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Research paper

Soybean saponin modulates nutrient sensing pathways and metabolism in zebrafish



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ABSTRACT

Soybean saponin (SA) is known as a major anti-nutritional factor that causes metabolic disturbances and growth reduction in fish. However, the mechanisms underlying these effects were far from fully understood. In particular, the influences of SA on nutrient sensing and downstream metabolic pathways remain largely unexplored. Using zebrafish as an animal model, this study was conducted to examine the phenotypic and molecular responses after dietary SA treatment for 2 weeks. SA at both 5 and 10 g/kg diet levels significantly reduced growth performance and feed efficiency, and damaged the morphology of the intestinal mucosa. SA stimulated AMP-activated protein kinase but reduced target of rapamycin (TOR) activities in both feeding trial and cellular studies. Furthermore, SA increased the mRNA expressions of growth axis genes including growth hormone, insulin-like growth factor 1, growth hormone receptor A, and growth hormone receptor B, but decreased insulin-like growth factor-binding protein 2 at both mRNA and protein levels. SA also increased the expressions of key metabolic enzymes involved in glutamine synthetase, glutamate dehydrogenase and lipolysis, hormone-sensitive lipase and lipoprotein lipase. Our results demonstrated that SA modulated nutrient sensing pathways and metabolism, thus provide new aspects on the explanation of the physiological effects of SA.

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1. Introduction

Soybean meal is one of the most commonly used plant protein sources in aquaculture due to its high protein content and low cost (Hardy, 2010). Soybean saponin (SA) is the major anti-nutritional factor (ANF) in soybean meal and known to be toxic for fish (Bureau et al., 1998; Chen et al., 2011; Francis et al., 2002; Gu et al., 2014; Iwashita et al., 2009; Knudsen et al., 2007, 2008): SA caused gastrointestinal disturbance in Atlantic salmon (*Salmo salar* L.) (Knudsen et al., 2008) and European sea bass (*Dicentrarchus labrax*) (Iwashita et al., 2009). High levels of SA reduced feed intake and feed efficiency ratio of fish (Bureau et al., 1998; Chen et al., 2011). Extensive studies have been carried out on the membrane

permeabilizing, immuno-stimulative, and hypocholesterolaemic properties of SA (Francis et al., 2002; Gu et al., 2014; Iwashita et al., 2009; Knudsen et al., 2007, 2008). However, the information on the underlying mechanism of SA has not been fully elucidated. A systematic characterization of signaling and metabolic pathways involved in its actions is warranted.

Animals respond to nutritional status through sophisticated signaling pathways at both cellular and systemic levels. At cellular level, target of rapamycin (TOR) signaling is the major controller that senses nutrient availability in cells (Jewell and Guan, 2013; Laplante and Sabatini, 2012). Chronic inhibition of TOR signaling significantly reduced nutrient utilization and growth in turbot (*Scophthalmus maximus* L.) (Wang et al., 2016a). The suboptimal performance of non-fishmeal proteins in aquafeeds was partially attributed to hypo-activated postprandial TOR signaling in turbot (Song et al., 2016; Xu et al., 2016). At systemic level, the growth hormone/Insulin-like growth factor (GH/IGF) axis senses nutritional status and regulates tissue growth (Cannata et al., 2010; Reindl and Sheridan, 2012). IGF signaling pathway was down

Abbreviations: AMPK, AMP-activated protein kinase; *cck*, cholecystokinin; *gh*, growth hormone; *igf1*, insulin-like growth factor 1; IGFBP2, insulin-like growth factor-binding protein 2; NPY, neuropeptide Y; SA, soybean saponins; TOR, target of rapamycin.

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regulated by dietary amino acid deficiencies, fishmeal replacement, and fasting in fish (Fuentes et al., 2012; Lansard et al., 2011; Seiliez et al., 2013; Song et al., 2016). It has been fully demonstrated that nutrient sensing signaling networks regulate feed intake and metabolism in both mammals (Jewell and Guan, 2013; Laplante and Sabatini, 2012) and fish (Dai et al., 2013; Song et al., 2016; Wang et al., 2016b; Xu et al., 2016). At central level, hypothalamic activities of AMP-activated protein kinase (AMPK)/TOR signaling play important roles in the regulation of food intake by altering the expression of orexigenic (neuropeptide Y and ghrelin) or anorexigenic (cholecystokinin) neuropeptides (Minokoshi et al., 2004; Xue and Kahn, 2006; Volkoff and Peter, 2006; Volkoff et al., 2005). At peripheral level, nutrient sensing cascades influence metabolism including glycolysis, lipogenesis, etc. in different tissues (Jewell and Guan, 2013; Song et al., 2016; Wang et al., 2016a; Xu et al., 2016). Therefore, these signaling networks link nutrient availability with metabolism to control homeostasis and growth at cell, tissue, and whole-body levels (Cannata et al., 2010; Reindl and Sheridan, 2012; Fuentes et al., 2012; Lansard et al., 2011; Seiliez et al., 2013; Dai et al., 2013; Minokoshi et al., 2004; Song et al., 2016; Wang et al., 2016b; Xu et al., 2016; Xue and Kahn, 2006; Volkoff and Peter, 2006; Volkoff et al., 2005). It is known that nutrients, including amino acids, glucose, lipids, etc., all generate inputs to nutrient sensing networks (Avruch et al., 2009; Chantranupong et al., 2015; Jewell and Guan, 2013; Laplante and Sabatini, 2012; Xiong et al., 2013). However, few studies were conducted on the effects of anti-nutritional factors on these signaling pathways.

Zebrafish is established as a vertebrate model for developmental, genetic, as well as nutritional studies because of its short generation interval, large number of offspring, and clear genetic background (Ulloa et al., 2011). In the present study, a two-week feeding trial was conducted by feeding zebrafish with diets containing 0, 5, and 10 g/kg of SA respectively. The molecular responses of nutrient sensing networks and metabolic consequences after dietary SA treatment were examined.

2. Materials and methods

2.1. Experimental diets

Soybean saponin (98% in purity) was obtained from Xi'an Chuankang Biotechnology Co., Ltd (Shaanxi, China). Casein and gelatin were used as the primary protein sources. Fish oil and starch were used as the lipid and carbohydrate sources, respectively. Three experimental isonitrogenic and isoenergetic purified diets were prepared, containing 0 (control diet), 5, or 10 g SA/kg diet (referred to as Con, SA5, and SA10 respectively). Similar SA inclusion levels had been used in other reports (Gu et al., 2014; Krogdahl et al., 2015). The composition of basal purified diet was adopted from previous study (NRC, 2011). The ingredients and approximate compositions of the diets were shown in Table 1. The ingredients were grounded into fine powder and passed through a 123- μ m mesh, mixed well, with fish oil added afterwards. Water (300 mL/kg dry ingredients) was added and mixed to produce flakes with tablet press machine. The flakes were dried for 8 h in a ventilated oven at 40 °C, crushed, and sieved into small pellets (250–425 μ m), and stored at –20 °C.

