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Effect of Aquafeed containing Soybean Meal on Lipid Metabolism and Immune Gene Expression in the Liver of Olive Flounder

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Abstract

In aquaculture, feed cost constitutes a large part of the total expense, and a dietary protein source is an important factor influencing both fish growth and feed cost. This study was performed to investigate the effects of extruded pellets containing soybean meal (SM) as a fish meal (FM) replacement on the liver of olive flounder, in terms of molecular biology. To investigate the effects of SM and FM on the expression of the genes involved in hepatic glucose and lipid metabolism and the immune response, real-time PCR was performed. SM increased the expression of genes involved in lipid metabolism and PPARγ. In addition, SM increased the expression of several immune-related genes, including *TLR2, IRF3, IRF7, RELA, IL1β, IL8*, and *TNF*. To identify the common factors regulating immune gene expression, we investigated the promoter regions of these genes. Comparative analysis of these factors showed that NF-κB binding elements were found on the promoters of all genes, except for *TLR3*. The promoter construct containing NF-κB mutant binding element was not activated by SM. These results suggest that gene expression may increase through activation of NF-κB signaling in the liver of olive flounder fed SM. This study will be helpful for developing improved extruded pellets.

Introduction

The aquaculture of various species, including flatfish, bass, black porgy, red seabream, rock bream, rockfish, and grey mullet, is actively developing. Among these species, olive flounder, mostly produced in flow-through systems at land-based facilities, plays a significant role in Korea's aquaculture. Its aquaculture production has ranged from 40,000–50,000 metric tons during the last 10 years (Yoo and Bai SC, 2014) [1]. In 2014, the output of olive flounder comprised about 52% of the total fish output in Korea. Oliver flounder is gaining popularity as a rich-production, low-fat, and low-caloric food in Korea. Furthermore, olive flounder has large amounts of collagen, which is beneficial for both skin and heart health.

Feed cost constitutes a large part of total aquaculture expenses. Because olive flounder is a carnivorous fish that uses protein rather than carbohydrates as an energy source (Kim et al., 2014) [2], having a dietary protein source is an important factor influencing its growth and health, as well as the cost of feed (Kim et al., 2012) [3]. The fish meal (FM) used as a dietary protein source comprises about 60% of the feed for olive flounder (Jasour et al., 2017; Kim et al., 2013) [4,5]. Many studies have been conducted to search for an alternative protein source to FM, due its expensive cost and some additional problems, including unstable supply and ecosystem destruction (Barrows et al., 2007; Yoo et al., 2006; Kim et al., 2014; Park et al., 2017) [2,6-8]. Although the use of plant protein sources are difficult due to the presence of anti-nutritional factors such as saponins, phytic acid, and trypsin inhibitors (Pham et al., 2008) [9], many studies have reported the use of plant protein sources as an FM replacement for olive flounder (Pham et al., 2005; Pham et al., 2007; Kim et al., 2006) [7,10,11].

The liver has many important functions that work to keep organisms healthy, in combination with other systems and organs. Using nutrients from food, the liver produces blood components, proteins, and enzymes to maintain homeostasis. The liver, therefore, plays an essential role in food digestion and various metabolisms, including carbohydrate, protein, amino acid, and lipid metabolisms (Rui, 2014) [12]. In particular, the liver plays several roles in lipid metabolism, such as in cholesterol synthesis, lipogenesis, and triglyceride production (Nguyen et al., 2008) [13]. The liver also removes various toxic metabolites from the bloodstream, and has an innate immune system that is selectively enriched in macrophages (Kupffer cells), natural killer (NK) cells, and natural killer T (NKT) cells (Racanelli and Rehermann, 2006; Woolbright and Jaeschke, 2018) [14,15].

To date, various studies of the effect of extruded pellets on olive flounder development have been reported (Jang et al., 2005; Cha et al., 2012; Choi et al., 2014) [2,16]. However, these studies focused on the measurement of physical aspects such as weight, blood component analysis, and the viability of cultured fish, rather than focusing on molecular biological aspects. The aim of this study was to investigate the molecular biological effects of extruded pellets containing soybean meal (SM) as a replacement of FM on the liver of olive flounder.

