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Substituting dietary fishmeal with soybean meal isolate influences hepatic lipid metabolism and gut microbiota in spotted seabass (*Lateolabrax maculatus*)

Zhe Wang, Linkun Yang, Chunxiao Zhang, Xueshan Li, Ling Wang, Kangle Lu, Kai Song*

Key Laboratory for Feed Quality Testing and Safety Evaluation, Fisheries College of Jimei University, Xiamen 361021, China

Key words: soy protein isolate; fishmeal replacement; lipid metabolism; gut microbiota; spotted seabass

Abstract

This study was conducted to investigate the effects of partial substitution of dietary fishmeal (FM) by soy protein isolate (SPI) on lipid metabolism and gut microbiota of spotted seabass. A diet containing 30% FM formed the basal diet (FM), and two SPI diets were formulated in which 25% (SPI25) and 50% (SPI50) of FM were replaced by SPI. Each diet was fed to triplicates of fish for 56 days. The results showed that replacing dietary FM with SPI reduced triglyceride and cholesterol contents in the liver and serum, and the hepatic lipid droplets area was also decreased by dietary SPI inclusion. Furthermore, replacement of dietary FM with SPI markedly down-regulated the mRNA expression of genes involved in lipogenesis (*srebpc-1c*, *fas*, *acc1*, *hmgcr*, *ppary* and *chrebp*) and lipolysis (*atgl*, *hsl*, *ppara*, and *cpt1*). Moreover, high-throughput sequencing analyses of gut microbiota revealed that dietary SPI inclusion dramatically decreased the Firmicutes/Bacteroidetes ratio. Overall, this study indicated that replacing 25-50% of dietary FM with SPI could reduce lipid accumulation in serum and liver of spotted seabass, which was associated with the suppressed hepatic lipogenesis and the remodeled gut microbiota.

* Corresponding author. Kai Song, e-mail: songkai@jmu.edu.cn

Introduction

Sustainable development of aquaculture depends on identifying viable alternatives to fishmeal (FM). Soy protein isolate (SPI), which is the most refined soy protein product, is produced through aqueous solubilization followed by isoelectric precipitation. Most of the anti-nutritional factors (ANFs) contained in SPI are eliminated during the production process, and the protein contents could be extended to over 90% (Deng et al., 2009). Accordingly, SPI has shown promise as a fishmeal replacer in the aquaculture industry. Previous studies in fish showed that replacing dietary SPI with FM affected the growth performance, feed utilization, nutrients digestibility, hematological parameters, ect. (Blaufuss and Trushenski, 2012; Nepal et al., 2018; Riche and Williams, 2011; Yaghoubi et al., 2016). In addition, the meta-analyses of human studies revealed that soy protein intake was associated with the decreases in serum triglycerides (TG), cholesterol, and low-density lipoprotein cholesterol (LDL-C) (Anderson et al., 1995; Zhan and Ho, 2005). Similarly, Nepal et al. (2018) noted that significantly reduced plasma cholesterol was caused by replacing dietary FM with SPI in common carp (*Cyprinus carpio*). Also, Riche and Williams (2011) found that the body lipid of Florida pompano (*Trachinotus carolinus*) dramatically decreased with increasing dietary SPI levels. The above findings indicated the potential lipid-lowering activity of SPI versus animal-origin proteins. However, the mechanisms through which SPI exerts its effects on lipid metabolism in fish have not been well investigated.

Spotted seabass (*Lateolabrax maculatus*) is a major aquaculture species in Asia due to its high economic value and delicious taste. In recent years, the intensification of aquaculture and high dietary lipid levels favors the occurrence of fatty liver in this species associated with abnormal lipid accumulation (Xie et al., 2021). To date, research concerning the effects of replacing FM with SPI in this species is lacking. Accordingly, the current study aimed to investigate substituting dietary FM with SPI on lipid metabolism and gut microbiota in spotted seabass.

Materials and methods

This research was approved by the Committee on the Ethics of Animal Experiments of Jimei University (Xiamen, China).