2.2. Experimental Animals

Zebrafish was provided by the Key Laboratory of Marine Drugs, Ocean University of China, and maintained in nine 5 L tanks (30 fish in each tank) at 28 °C under a 14 h light: 10 h dark cycle and fed *ad libitum* twice daily with yolk or brine shrimp (*Artemia*) until

Table 1

Composition and proximate analysis of the experimental diets.

Ingredients	Soybean saponin level (g/kg diet)		
	0	5	10
Casein	388	388	388
Gelatin	97	97	97
Fish oil	115	115	115
Starch	280	280	280
Cellulose	55	50	45
Monocalcium phosphate	10	10	10
Vitamin premix ¹	10	10	10
Mineral premix ²	40	40	40
Choline chloride	5	5	5
Soybean saponin	0	5	10
Total	1000	1000	1000
<i>Proximate composition (g/kg diet)</i>			
Dry matter	916.4	917.0	917.6
Crude protein	485.2	483.5	489.5
Crude lipid	115.8	115.1	115.8
Crude ash	32.2	33.4	31.2

1. Vitamin premix contained (mg/g mixer) thiamin hydrochloride, 5 mg; riboflavin, 5 mg; calcium pantothenate, 10 mg; nicotinic acid, 6.05 mg; L-ascorbyl-2-monophosphate-Mg, 3.95 mg; pyridoxine hydrochloride, 4 mg; folic acid, 1.5 mg; inositol, 200 mg; menadione, 4 mg; alpha-tocopherol acetate, 50 mg; retinyl acetate, 60 mg; biotin, 0.6 mg. All ingredients were diluted with alpha-cellulose to 1 g. 2. Mineral premix contained (g/kg diet) calcium biphosphate, 13.58 g; calcium lactate, 32.7 g; FeSO₄·6H₂O, 2.97 g; magnesium sulfate, 13.7 g; potassium phosphate dibasic, 23.98 g; sodium biphosphate, 8.72 g; sodium chloride, 4.35 g; AlCl₃·6H₂O, 0.015 g; KI, 0.015 g; CuCl₂, 0.01 g; MnSO₄·H₂O, 0.08 g; CoCl₂·6H₂O, 0.1 g; ZnSO₄·7H₂O, 0.3 g.

28 days post-fertilization (dpf). The rearing water was run continuously through a gravel-activated charcoal filter in a recirculation system. The mean pH and dissolved oxygen concentration were 7.1 and 6.2 mg/L, respectively. All experiments were conducted with approved protocol in Ocean University of China (Permit Number: 20001001).

2.3. Zebrafish feeding experiment and sample collection

Before the experiments started, zebrafish (28 dpf) were conditioned with control diet for 7 days. The fish were then fasted for 24 h, anesthetized with 80 mg/L MS-222, weighed, and randomly assigned to 12 tanks (5 L), with 20 fish per tank. Each diet was randomly assigned to four tanks. The initial weight for these 35 dpf mixed-sex zebrafish was 9.02 ± 0.16 mg. To reduce pellet waste, the fish were slowly hand-fed until apparent satiety. The fish were fed four times a day, at 08:45, 12:45, 16:45, and 20:45. The feeding trial lasted for 2 weeks, a period that has been used in previous nutritional studies in zebrafish (Gómez-Requeni et al., 2011).

After 2 weeks of feeding trial, fish were fasted for 24 h. Three fish from each tank were removed with a dip net and submerged in chilled water (2–4 °C) for euthanization. These sampled fish were first weighed to determine the specific growth ratio (SGR) and photographed by stereoscope (SMZ-1000, Nikon, Tokyo, Japan) to measure the body length. Three fish in each tank were sampled for histological evaluation. They were placed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 24 h and then stored in 70% ethanol until further processing. Three other fish from each tank were placed in 2 mL microcentrifuge tubes, immediately placed in RNAlater (Ambion® Life Technologies, Carlsbad, CA, USA) at 4 °C for 24 h, and finally were stored at –80 °C until RNA analysis. The remaining fish were then fed a single meal of their allocated diet until visible satiety. Three sampled fish from each tank were collected at 3 h after refeeding, and were frozen in liquid N₂ and stored at –80 °C for Western blotting.

2.4. Zebrafish liver cell culture and treatment

Zebrafish liver cell line (ZFL, ATCC CRL-2643) was grown in a standard culture medium of Leibovitz's L-15, Dulbecco's Modified Eagle's Medium (DMEM), and Ham's F12 (50:35:15) supplemented with 10 mg/ml insulin, 5% fetal bovine serum, and 50 ng/ml epidermal growth factor (EGF) at 28 °C, as recommended by ATCC. For experiments, ZFL cells were grown in L-15 medium without amino acid with a gradient levels (0, 50, 100, 150, 200 µM) of SA (Sigma-Aldrich, S9951) for 12 h. Subsequently, the medium was replaced with standard L15 medium with different concentrations of SA (0, 50, 100, 150, 200 µM) and 1% insulin for 15 min to stimulate TOR activity. Cells were lysed in RIPA (50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 1 mM EDTA, pH 7.5), with 1 mM/ml phenylmethanesulfonyl fluoride (Amresco, Ohio, USA) and 1 mM/ml sodium orthovanadium (Beyotime, Shanghai, China) for 1 h. The cell lysates were cleared by centrifugation and subjected for Western blotting. All cell culture reagents were purchased from Invitrogen (USA). Experiments were conducted for at least 6 repeats.

2.5. Sample analyses

2.5.1. Histology

Three fish from each treatment group were used for histological evaluation. The whole fish were dehydrated in ethanol, equilibrated in xylene, embedded in paraffin, and sectioned (5 µm), according to standard histological techniques. The tissues were stained with hematoxylin and eosin. A blind histological examination was performed under a light microscope, with particular attention to the intestinal structure, and morphological appearance of the intestine was assessed using a categorical scoring system with grades of normal, moderate and abnormal, and the semiquantitative scoring system was adapted from Iwashita et al. (2009).