Materials and Methods

Fish and feeding conditions

The feeding experiment was performed at the Aquafeed Research Center, National Fisheries Research & Development Institute (NFRDI), Pohang, South Korea. In total, 120 olive flounder (initial body mass of 55.7 ± 0.6 g (mean ± SD)) were randomly distributed into four tanks at a stocking density of 30 fish/tank (1000 L capacity, duplication per treatment) and hand-fed to satiation twice a day (09:00 and 17:00h) for 12 weeks. Oxygen was supplied through air dispersion near saturation level. Other experimental conditions were controlled, such as: photoperiod of 12:12h (light: dark), water flow rate of 3–5L min^{-1}, and water temperature of 22 ± 3.7°C. The uneaten feed was removed 1h after every meal for the calculation of feed efficiency, and survival was monitored daily during the feeding experiment.

Experimental diets

The compositions of the experimental diets are presented in Table 1. The experimental diets were formulated using FM or SM. The experimental diets were made according to the methods of Lee et al. (Lee et al., 2009) [17]. After mixing the ingredients thoroughly, oil and 30% distilled water were added and further mixed. The wet dough was pelletized at a particle size of 4mm using a chopper machine. The experimental diets were freeze-dried, sieved into the desired particle size (1 to 4mm), and then stored at −20°C. The experimental diets were stored in freezer not to be deteriorated. When it is necessary, diets were warm-up at room temperature. Then diets were allotted into three (replicates) small feeding container. We check the amount of consumption by weighing each container before and after feeding.

Ingredients	Diet Group	
	FP	SP
Fish meal (Chile)	62	37.2
Squid liver powder	2	1
Defatted soybean meal	0	37.5
Wheat flour	23	0.5
Corn gluten wheat	3	6.3
Lysine	0	1.8
Methionine	0	0.6
Taurine	0	1
Mono-calcium phosphate	0	1
Fish oil	4	6.1
Vitamin	2	2
Mineral	2	2
CMC	1	1
Cellulose	1	1
Total	**100**	**100**

Table 1: Ingredients composition of the experimental diets.

Sampling

At the end of the one and two month feeding experiments, 2 fish per tank were randomly selected and anesthetized with 100mgkg^{-1} tricaine methanesulfonate. Liver tissues were soaked in RNAlater (Ambion) and stored at 4°C for 24h. The samples were stored at −80°C before use.

Preparation of RNA and real-time PCR

Total RNA was prepared from liver tissues and HINAE cells using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized using Moloney Murine leukaemia virus (MMLV) reverse transcriptase (Promega) with an oligo-dT at 37°C for 1h. The cDNA was subjected to real-time PCR using specific primers (Table 2). 18s rRNA was used to normalize the expression level of the target genes. Quantitative real-time PCR was performed using TOPreal^{TM} qPCR 2X PreMIX (Enzynomics). Following an initial 10-min Taq DNA polymerase activation step at 95°C, real-time PCR was performed for 40 cycles using the following cycling conditions: 95°C for 10 s, 60°C for 15s, and fluorescence reading in a SDS 7500 thermal cycler (Applied Biosystems, Inc.). After the PCR cycles were complete, melting curve analysis was performed by gradually increasing the temperature and assaying changes in fluorescent emission intensity (0.1°C/s). The relative expression of each gene was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) [18].

Target gene	Sense primer (5'-3')	Antisense primer (5'-3')	References
TLR2	gctacatctgcgactctcct	cacagggacacgaacaaatc	[31]
TLR3	aacgcctggttcatcaagtg	cgaatgtcgaagtgcaagag	[31]
TLR7	cctgggaaatctggaagaac	tttgagggaggagaaactgc	[31]
IRF3	acaccatgaaccagagcaac	tgtccaaaagtgtccctgtg	[31]
IRF7	tctgatctgtcggcactttc	ccgaacacggagttaatgag	[31]
RELA	gaatcaaccgcaactctggaag	gctcggtgagattagtgtcg	
IL1β	aaagaagcatcaccactgtct	ctactcaacaacgccaccttt	[31]
IL8	tccgtgggtgaagagagt	attagggtcgtgttgagttgt	[31]
TNF	ggtagagacgaggaagacga	cctggctgtagacgaagtag	
PPARG	ctgggaagaccggaacaac	gtccgctgatgctcgtcatt	
SREBP1	ctccctccctcctttctgtc	ggagaggcagtccatgaaga	[32]
FASN	ccacagcgcatcacatcct	gtcagccaatgacgcatcag	
18s rRNA	cggctaccacatccaaggaa	atacgctattggagctggaattacc	

Table 2: Primers used for real-time PCR.