Diet formulation and feeding trial

Three experimental diets were formulated to contain 44% crude protein and 11% crude lipid (**Table 1**). A basal diet was prepared to include 30% FM. We designed two other experimental diets prepared by replacing 25 or 50% of FM with SPI (referred to as FM, SPI25, SPI25, respectively). The procedures of diet formulation were conducted as described in our recent work (Cheng et al., 2021).

Spotted seabass juveniles were obtained from a commercial hatchery (Zhangzhou, China) and transported to the fisheries laboratory of Jimei University. All fish were fed a basal diet for 14 days to become acclimated to the conditions and experimental diets. Then, a total of 225 fish (similar initial weight, 8.75 ± 0.20 g) were distributed into nine 20-L tanks supplied with fresh water at a density of 25 fish per tank. Three tanks were randomly assigned to each group, and fish were hand-fed to apparent satiation twice daily (8:00, 18:00) for 8 weeks. Almost 35% of water in each tank was renewed daily. The feeding trial was conducted in the recirculating culture system, and water quality was measured weekly. Generally, the water temperature was maintained at 27.0 ± 1.0℃, the dissolved oxygen content was approximately 8.0 mg/L, water pH was 7.5-8.5, ammonia was below 0.1 mg/L, and a 12 h light/12 h dark photoperiod was maintained with fluorescent lights during the feeding trial.

Table 1 Formulation and proximate composition of the experimental diets.

Ingredients (g/kg)	Diet designation		
	FM	SPI25	SPI50
Brown fishmeal	300.0	225.0	150.0
Soy protein isolated	0.0	60.0	120.0
Wheat flour	312.4	321.7	329.9
Wheat gluten	130.0	130.0	130.0
Poultry by-product meal	80.0	80.0	80.0
Fish oil	20.0	24.0	29.0
Soybean oil	25.0	25.0	25.0
Lecithin	20.0	20.0	20.0
Squid paste	20.0	20.0	20.0
Microcrystalline cellulose	50.0	50.0	50.0
Vitamin C	0.5	0.5	0.5
Monocalcium phosphate	20.0	20.0	20.0
DL-Methionine	3.2	4.2	5.3
Lysine monohydrochloride	4.9	5.6	6.3
Choline chloride	5.0	5.0	5.0
Mineral premix[1]	5.0	5.0	5.0
Vitamin premix[2]	3.0	3.0	3.0
Y_2O_3	1.0	1.0	1.0
Proximate compositions (%)			
Crude protein	43.68	43.74	43.97
Crude lipid	11.07	11.21	11.29

[1, 2] Mineral premix and vitamin premix were prepared according to our recent research on spotted seabass (Cheng et al., 2021).

Sample collection

At the end of the feeding trial, twelve fish per tank were anesthetized with eugenol (1: 10000). The total length was determined, and fish blood was collected from the caudal vein using a sterile syringe and stored at 4℃ overnight. Serum samples were then separated by centrifugation (4000 ×*g*, 10 min, 4℃) and stored at −80℃. Liver samples of two fish per tank and fixed into 4% paraformaldehyde for histology analysis. Livers were collected from additional fish in each tank were sampled for RNA extraction and biochemical

parameters. Mid intestine samples of two fish in each tank were collected and pooled for microbiota analysis. All analyses were performed in triplicates.

Histology analysis and biochemical parameters

Liver tissues were stained with Oil Red O by Service Biotechnology Co., Ltd. (Wuhan, China) following the standard histological procedures for histology analysis. Besides, liver samples were homogenized in phosphate-buffered saline (PBS) and centrifuged (4000 ×*g*, 10 min, 4℃) later. Then, the supernatant was collected and stored at −80℃ for subsequent analysis. Liver samples were used to detect the contents of TG, total cholesterol (TC), and total bile acid (TBA), as well as activities of lipoprotein lipase (LPL) and hepatic lipase (HL). Besides, serum samples were used to determine the contents of TC, TG, LDL-C, and high-density lipoprotein cholesterol (HDL-C). According to the manufacturer's instructions, all the above biochemical parameters were analyzed by colorimetric method with commercial kits (Nanjing Jiancheng Biological Company, China).