2.5.2. Quantitative real-time PCR

qRT-PCR reactions were conducted as described in previous study (Tian et al., 2015). Elongation factor 1 alpha 1 (*ef1*) was selected as the normalization gene as no expression differences were observed among treatments. The primers used for these genes were given in Table 2. The threshold cycle (C_T) values were determined using the LightCycler software. The data (normalized C_T values) from the treated and control templates were compared and the $2^{-\Delta\Delta CT}$ method was used for the relative quantification of transcript expression. All primers were synthesized by BGI (Beijing, China), and all kits were provided by Takara Biotech (Dalian, China).

2.5.3. Protein extraction and Western blotting analysis

The whole fish tissues were homogenized with glass Tenbroeck tissue grinder (Kimble Chase, New Jersey, USA) on ice and lysed in RIPA with 1 mM/ml phenylmethanesulfonyl fluoride (a protease inhibitor; Amresco, Ohio, USA) and 1 mM/ml sodium orthovanadium (Beyotime, Shanghai, China). The protein concentrations were measured with the bicinchoninic acid assay method (Beyotime). All samples were adjusted to equal concentrations. Tissue lysates (20 µg) were separated by SDS-PAGE and transferred to 0.45 µm polyvinylidene difluoride membranes (Millipore) for Western blotting analysis. The membranes were blocked for 2 h with 5% nonfat dry milk (Bio-Rad) in wash buffer (Beyotime), and then probed with primary antibody overnight at 4 °C. The membranes were washed in wash buffer and probed with the appropriate HRP-conjugated secondary antibody and detected with ECL Western blotting substrate (Thermo, Massachusetts, USA). Densitometry analyses were performed with the Image J software (National

Institutes of Health). Antibodies directed against NPY (Cat. No. 11976), IGFBP-2 (Cat. No. 3922), AMPK α (Cat. No. 2532), phosphor-AMPK α (Thr172) (Cat. No. 2531), phospho-TOR (Ser2448, P-TOR) (Cat. No. 2971), TOR (Cat. No. 2972), phospho-ribosomal protein (Ser235/236, P-S6) (Cat. No. 4856), S6 (Cat. No. 2217), phospho-ribosomal protein S6 kinase (Thr 389; P-S6K1) (Cat. No. 9205), S6K1 (Cat. No. 9206), phospho-4E-binding protein 1 (Thr37/46; P- 4E-BP1) (Cat. No. 9459), 4E-BP1 (Cat. No. 9452), and β -tubulin (Cat. No.2146) were purchased from Cell Signaling Technology Inc. They have been used successfully in previous studies in zebrafish (Mendelsohn et al., 2008; Yuan et al., 2013) and all the antigenic regions against these antibodies were conserved in zebrafish.

2.6. Statistical analysis

Each parameter was tested for each sampled fish and tested in triplicate. The data were analyzed with one-way ANOVA and Tukey's multiple range test. The data were expressed as means and their SD in the tables or figures. Statistical significance was defined as $P < .05$. All statistical analyses were performed with SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of SA on growth performance and feed efficiency

Dietary SA significantly reduced final bodyweight, SGR, the survival rate, and the feed efficiency rate (FER) compared with those of fish fed with the SA-free diet (Table 3). Compared to control diet, SA10, but not SA5, significantly reduced body length and feed intake ($P < .05$). The final body weight, survival rate, SGR, and FER were decreased by 16.72%, 14.02%, 9.03%, and 17.53% respectively, in the SA5; and decreased by 34.75%, 20.93%, 22.64%, and 28.47%, respectively, in SA10 when compared to those of the control group ($P < .05$).

3.2. Effects of SA on intestinal morphology

The morphology of the intestine was significantly affected in the SA-fed groups (Table 4, Fig. 1). Fish fed the control diet have a simple fold height and less enterocyte vacuolization than the fish fed the SA-supplemented diets. Feeding the SA5 diet resulted in moderate morphological changes, and had a shortening of the height of the mucosal folds compared with feeding the control diets. Most of the samples (9/12) from fish fed diet SA10 showed serious abnormalities in the epithelial cells, and hyperplastic connective tissue in the mucosal folds and submucosa occurred extensively.

3.3. Effects of SA on daily feed intake and related genes

In the present study, daily feed intake was not influenced by SA5 but reduced during the late stage of trial (10th day–14th day) in SA10 ($P < .05$) (Fig. 2A). Meanwhile, the levels of major appetite-control hormones were analyzed. The mRNA expressions of neuropeptide Y (*npy*) and ghrelin (*ghrl*) were significantly increased, while that of cholecystokinin (*cck*) was significantly decreased ($P < .05$) in SA groups (Fig. 2B). The protein level of NPY was also increased in SA groups (Fig. 2C) ($P < .05$).

3.4. Effects of SA on cellular and systemic nutrient sensing networks

The activities of TOR signaling were found to be significantly influenced by SA in the present study. As shown in Fig. 3, SA signif-

Table 2
Nucleotide sequences of primers and cycling conditions used for PCR amplification.

Gene ¹	Accession no. 2	Forward primer (5'–3')	Reverse primer (5'–3')	Tm (°C)	Amplicon size (bp)
<i>Appetite</i>					
<i>npy</i>	NM_131074	CGCGTTTCTCTGTTCGTC	ACCTTTTCCCATACCTCTGC	58	161
<i>ghrl</i>	EU908735	GTCCTGTGTGTTCTCTTTCC	TCTCTTCTGCCACTCTTGG	59	114
<i>cck</i>	XM_001346104	GTTCACTTAATGTCCGGCTCC	TAGTTCGGTTAGGCTGCTGC	59	129
<i>GH/IGF-I axis</i>					
<i>gh</i>	AJ937858	AGGTCTTATGCCTGAGGAACG	AAGGTCTGGCTGGGAAACTC	59	177
<i>ghra</i>	NM_001083578	AAGCCAGACTTACCACGAG	GCTCTCCCTGTTCAAGTGGT	59	241
<i>ghrb</i>	NM_001111081	CTAATACACCAACAGCCCCAC	CCTGGAGAAGGCTTGAGAAC	58	220
<i>igf1</i>	NM_131825	ACACAGGGGGCAGAACTAT	AAGATGGGGCTTAAACGTC	58	196
<i>igfbp-2</i>	NM_131458	TACCGCTCGCTCAGAAATG	GGATAACACCCGAGACCACT	58	198
<i>Key enzymes of nutrient metabolism</i>					
<i>glut</i>	NM_181559	TGTGAAGACTTCGGTGTGGT	TGCGGATGTAGATTGTGC	59	175
<i>glud</i>	NM_199545	CCATCCCTATTGTACCCACTG	TTGACGTAAGCCGCTGTTT	58	172
<i>fas</i>	XM_682295.5	ATGGAGTTTTACAGGCGAG	GGGAATAATATGCGGTGGC	58	175
<i>lpl</i>	BC064296	GAATACACGCGAGAAGGA	CAGTTTCCGAATGTGAAGG	58	188
<i>hsl</i>	XM_003200544	CAGGCACGGAAGAGGATACA	TGACAGCATACCGAACGCG	59	126
<i>hmgcr</i>	NM_001079977	CATTTTCTTTGAGCAGTGG	GCTTGGTGAATAGGGGAT	58	128
<i>gck</i>	BC122359	TGAGGATGAAGAGCGAGGC	AGAGAAGGTGAATCCAGCG	58	178
<i>pk</i>	NM_201289.1	CAAAGGACACTTCCCTGTAGAG	GGACAACGAGGACGATAACG	58	249
<i>pck</i>	NM_214751	GTGAAGTGAACGAGACTG	AGCACTTGAGAGCAACGAT	59	192
<i>fbp</i>	NM_199942	CATCTGTATGGGATGCTGG	TTACCCCTCTATCTGGCTC	59	173
<i>g6pd</i>	XM_692728.6	GCCTCCCTCAGCACATAGA	ATGGGGATGCCCTCGTATT	59	170
<i>Reference gene</i>					
<i>efz1</i>	XM_005173785	TGTCCTCAAGCCTGGTATGG	TGGGTCGTTCTTGCTGTCTC	58/59	190