Promoter analysis

The genome information of olive flounder was provided by NFRDI. To search for putative transcription factor binding elements of the genes affected by SM diets (soy bean extract), the gene promoters were analyzed at the PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) website. HINAE cells were seeded in 24-well culture plates and transfected with the reporter vector and β-galactosidase expression plasmid, along with treatment with SM extract. After 24 hours of transfection, the cells were lysed in cell culture lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

Construction of a mutated plasmid without an NF-κB binding site

To investigate the function of the NF-κB binding site for gene transcription, the binding site was deleted from the 617-bp NOD2 promoter fragment using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primer set was designed as follows: forward, 5′-CCGGCTTTTCCTTTCTTGAAGGTGGGGT-3′ and reverse, 5′-ACCCCACCTTCAAGAAAGGAAAAGCCGG-3′. The mutant promoter (607 bp) without the NF-κB binding site replaced the CMV promoter in pEGFP-N3, using the same method described above. The mutated plasmid was constructed and designated as pmNOD2 (607)-EGFP.

Statistical analysis

Statistical analysis was carried out by an unpaired or paired *t*-test as appropriate. All data are reported as mean ± SD. P value <0.05 was considered significant.

Results

SM increases the expression of lipid metabolism-related genes in the liver of olive flounder

The liver is an essential metabolic organ, especially for bile acid, glucose, and lipid production (Rui, 2014) [12]. We, therefore, used real-time PCR to investigate the effects of SM on the expression of the genes involved in hepatic glucose and lipid metabolism. The real-time PCR data for the pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (*PEPCK*) enzymes involved in glycolysis and gluconeogenesis, respectively, did not show any significant changes in mRNA levels in the liver of olive flounder fed FM or SM for 1 or 2 months (data not shown).

In the fed state, the glycolytic products derived from glucose and lipid in hepatocytes are used to synthesize fatty acids through *de novo* lipogenesis, in which sterol regulatory element-binding protein 1 (*SREBP1*) and fatty acid synthase (*FASN*) play critical roles (Nguyen et al., 2008) [13]. The expression of *SREBP1* is regulated by transcription factor liver X receptor (*LXR*) induced by peroxisome proliferator-activated receptor γ (*PPARG*), known as a master gene of lipid metabolism (Nguyen et al., 2008) [13]. To investigate the effects of SM and FM on the expression of the genes involved in hepatic lipid metabolism, real-time PCR was performed. As shown in Figure 1, the mRNA levels of *PPARG, SREBP1*, and *FASN* in the liver of olive flounder fed SM for 1 month were up-regulated by an average of 20%, 23%, and 39%, respectively, compared with those of olive flounder fed FM for 1 month. Furthermore, the mRNA levels of *PPARG* in the liver

of olive flounder fed SM for 2 months increased by almost half. In the case of *SREBP1* and *FASN,* the mRNA levels in the liver of olive flounder fed SM for 2 months significantly increased by more than half, compared with olive flounder fed FM for 2 months. Although the mRNA levels of *PPARG, SREBP1*, and *FASN* in the liver of olive flounder fed FM for 2 months were slightly down-regulated compared with those fed FM for 1 month, a significant difference was not observed (Figure 1). In short, SM affected the expression of the genes involved only in lipid metabolism in the liver of olive flounder. These results suggested that SM intake may induce physiological changes in the liver of olive flounder by up-regulating lipid metabolism-related gene expression.

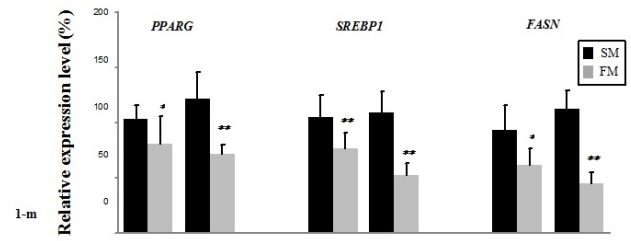

Figure 1: Effect of SM on the expression of the genes involved in lipid metabolism in the liver of olive flounder.