Quantitative real-time PCR

Protocols for hepatic total RNA extraction, RT-PCR, and quantitative PCR were performed according to our established work (Cheng et al., 2021). Primers of genes (*srebpc-1c*, *fas*, *acc1*, *hmgcr*, *pparγ*, *chrebp*, *atgl*, *hsl*, *pparα*, and *cpt1*) were designed with the Primer Premier 5.0 software based on nucleotide sequences of spotted seabass (**Table 2**). The relative expression levels of genes were calculated according to the comparative Ct method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Table 2 Sequences of the PCR primers used in this study.

Genes	Forward (5'-3')	Reverse (5'-3')
acc1	AAGGCGGTGGTGATGGATTT	GGCCATGTCGCCTTTGTTTT
fas	AAACTGAAGCCCTGTGTGCC	CACCCTGCCTATTACATTGCTC
srebp-1c	CCTCACTCTGCAGCCAATCA	CGTAGTCCCACCCTCAAACC
pparγ	TGTGCGTCTGAAGAAACCCT	TACGTCAACGGCATCGCTAA
hmgcr	GACCGTGCATACGGAACAGA	AGTGTGTGGGTTGAGACCG
chrebp	GTGACAACGCTCAGCTCTCA	TGATGGCAGAGTTCAGGAGC
atgl	CTTCCTCTCCGCAACAAGTC	TGGTGCTGTCTGGAGTGTTC
hsl	CGAAACACAGAGACGGTCCA	TCATGACATCTACCAGCCGC
pparα	CCGTGCGTGTTTTCACCATT	AGACCAAATACATCGCCCCC
cpt1	CCTCAATGATACATCGGAACCC	CTGCGGCTCATCATCTAACG
cyp7a1	GTGTCGTATCCCCAAAGCGA	GGTCCTGCCGTGTAATCTGT
cyp8b1	AGGAGAACCCCCGTTGGATA	CCCTGCCAACTGTATCGTGA
β-actin	CGCCGCACTGGTTGTT	TTTGGGGTTCAGGGGG

Abbreviations: *acc1*, acetyl-CoA carboxylase 1; *fas*, fatty acid synthase; *srebp*, sterol regulatory element-binding protein; *ppar*, proliferator-activated receptor; *hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *chrebp*, carbohydrate responsive element binding protein; *atgl*, adipose triglyceride lipase; *hsl*, hormone sensitive lipase; *cpt1*, carnitine palmityltransferase 1; *cyp7a1*, cholesterol 7α hydroxylase; *cyp8b1*, sterol 12α-hydroxylase.

Gut microbiota analysis

Total gut bacterial DNA was extracted with a HiPure Soil DNA Kit (Magen, Guangzhou, China). The sequence analyses of the V3-V4 region of 16S rRNA gene were performed by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China) using Illumina MiSeq PE 250.

Statistical analysis

The homogeneity of variances of data was identified, and data were then analyzed using one-way ANOVA followed by Tukey's test (SPSS 22.0). Statistical significance was set at $P < 0.05$, and results were presented as mean ± SEM of triplicate groups.

Results

Liver histology and biochemical parameters

Oil red O staining results indicated the accumulation of liver lipid droplets decreased with dietary SPI inclusion (**Figure 1**). Serum TG, TC, and LDL-C contents were markedly decreased by replacing 50% of dietary FM with SPI ($P < 0.05$) (**Table 3**), while these traits did not differ between FM and SPI25 groups ($P > 0.05$). No significant difference in serum HDL-C content was observed among all groups ($P > 0.05$). Hepatic TG and TC contents significantly decreased with the increase of dietary SPI levels ($P < 0.05$) (**Table 4**), while an opposing trend was detected in HL activity among all treatments ($P < 0.05$). No significant difference in hepatic TBA content and LPL activity was detected among all groups ($P > 0.05$). These results indicated that substituting dietary FM with SPI reduced lipid accumulation in spotted seabass.