1. The gene symbols are from <http://zfin.org>. *cck*, cholecystokinin; *efz1*, elongation factor 1 alpha 1; *fas*, fatty acid synthetase; *fbp*, fructose-1,6-bisphosphatase; *gck*, glucokinase; *gh*, growth hormone; *ghra*, growth hormone receptor A; *ghrb*, growth hormone receptor B; *ghrl*, ghrelin; *glud*, glutamate dehydrogenase; *glut*, glutamine synthetase; *g6pd*, glucose-6-phosphate dehydrogenase; *hmgcr*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *hsl*, hormone-sensitive lipase; *igf1*, insulin-like growth factor I; *igfbp-2*, insulin-like growth factor-binding protein 2; *lpl*, lipoprotein lipase; *npy*, neuropeptide Y; *pck*, phosphoenolpyruvate carboxykinase; and *pk*, pyruvate kinase.
2. Accession numbers are from www.ncbi.nlm.nih.gov.

Table 3
Growth performance and biometric parameters in zebrafish fed diets containing different SA levels for 2 weeks.

Groups	Initial body weight (mg)	Final body weight (mg)	Feed intake (mg/fish)	Survival Rate (%) ^{a)}	Length (cm)	SGR ^{b)}	FER ^{c)}
Con	9.06 ± 0.18	66.42 ± 5.40 ^a	91.1 ± 4.2 ^a	95.6 ± 3.85 ^a	1.58 ± 0.07 ^a	14.4 ± 0.72 ^a	63.3 ± 4.05 ^a
SA5	9.06 ± 0.11	55.29 ± 5.56 ^b	87.5 ± 3.1 ^a	82.2 ± 3.85 ^b	1.50 ± 0.10 ^{ab}	13.1 ± 0.96 ^b	52.2 ± 3.66 ^b
SA10	9.06 ± 0.10	43.34 ± 3.85 ^c	76.2 ± 2.7 ^b	75.6 ± 3.85 ^b	1.42 ± 0.19 ^b	11.14 ± 0.70 ^c	45.3 ± 4.13 ^b

a). Survival rate = 100 × final fish number / initial fish number.

b). Specific growth ratio (SGR) = 100 × ln(final weight / initial weight) / days.

c). Feed efficiency rate (FER) = 100 × wet weight gain / dry feed consumed.

Data was shown as means ± SD. Figures in the same column with different letters indicate a significant difference using Tukey's multiple range test ($P < .05$).

Table 4
Occurrence of morphological changes in the intestine of zebrafish fed diets containing different SA levels for 2 weeks (n = 12).^{a)}

Groups	Normal	Moderate	Abnormal
Control	10	2	0
SA5	2	7	3
SA10	0	3	9

a). The numbers represent the number of fish. In each treatment group, twelve fish were used for histological evaluation.

icantly increased the phosphorylation of AMPK α ($P < .05$), while reduced that of TOR, S6K1, S6, and 4E-BP1 in zebrafish ($P < .05$). As shown in Fig. 4, when ZFL cells were treated with gradient levels of SA, the phosphorylation of TOR, S6K1, S6 and 4E-BP1 was decreased while that of AMPK α was increased.

SA also influenced the activities of GH/IGF signaling axis. As shown in Fig. 5, SA increased the mRNA expressions of growth axis genes including *gh*, *igf1*, growth hormone receptor A (*ghra*), and growth hormone receptor B (*ghrb*) ($P < .05$), but decreased insulin-like growth factor-binding protein 2 (*IGFBP-2*) at both mRNA and protein levels ($P < .05$).

3.5. Effects of SA on metabolism

The effects of SA on the expression levels of key enzymes in metabolism were analyzed. On protein metabolism related genes, dietary SA increased mRNA expressions of glutamine synthetase (*glut*) and glutamate dehydrogenase (*glud*) ($P < .05$) (Fig. 6A). Among the lipid metabolic enzymes, hormone-sensitive lipase (*hsl*) and lipoprotein lipase (*lpl*) mRNAs were up regulated with increasing dietary SA levels ($P < .05$). 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*hmgcr*) was downregulated in SA10 ($P < .05$), but was unchanged in SA5 ($P > .05$). No significant change of fatty acid synthetase (*fas*) expression was observed among groups ($P > .05$) (Fig. 6B). On glucose metabolism related genes, SA significantly increased the mRNA expressions of fructose-1,6-bisphosphatase (*fbp*). SA5 had no effect on the expression of the genes encoding glucose-6-phosphate dehydrogenase (*g6pd*) expression ($P > .05$), but increased the expression of the genes encoding glucokinase (*gck*) ($P < .05$). SA10 up regulated the expression of *g6pd* ($P < .05$), but down regulated that of *gck* ($P < .05$). Dietary SA had no significant effect on pyruvate kinase (*pk*) or phosphoenolpyruvate carboxykinase (*pck*) expressions ($P > .05$) (Fig. 6C).