SM increases the expression of inflammatory genes in the liver of olive flounder

The liver has unique immunological properties as an immunological organ (Racanelli and Rehermann B, 2006) [14]. To investigate the effect of SM on the liver's innate immune response, we analyzed the expression of the genes involved in the immune response through real-time PCR. Toll-like receptors (TLRs) play an essential role in the innate immune response by recognizing conserved molecules derived from microbes (Seki and Brenner, 2008) [19]. In the real-time PCR data for TLRs, *TLR3* and *TLR7* did not show any remarkable differences in the transcript levels on the liver of olive flounder fed FM or SM for 1 and 2 months (Figure 2A). The transcript levels of *TLR2* in the liver of olive flounder fed SM significantly increased compared with those of olive flounder fed FM during the same time period.

Interferon regulatory factor (*IRF*) 3 and 7, which regulate interferon transcription, are the downstream factors of *TLR3* and *TLR7*, respectively (Seki and Brenner, 2008) [19]. On the transcript level, *IRF3* and *IRF7* showed remarkable differences in the livers of olive flounders fed FM or SM for 2-month (Figure 2B). *RELA*, also known as p65, is a subunit of nuclear factor-kappa B (NF-κB) heterodimer, which plays a key role in the regulation of the immune response to infection (Hayden, Ghosh, 2012) [20]. The transcript levels of *RELA* in the liver of olive flounder fed SM for 1 or 2 months were significantly up-regulated compared with those of olive flounder fed FM for 1 or 2 months, respectively (Figure 2B).

Interleukin 1β (*IL1β*), *IL8*, and tumor necrosis factor (*TNF*) are cytokines, and are the target genes of NF-κB (Hayden, Ghosh, 2012) [20]. The transcript levels of *IL1β, IL8*, and *TNF* in the liver of olive flounder fed SM showed a significant increase compared with those of olive flounder fed FM for the same time period (Figure 2C). In addition to *IL8*, the transcript levels of *IL1β* and *TNF* in the liver of olive flounder fed FM for 2 months were down-regulated compared with those of olive flounder fed FM for 1 month. These results suggested that SM intake may increase the immune response in the liver of olive flounder by up-regulating the expression of various immune-related genes.

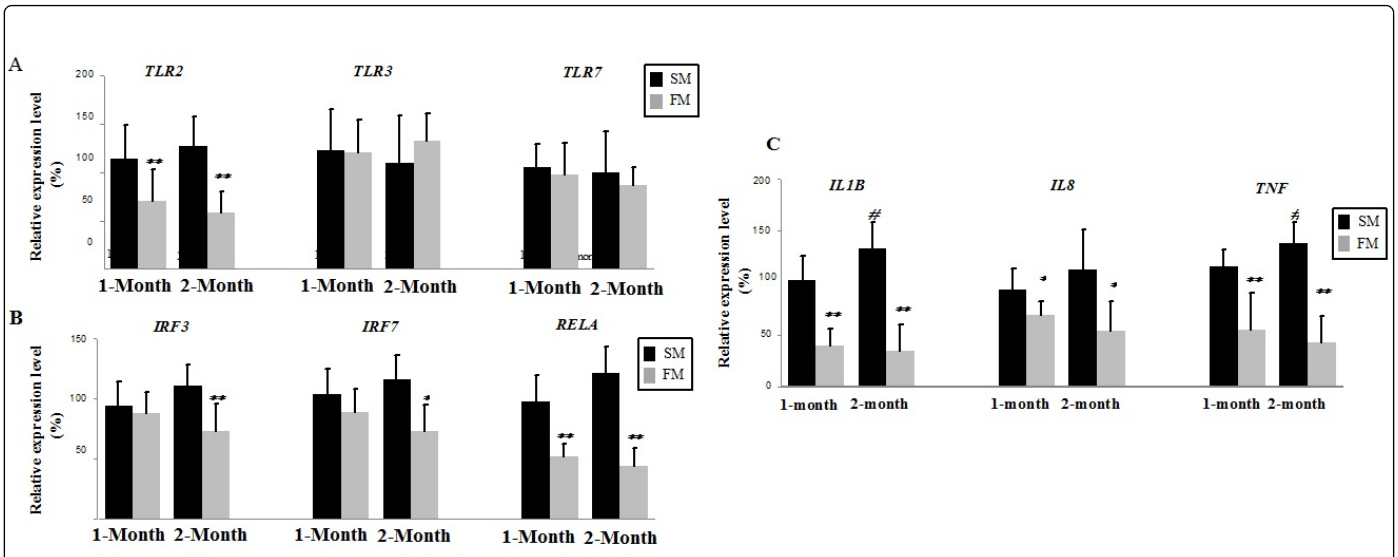

Figure 2: Effect of SM on the expression of the immune genes in the liver of olive flounder.