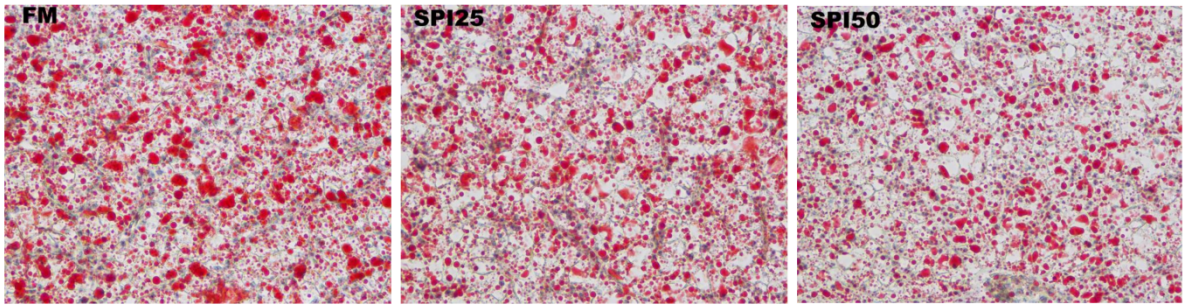

Figure 1 Oil O red staining sections of liver for spotted seabass fed experimental diets for 8 weeks (scale bar = 100 μm).

Table 3 Serum biochemical indexes (mmol/L) of spotted seabass fed experimental diets for 8 weeks.

Items	Experimental diet treatments		
	FM	SPI25	SPI50
TG[1]	5.72 ± 0.11^{b}	5.39 ± 0.13^{b}	4.14 ± 0.08^{a}
TC[2]	17.08 ± 0.17^{b}	15.87 ± 0.64^{b}	13.04 ± 0.18^{a}
LDL-C[3]	5.14 ± 0.53^{b}	4.51 ± 0.11^{b}	3.40 ± 0.11^{a}
HDL-C[4]	6.66 ± 0.13	6.13 ± 0.13	5.73 ± 0.35

Values are mean ± SEM of three groups of fish. Values in the same row with different superscripts are significantly different ($P < 0.05$).

[1] TG: triglyceride.

[2] TC: total cholesterol.

[3] LDL-C: low-density lipoprotein cholesterol.

[4] HDL-C: high-density lipoprotein cholesterol.

Table 4 Hepatic biochemical indexes of spotted seabass fed experimental diets for 8 weeks.

Items	Experimental diet treatments		
	FM	SPI25	SPI50
TG[1]	14.85 ± 0.54[c]	12.99 ± 0.10[b]	11.35 ± 0.46[a]
TC[2]	2.08 ± 0.15[b]	1.88 ± 0.08[ab]	1.69 ± 0.02[a]
TBA[3]	32.29 ± 0.07	33.06 ± 0.04	33.73 ± 0.54
LPL[4]	4.01 ± 0.01	4.55 ± 0.17	4.68 ± 0.31
HL[5]	3.66 ± 0.16[a]	3.62 ± 0.33[a]	4.34 ± 0.11[b]

Values are mean ± SEM of three groups of fish. Values in the same row with different superscripts are significantly different ($P < 0.05$).
[1] TG: triglyceride (mmol/gprot).
[2] TC: total cholesterol (mmol/gprot).
[3] TBA: total bile acid (mmol/gprot).
[4] LPL: lipoprotein lipase (U/mgprot).
[5] HL: hepatic lipase (U/mgprot).

Relative mRNA expression of genes involved in lipid and bile acid metabolism

The expressions of lipid synthesis-related genes including *srebpc-1c*, *fas*, *acc1*, *hmgcr*, *ppary* and *chrebp* dramatically decreased by replacing dietary FM with SPI ($P < 0.05$) (**Figure 2A**). Consistently, dietary SPI inclusion also down-regulated the expressions of genes involved in lipid catabolism, including *atgl*, *hsl*, *ppara*, and *cpt1* ($P < 0.05$) (**Figure 2B**). No marked differences in bile acid synthesis-related genes *cyp7a1* and *cyp8b1* were observed among all treatments ($P > 0.05$) (**Figure 2C**). These results indicated that substituting dietary FM with SPI suppressed lipid synthesis and catabolism in spotted seabass.