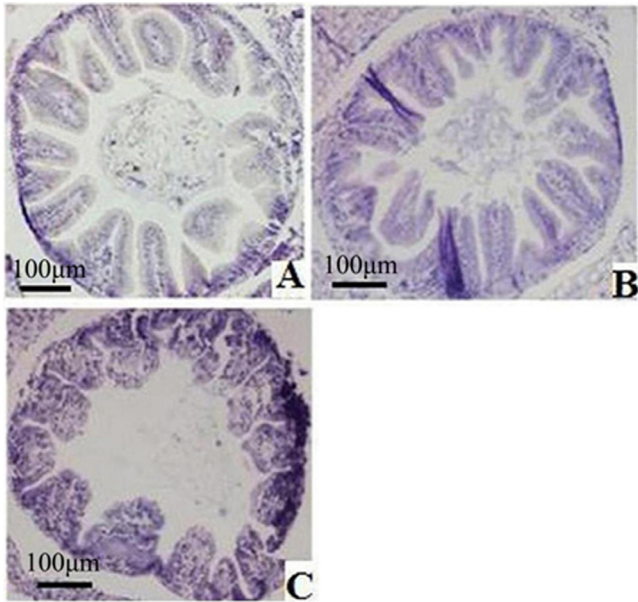


Fig. 1. Histological sections of zebrafish. (A) The fish fed control diet; (B) the fish containing 5 g soybean saponin per kg diet; (C) the fish fed containing 10 g soybean saponin per kg diet.

4. Discussion

The present study, as well as other studies, clearly demonstrated that dietary SA reduced feed intake and growth performance in fish, and damaged the morphology of the intestinal mucosa (Bureau et al., 1998; Chen et al., 2011; Iwashita et al., 2009; Knudsen et al., 2008). Contrasted to previous reports that mainly focused on the effects of SA on cell permeability and membrane homeostasis (Bureau et al., 1998; Chen et al., 2011; Francis et al., 2002; Gu et al., 2014), the present study was designed to examine the effects of SA on the molecular responses of nutrient sensing, appetite control, and metabolism after dietary SA treatment for 2 weeks in zebrafish.

TOR was the major checkpoint controlling cellular protein synthesis and metabolism. Upon postprandial activation, TOR phosphorylates downstream effectors including S6K1 and 4E-BP1, which regulate protein translational initiation (Jewell and Guan, 2013; Laplante and Sabatini, 2012), while GH/IGF-I axis acts as the systemic nutrient sensor and controls somatic growth (Cannata et al., 2010; Reindl and Sheridan, 2012). In the present study, we found that SA significantly inhibited TOR signaling with reduced phosphorylation levels of TOR, 4E-BP1, S6K1 and S6 in feeding trial as well as in ZFL cells. This is the first report on the effect of SA on TOR signaling. However, several other structurally distinct saponins have been demonstrated to inhibit TOR signaling and growth of cancer cell lines (Auyeung et al., 2010; Tai et al., 2016; Zhan et al., 2016a,b). In the present study, SA was also found to stimulate the phosphorylation of AMPK. Similar result was also found in other saponins (Duan et al., 2016). AMPK is a key regulator of energy balance expressed ubiquitously in eukaryotic cells, and acts to restore energy homeostasis by promoting catabolic pathways, resulting in ATP generation, and inhibiting anabolic pathways that consume ATP (Shaw et al., 2004). In general, inhibition of TOR signaling and activation of AMPK would consequently switch on catabolic and anabolic pathways. This was further confirmed by examination of the levels of key enzymes in metabolism, as discussed below.

GH/IGF signaling has a major role in the control of growth and metabolism in fish (Cannata et al., 2010; Reindl and Sheridan,

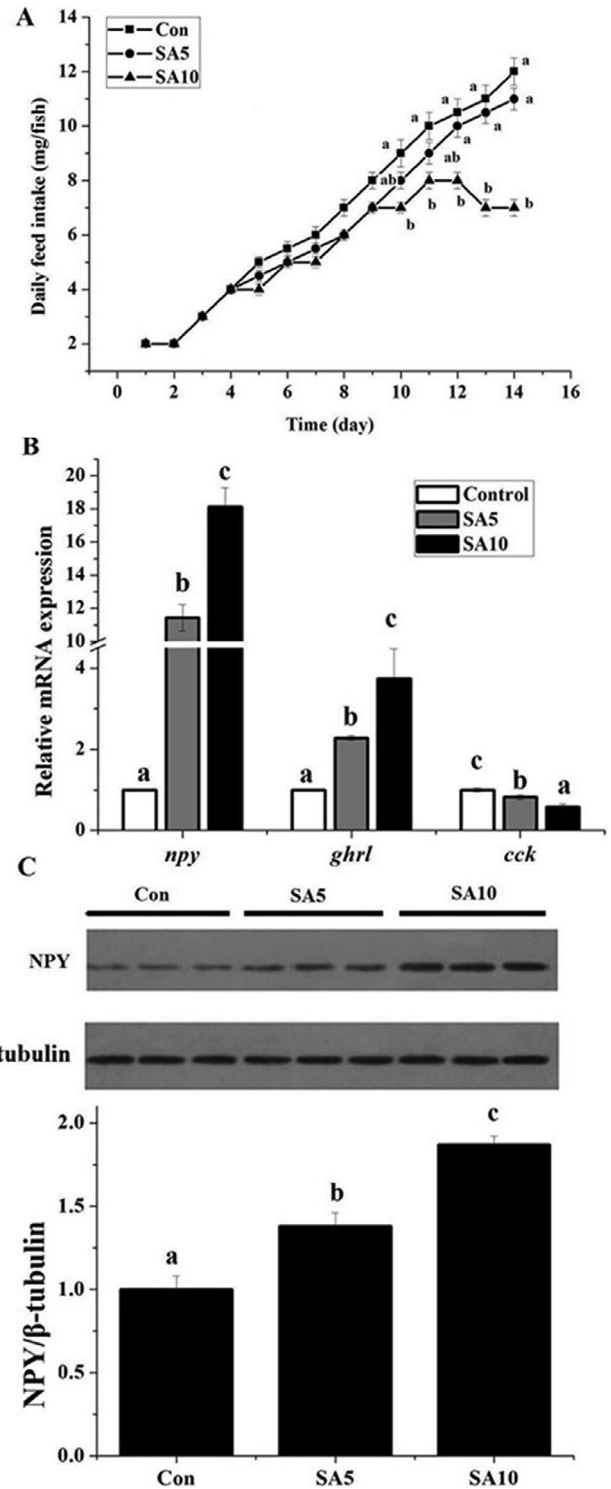


Fig. 2. The effects of dietary soybean saponins on feeding in zebrafish. A: daily feed intake ($n = 4$ tanks); B: the relative mRNA expression of feeding-related genes ($n = 12$ fish); C: the ratio between the treatment group and the control groups of NPY/ β -tubulin protein level ($n = 12$ fish). Data was shown as mean \pm SD. Means with different letters in the same time or columnar were significantly different using Tukey's multiple range test ($P < .05$). Error bars represented standard deviation (SD).