Identification of the putative transcription factor binding elements and promoter activation

To identify common transcription factors that can regulate lipid metabolism and immune-related gene expression, the olive flounder genome was analyzed. Through pair-wise alignment with the genome and mRNA sequences of *PPARG, TLR2, TLR3, IRF3, IRF7,* and *RELA*, we obtained promoter sequences of the genes. On the PROMO website, we investigated the putative transcription factor binding elements which can regulate gene expression. Comparative analysis of the factors showed that there are NF-κB binding elements on the promoters of all genes, except for *TLR3* (Figure 3A). These results suggested that the expression of *PPARG, TLR2, IRF3, IRF7,* and *RELA* genes may be up-regulated by NF-κB activation in the liver of olive flounder fed SM.

To confirm the up-regulation of IRF3, RELA, and TLR2 by NF-κB-dependent transcriptional activation, we constructed promoter-directed luciferase reporter plasmids for IRF3, RELA, and TLR2. In addition to these gene promoters, a TLR3 promoter was prepared for comparing with a promoter without the NF-κB response element. As shown in Figure 3B, SM extract enhanced the promoter activation of IRF3, RELA, and TLR2 compared to FM-fed samples. However, the promoter activity of TLR3 was not up-regulated by SM components (Figure 3B). The up-regulated gene promoters were consistent with the presence of the NF-κB-response element in their promoter region, as shown in Figure 3A. These results indicated that SM components enhance NF-κB-dependent gene expression.

NF-κB response element is essential for SM-mediated gene induction

NF-κB exerts its fundamental role as a transcription factor by binding to variations of the consensus DNA sequence of 5′-GGGRNYY YCC-3′ (in which R is a purine, Y is a pyrimidine, and N is any nucleotide), known as κB sites (Fengyi et al, 2009) [21]. To examine whether SM components direct the transactivation of their cognate gene expression via the NF-κB response element, we transfected the NF-κB consensus binding site (GGGAATTTCC)-conjugated luciferase reporter or mutant binding site (GATAATTTCC) plasmids in the presence of FM or SM extracts. As shown in Figure 4, SM components largely increased NF-κB binding site-conjugated luciferase expression compared with samples that received FM treatment. However, the mutant binding site was not affected by SM treatment. These results indicate that SM components activate transcription of the NF-κB response element-dependent promoter.

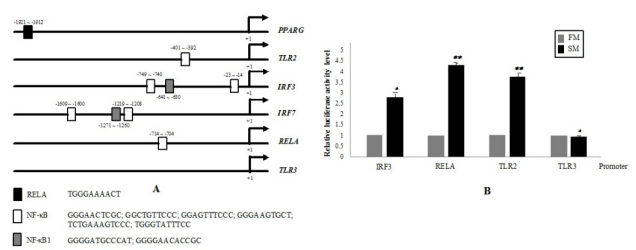

Figure 3: Promoters of the genes affected by SM diets and transcriptional activation.

Discussion

The use of raw fish-based pellets is preferred in aquaculture, due to a high growth rate and the enhanced quality of olive flounder meat. However, their usage results in various problems, including environmental pollution, unstable supply, the depletion of fishery resources, and the induction of fish diseases. To solve these problems, many experts have made an effort to develop various extruded pellets. Soybean has several advantages as a dietary protein source, such as abundant protein content over 40%, stable supply, and inexpensive prices (Lee and Jeon, 1996) [22].

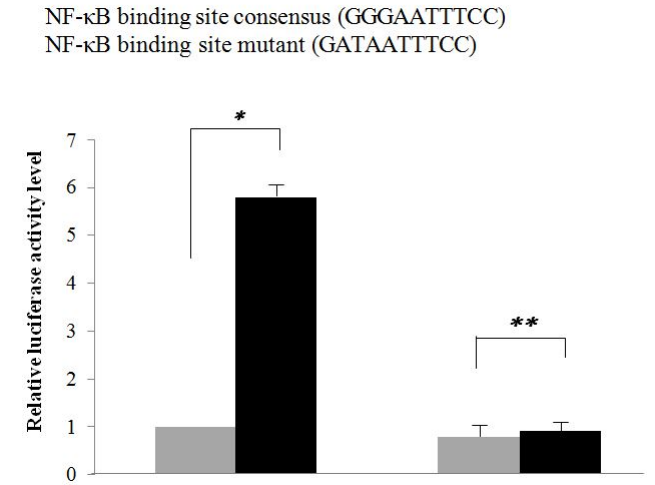

Figure 4: Transcriptional activation of SM-mediated gene induction dependent on NF-κB response element.