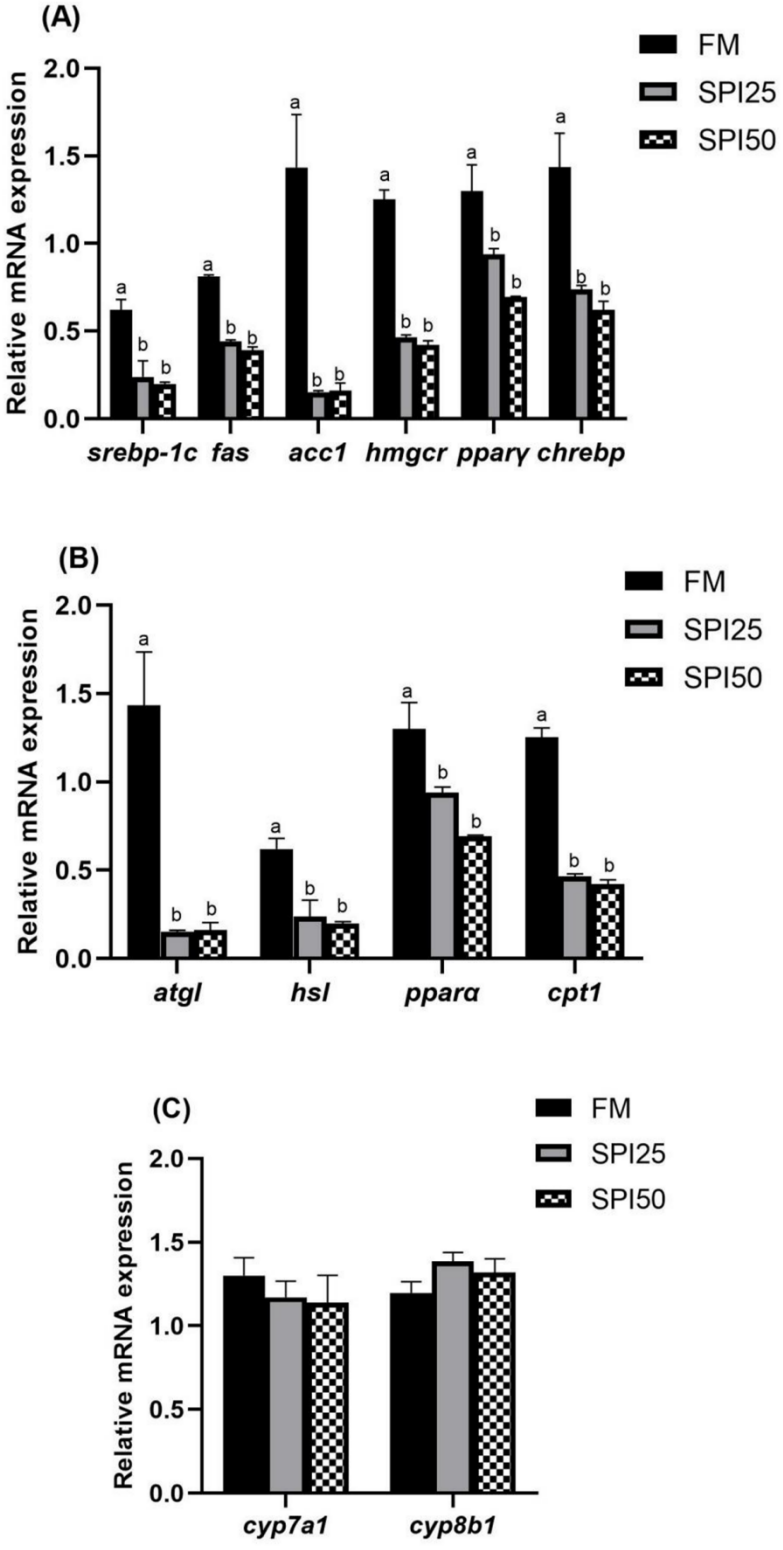

Figure 2 Relative mRNA expression of genes involved in (A) Lipid synthesis, (B) Lipid catabolism, and (C) Bile acid synthesis in the liver of spotted seabass fed experimental diets for 8 weeks. Bars with different letters are significantly different ($P < 0.05$).