2012). GH/IGF signaling was down regulated during fasting in fine flounder (Safian et al., 2012) and after dietary fishmeal replacement in turbot (Safian et al., 2012). In the present study, SA was found to increase the mRNA expressions of GH/IGF axis genes,

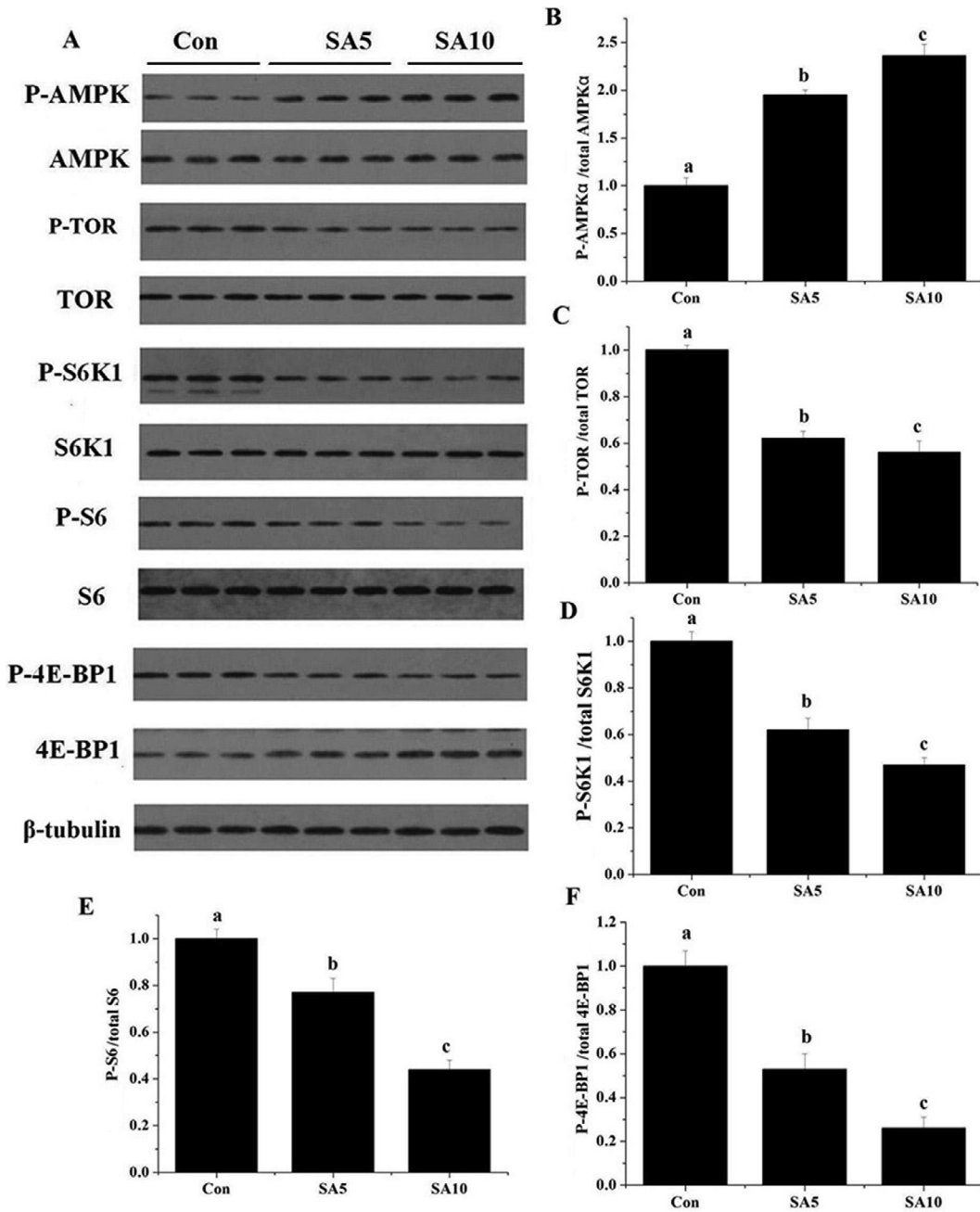


Fig. 3. The effects of dietary soybean saponins on TOR signaling pathway. Data was shown as mean \pm SD (n = 12). Means with different letters in the same pattern were significantly different using Tukey’s multiple range test ($P < .05$). Error bars represented SD.

including *gh*, *igf1*, *ghra*, and *ghrb*. On the other hand, SA down regulated both mRNA and protein levels of IGFBP-2, which served as an antagonist for IGF1 binding to its receptor (Duan, 2002). Such GH/IGF signaling stimulatory effect was also found with *Trigonella foenum-graecum* saponins in tilapia (Khalil et al., 2008) and *Acanthopanax senticosus* saponins in weaned pigs (Kang et al., 2010). While saponins are generally considered as anti-nutrient, it has been shown in several experiments that low dietary *Quillaja saponaria* (South American soap-bark tree) saponins increased nutrient utilization and growth performance of carp (*Cyprinus carpio*) (Serrano, 2013) and tilapia (*Oreochromis niloticus*) (Francis et al., 2001) respectively. It seems that dietary inclusion of saponins might be beneficial at low level but detrimental when it was high, which was the case in the present study and previous

reports (Bureau et al., 1998; Chen et al., 2011; Gu et al., 2014, 2015; Iwashita et al., 2009; Knudsen et al., 2007, 2008; Krogdahl et al., 2015)

Animals generally respond to nutritional status by triggering either energy-consuming anabolic pathways or energy-producing catabolic pathways (Song et al., 2016; Tian et al., 2015; Shaw et al., 2004). Such metabolic switches are modulated by nutrient sensing such as AMPK/TOR pathways (Dibble and Manning, 2013). In the present study, SA was found to down regulate the expression of *hmgcr*, consistent with previous reports on the hypocholesterolemic effect of SA in fish (Gu et al., 2014; Chantranupong et al., 2015; Kortner et al., 2012, 2013). Furthermore, the up regulated levels of amino acid degradation (*glu* and *glud*) and lipolysis (*lpl* and *hsl*) enzymes suggested the increased

