene [85]. S6K1 was one of the important downstream regulators in TOR signaling pathway [86], which mainly regulated the synthesis of proteins [87]. In our study, IGF-1, TOR and S6K1 mRNA levels were affected by dietary FSE levels, suggesting that FSE diets might activate TOR signaling pathway. This was the first study on the effect of FSE on TOR signaling pathway, and provided a basis for further studies on the effects of FSE on TOR signaling pathway and mechanisms.

Studies had shown that fenugreek seeds had antioxidant activity [88]. S. Kaviarasan reported that the antioxidant qualities of fenugreek was due to the scavenging of hydroxyl radicals by some of its chemical components [17]. Several studies had shown that owing to it possessed strong antioxidant activity, plant feeding could lessen oxidative injury on fish [89,90]. Related study also demonstrated that the antioxidant properties of fenugreek and garlic have profound nutritional potential [91]. Antioxidant enzymes and genes played a critical role in anti-oxidative stress in fish tissues [92]. GSH is considered as non-enzymatic antioxidant while SOD, CAT and GPx are considered as antioxidant enzymes [93]. In this experiment, SOD activities in the fish fed with 0.04% FSE were significantly increased, CAT and GPx activities in the fish fed with 0.08% FSE were also significantly increased. In addition, the antioxidant capacity of fish could be directly reflected by T-AOC [94], in this experiment, T-AOC activities were significantly increased in the fish fed with FSE diets. Collectively, these findings suggest that dietary FSE have the potential in improving the antioxidant capacity of the blunt snout bream.

The activities of antioxidant enzymes were linked to their related mRNA levels in fish [95]. Nrf2 occupied a vital position in regulating the gene expression of antioxidant enzymes in fish [96]. Nrf2 could also increase the expression of GPx which occupied an important position in antioxidant system [29]. In our study, Nrf2 and GPx1 mRNA levels exhibited similar trends that 0.04% dietary FSE significantly up-regulated Nrf2 and GPx1 mRNA levels. A similar phenomenon was reported by Wang et al. who also observed that the expression of GPx gene levels in juvenile grass carp was increased by the up-regulation of Nrf2 expression [97]. In our study, 0.02%, 004%, 0.08% and 0.16% FSE diets also resulted in down-regulation of Keap1 mRNA expression in juvenile blunt snout bream liver. Current results implied that dietary FSE could promote nuclear translocation of Nrf2 and increase GPx1 gene expression by down-regulating the expression of Keap1 gene in fish liver. In our study, 0.02%, 0.04%, 0.08% and 0.16% FSE diets resulted in the down-regulation of Keap1 mRNA expression in juvenile blunt snout bream liver. In addition, it was reported that S6K1 could be activated by TOR to promote the production of anti-inflammatory cytokines in mice [98]. Some upstream signaling molecules, such as TOR, could regulate Nrf2 gene expression [99]. Nrf2 in mice liver could be activated by TOR and S6K1 [100]. In our experiment, 0.04% FSE significantly increased TOR, S6K1 and Nrf2 mRNA levels indicating that FSE had great potential to improve antioxidant activity in juvenile blunt snout bream. Our results were in agreement with those of Guardiola who reported that fenugreek supplement could enhance antioxidation ability of gilthead seabream (*Sparus aurata* L.) [67]. However, the mechanism of how dietary FSE regulated Nrf2 antioxidant signaling pathway in juvenile blunt snout bream was still unclear and needed further investigations.

Overall, our present study did not show evidence to suggest that dietary FSE supplementation significantly affect growth performance, however, it was evident that it can improve plasma biochemical parameters, regulate lipid metabolism, promote Nrf2 antioxidant capacity and enhance immune response of juvenile blunt snout bream (*Megalobrama amblycephala*).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.018.

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