Gut microbiota

Venn diagram of bacterial communities showed that the core OTUs among all groups was 45, and independent OTUs of FM, SPI25, and SPI50 groups were 601, 383, and 451, respectively (**Figure 3A**). Alpha-diversity indexes including ACE, Chao1, Shannon and Simpson were shown in **Table 5**. It could be observed that ACE and Chao1 indexes dramatically increased with the increase in dietary SPI levels ($P < 0.05$), while Shannon and Simpson's indexes did not affect ($P > 0.05$).

In general, Proteobacteria, Bacteroidetes, and Firmicutes were the three major bacterial phyla in the mid intestinal of spotted seabass (**Figure 3B**). The Proteobacteria abundance in the FM group was significantly higher than that in both SPI25 and SPI50 groups ($P < 0.05$) **(Figure 3D**), and the abundance of Bacteroidetes ($P < 0.05$) and Firmicutes ($P > 0.05$) increased along with dietary SPI inclusion. The dominant genus in FM and two SPI groups was *Plesiomonas* and *Prevotella*, respectively (**Figure 3C**). Replacing dietary FM with SPI significantly increased the abundance of *Prevotella*, *Succiniclasticum,* and *Desulfovibrio* in the gut of spotted seabass ($P < 0.05$) (**Figure 3E**). These results indicated that substituting dietary FM with SPI changed gut microbiota profile in spotted seabass.

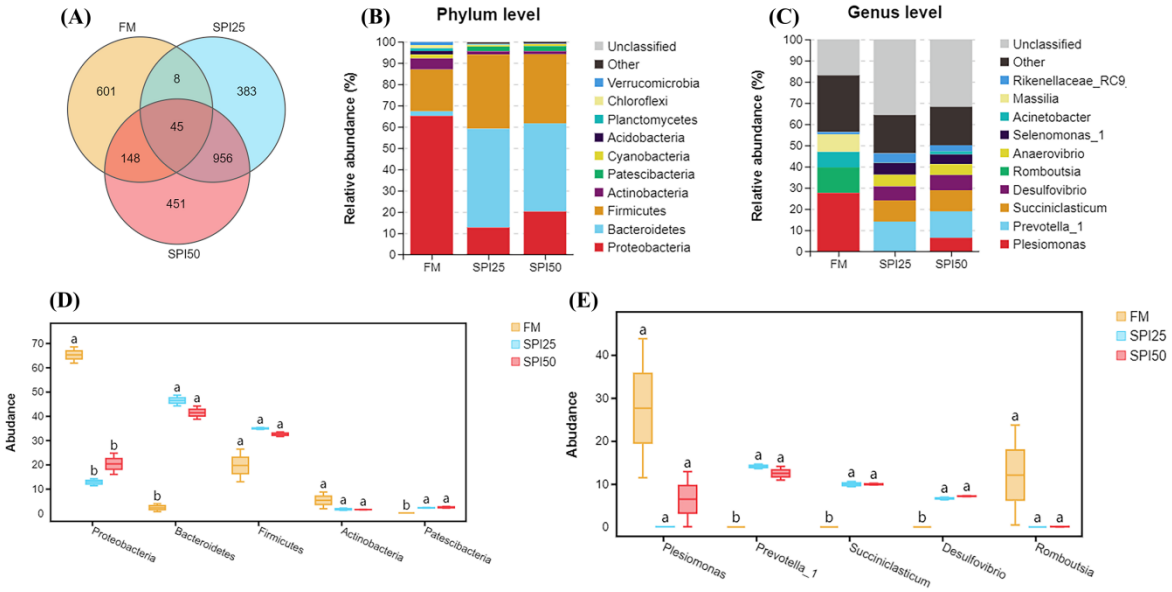

Figure 3 16S rRNA gene sequencing analysis of the gut microbiota. (A) Venn diagram; Percent of community abundance at (B) phylum and (C) genus level; The relative abundance of major bacterial (D) phyla and (E) genera in boxplot; Bars with different letters are significantly different ($P < 0.05$).