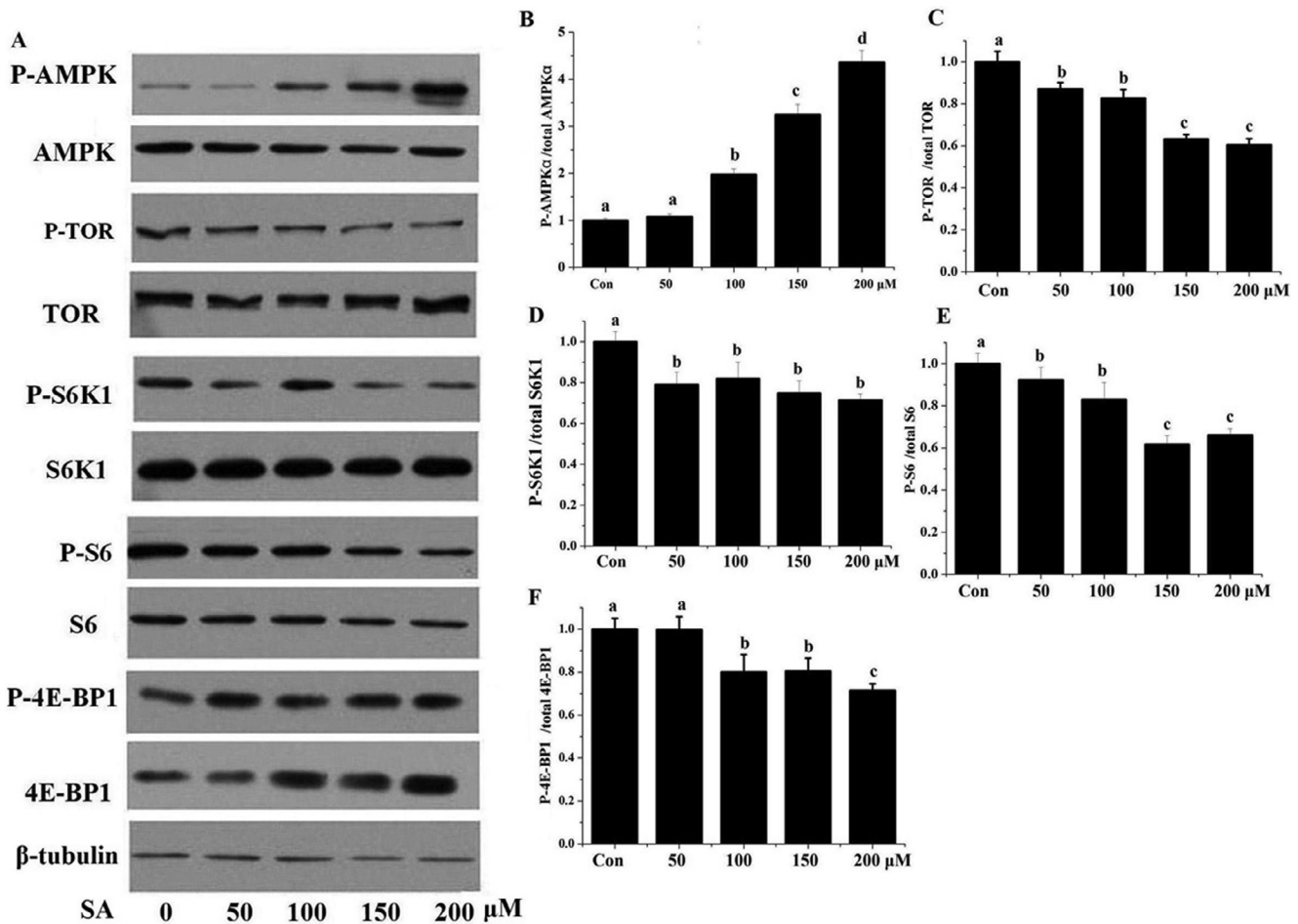


Fig. 4. Effects of soybean saponins on the TOR signaling pathways in the ZFL cells. Data were shown as mean \pm SD ($n = 6$). Means with different letters in the same pattern were significantly different using Tukey's multiple range test ($P < .05$). Error bars represent SD.

catabolism under SA treatment. Meanwhile, the mRNA levels of genes involved in glucose synthesis and the pentose phosphate shunt increased, suggesting the stimulated need for energy production, a metabolic consequence of activation of AMPK, and finally cause leading to growth performance decreasing under SA treatment.

In the present study, daily feed intake was reduced during the late experimental period in SA10 group. This was consistent with previous reports (Bureau et al., 1998; Chen et al., 2011; Iwashita et al., 2009; Knudsen et al., 2008) that demonstrated that SA inclusion reduced feed intake. It is well known that soybean saponin is the primary cause of the bitterness and astringency tastes of soybeans (Okubu et al., 1992). This should also be the likely reason for the reduced feed intake in this experiment by SA. We also examined the neuroendocrine pathways including the peripheral satiety system (short-term system) and the central feeding system (long-term system) (Volkoff and Peter, 2006; Volkoff et al., 2005), which are regulated by AMPK/TOR pathways within the hypothalamus (Jewell and Guan, 2013; Laplante and Sabatini, 2012; Minokoshi et al., 2004). NPY is the key molecule in the central feeding system, while CCK and ghrelin are neuropeptides involved in the peripheral satiety system (Volkoff et al., 2005). Both NPY and ghrelin are orexigenic, while CCK is anorexigenic in fish (Volkoff and Peter, 2006; Volkoff et al., 2005; Tian et al., 2015). In the present study the levels of *npy* and *ghrelin* were upregulated, and that of *cck* was downregulated by SA. This can be a consequence of

inhibited TOR signaling and increased AMPK activity (Minokoshi et al., 2004; Cota et al., 2006).

5. Conclusions

The present study, along with our recent report on gossypol (Bian et al., 2017), clearly demonstrated that certain ANFs significantly influenced nutrient sensing signaling cascades and metabolism. These results should provide new aspects on the understanding how ANFs influenced the feed utilization. On the other hand, these studies add ANFs into the expanding list of dietary parameters, including protein levels, amino acid composition, lipids, carbohydrate, etc. (Song et al., 2016; Xu et al., 2016; Avruch et al., 2009; Chantranupong et al., 2015; Xiong et al., 2013; Bian et al., 2017), that can modulate nutrient sensing networks. A better evaluation of diet quality at molecular level should be very useful for the improvement of feed utilization in animals.