Table 5 Alpha-diversity indexes (including ACE, Chao1, Shannon and Simpson) of spotted seabass fed experimental diets for 8 weeks.

Items	Experimental diet treatments		
	FM	SPI25	SPI50
ACE	967.09 ± 33.35[a]	2239.01 ± 59.14[b]	2540.91 ± 88.42[c]
Chao1	936.76 ± 50.32[a]	2170.06 ± 21.06[b]	2409.72 ± 119.31[b]
Shannon	5.33 ± 1.04	7.14 ± 0.19	7.06 ± 0.11
Simpson	0.90 ± 0.08	0.99 ± 0.00	0.98 ± 0.00
Coverage (%)	99.77 ± 0.03[b]	99.38 ± 0.00[a]	99.33 ± 0.04[a]

Values are mean ± SEM of three groups of fish. Values in the same row with different superscripts are significantly different ($P < 0.05$).

Discussion

Plant-derived protein has been proven to exert hypolipidemic effects on animals in comparison to animal protein sources (Torres et al., 2006; Ijaz et al., 2018). In the present study, substituting dietary FM with SPI led to reduced lipid droplets area, TG, and TC contents in both serum and liver. Besides, HL, a key hydrolytic enzyme of TG, decreased along with dietary SPI inclusion. These results revealed that substitution of dietary FM with SPI could reduce lipid accumulation in spotted seabass. Similar results were also observed in Japanese flounder (*Paralichthys olivaceus*) (Deng et al., 2009). Research on rats showed that soy protein participated in lipid homeostasis by regulating several transcription factors such as the srebp family (Torres et al., 2006). The srebp1 isoform was preferentially involved in *de novo* lipogenesis of fatty acids via activating lipogenic genes, *fas* and *acc*, required for lipid synthesis; whereas srebp2 isoform controls the cholesterol homeostasis by activating *hmgcr* gene, required for cholesterol synthesis (Dihingia et al., 2018). Besides, *pparγ* was proven to regulate adipocyte differentiation and adipocyte hypertrophy (Li et al., 2018). In the current study, replacing dietary FM with SPI resulted in lower mRNA expressions of *srebp1*, *fas*, *acc1*, *hmgcr*, and *pparγ*. A generally accepted biochemical principle is that energy metabolism is coordinately regulated by multiple pathways (Sharpe and Brown, 2013). chrebp was a key factor in converting glucose to TG for storage, and it was demonstrated that chrebp and srebp factors could act in synergy to induce transcription of lipogenic genes (Dentin et al., 2005). In the present study, the expressions of both *chrebp* and *srebp-1c* genes decreased along with dietary SPI inclusion. Therefore, it could be concluded that the substitution of dietary FM with SPI suppressed the hepatic lipogenesis of spotted seabass.

Besides lipogenesis, lipolysis also played a crucial role in lipid metabolism for animals (Peng et al., 2014). atgl and hsl, two major lipid hydrolases, were proven to directly regulate lipolysis in a step-wise approach. More specifically, the lipolysis was initiated by atgl via cleaving one fatty acid from TG, and hsl then acted on diacylglycerol releasing two additional fatty acids and one glycerol molecule (Jin et al., 2017). Additionally, the oxidation of fatty acids, referred to as β-oxidation, was regulated by pparα and cpt1 (a crucial rate-limiting enzyme of β-oxidation) (Lai et al., 2021). The information about the role of SPI in the regulation of hepatic lipolysis is limited. Research on rats indicated that soy protein-derived peptide simulated fat oxidation via activating lipolysis-related transcription factors (Torres et al., 2006). In contradiction to the above result, research on