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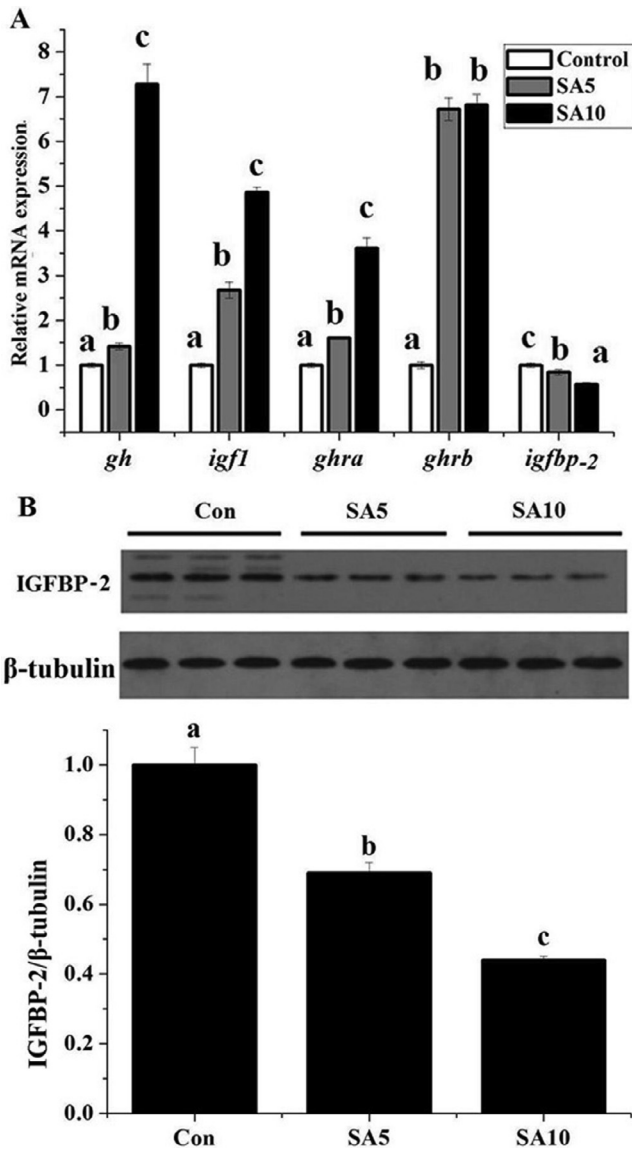


Fig. 5. The effects of dietary soybean saponins on GH/IGF signaling in zebrafish. A: the relative mRNA expression of growth axis genes; B: the ratio between the treatment group and the control groups of IGFBP-2/β-tubulin protein level. Data was shown as mean ± SD (n = 12 fish). Means with different letters in the same gene or protein were significantly different using Tukey's multiple range test ($P < .05$). Error bars represented SD.

Yun Li, Jianfeng Zhou, and Yunzhang Liu for help with the feeding trial. G. H., J. T. and K. M. designed the research; J.T., K. W., X. W., H. W., and C. L. conducted the experiments and analyzed the data; G. H., J.T. and H. Z. wrote the paper; all the authors have read and approved the final manuscript.

The authors declare no conflict of interest.

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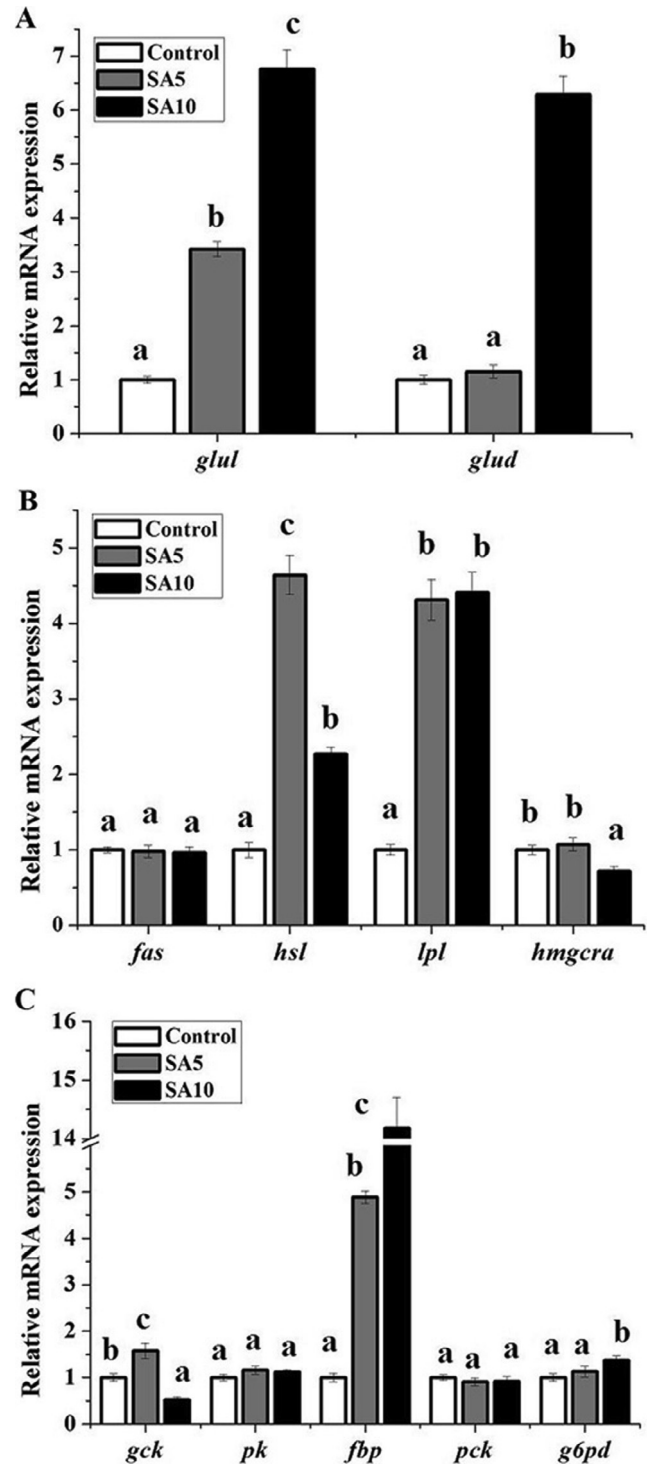


Fig. 6. Effects of dietary soybean saponins on metabolic enzymes in zebrafish. (A) protein metabolism enzymes; (B) lipid metabolism enzymes; (C) carbohydrate metabolism enzymes. Data was shown as mean ± SD (n = 12 fish). Means with different letters in the same gene were significantly different using Tukey's multiple range test ($P < .05$). Error bars represented SD.

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