fish showed that replacing dietary FM with plant-protein source suppressed the expression of *ppara* and *cpt1*, and the LPL activity in the liver (Ye et al., 2019). Consistently, the current study revealed that replacing dietary FM with SPI down-regulated the expression of lipolysis-related genes including *ppara*, *cpt1*, *atgl*, and *hsl*. It might be due to that the liver lipolysis was suppressed via a feedback regulatory mechanism in response to a low-fat statue of SPI-fed fish, thus achieving a dynamic equilibrium between lipid synthesis and catabolism. In line with this point, previous studies demonstrated the expression of aforementioned genes (*ppara*, *cpt1*, and *atgl*) increased along with hepatic lipid contents (Gou et al., 2019). Moreover, the regulation mechanisms of lipid metabolism vary from terrestrial and fish, and it should be investigated on a case-by-case basis.

A previous study revealed that SPI-derived 7S protein involved cholesterol metabolism via stimulating bile acid synthesis (Liu et al., 2017). Thus, we determined the TBA content and expression of *cyp7a1* and *cyp8a1* in the liver. However, no significant difference was obtained in these traits among all groups. Replacing dietary FM with SPI suppressed lipolysis and lipogenesis in the liver indicated that a systematic fatty acid cycle was inactive in SPI-fed fish versus FM-fed fish. In addition, Lipids synthesis, catabolism, and transport were generally regulated by controlling key enzymes through allosteric and covalent modifications (Dentin et al., 2005). Thus, the lipid metabolism-related enzymes still need further investigation at the post-translational level.

Diet intake establishes a central determinant of the compositions of trillions of microorganisms residing in the gut. Furthermore, diet-induced modulation of gut microbiota directly influences the host physiological state (Xu et al., 2021; Wang et al., 2021). Thus, it is imperative to understand how protein intake from different sources affects gut microbiota. Soy protein was proven to have particular potential in increasing the gut microbial diversity in animals (Butteiger et al., 2016). The current study showed that substitution of dietary FM with SPI led to high microbial diversity and richness, which ACE and chao1 indexes indicated. The gut microbiota compositions have also been remolded by dietary SPI inclusion. Specifically, The SPI-fed group had a relatively higher abundance of Bacteroidetes and Firmicutes than the FM-fed group, which was dominant by Proteobacteria. Consistently, Ingerslev et al. (2014) reported that different dietary types determined gut microbiota community in rainbow trout (Oncorhynchus mykiss). Firmicutes was dominant in the gut of plant based-fed fish, whereas Proteobacteria in marine based-fed fish. The Firmicutes/Bacteroidetes (F/B) ratio was usually used to quantify the gut microbiota of obese and lean individuals. A high proportion of F/B resulted in more effective energy absorption and subsequent weight gain (Kong et al., 2021). A previous study showed that replacing 20% of dietary animal protein with soy protein for 6 weeks increased the F/B ratio in fecal microbiota (Lee et al., 2015). Similarly, the present study showed a markedly lower F/B ratio in both SPI25 and SPI50 groups (0.75 and 0.78) compared with the FM group (8.94 . This observation was supported by the lean phenotype of SPI-fed fish versus FM-fed fish.

A bloom of the *Prevotella* genus characterized the SPI-fed group. This species was reported to negatively correlate with obesity and metabolic disorders in numerous studies (Arnoriaga-Rodriguez et al., 2020). Nutritional interventions with fiber-rich foods usually led to an increased *Prevotella* abundance, which was capable of breaking down dietary-derived plant polysaccharides but not animal polysaccharides (Tett et al., 2021). In addition, SPI-fed fish were unique in showing a greater abundance of *Desulfovibrio*. It has been reported that *D. Vulgaris* within *Desulfovibrio* spp. could prevent fatty liver disease by inhibiting fatty acid *de novo* synthesis (Hong et al., 2021). Accordingly, the alterations in spotted seabass's lipid metabolism patterns partially resulted from the remodeled gut microbiota caused by dietary SPI inclusion.

In conclusion, results of the present study showed that replacing 25-50% of dietary FM with SPI reduced lipid accumulation in the serum and liver of spotted seabass, which was associated with the down-regulation of genes involved in lipolysis and lipogenesis, as well as the alterations in gut microbiota.

Acknowledgments

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