However, olive flounder is a carnivorous fish, and therefore, soybean, containing plant protein, may be unsuitable as a source of dietary protein for extruded pellets for olive flounder. According to a previous report, soybean has anti-quality factors, such as phytic acid and trypsin inhibitor, which inhibit olive flounder growth (Rumsey, 1993) [23]. Kim et al. reported that fermented SM has similar efficiency to FM when considering growth of olive flounder (Kim et al., 2013) [24]. In addition, soybean is processed through defatted or zymolytic courses to overcome the problem induced by anti-quality factors. Defatted soybean processed by pressure and heat treatment was used as a dietary protein source, as the trypsin inhibitor is easily destroyed by heat.

TLRs are widely expressed on the membranes of immune cells, and induce the innate immune response by recognizing structurally conserved molecules derived from microorganisms. These are referred to as pathogen-associated molecular patterns (PAMPs). In addition to immune cells, TLRs are expressed on intestinal epithelial cells as well as hepatocytes, which express mRNA for all TLRs (Seki E and Brenner, 2008) [19]. To date, 13 *TLRs* have been identified in mammalian species. However, more than 15 TLRs have been identified in fish, including mammalian TLR homologues and fish-specific *TLRs*, such as *TLR5* soluble form (5S), *14, 20, 21, and 22* (Hwang et al., 2011) [25]. In olive flounder, 11 TLRs have been identified, including *TLR1, 2, 3, 5, 5S, 7, 8, 9, 14, 21, and 22* (Hwang et al., 2011) [25]. While some *TLRs* such as *TLR1, 2, 4, 5, and 6* are expressed on the cell surface, other *TLRs* including *TLR3, 7, 8, and 9* are expressed on the

endosome-lysosome membrane (Akira et al., 2006) [26]. Heterodimers of TLR2 with TLR1 or TLR6 recognize bacterial triacylated lipopeptides or diacylated liopeptides, respectively. Once the heterodimers recognize the ligands, the common adaptor molecule MyD88 activates activating protein 1 (AP1) and NF-κB by recruiting the complex, including IL1R-associated kinase (IRAK) 1 and 4, interferon regulatory factor 5 (IRF5), tumor necrosis receptor-associated factor 6 (TRAF6), and transforming growth factor-β-activated kinase 1 (TAK1) (Akira et al., 2006) [26]. Intracellular TLR3 and TLR7 recognize viral-derived double- and single-stranded RNA, respectively (Alexopoulou et al, 2001; Diebold et al, 2004) [27,28]. TLR7 is also MyD88-dependent. Upon TLR7 activation, MyD88 recruits the complex containing IRF 7 instead of IRF5, resulting in IFNα gene expression (Honda et al., 2005) [29]. TLR3 is a TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent TLR. Upon TLR3 activation, TRIF recruits TRAF3 associated with NF-κB activator binding kinase 1 (TBK1) and IKKε, leading to IFN-β transcription through IRF3 activation (Bradbury, 2006) [30]. Among the *TLRs*, only the expression level of *TLR2* was significantly up-regulated in the liver of olive flounder fed SM (Figure 2A). Interestingly, the expression levels of *IRF3* and *IRF7*, which are downstream factors of TLR3 and 7 signaling, respectively, significantly increased in the liver of olive flounder fed SM (Figure 2B).

Due to the induction of *RELA* expression, the expression levels of NF-κB target genes may be up-regulated in the liver of olive flounder fed SM (Figure 2C). These results suggested that SM may increase the expression levels of *TLR2, IRF3, IRF7*, and *RELA* via activation of novel common factors. To identify the common factors that can regulate *TLR2, IRF3, IRF7, and RELA* expression, we investigated promoter regions of the genes on the PROMO website. As a result, NF-κB or its subunit binding elements were jointly found on the promoters of the genes up-regulated by SM diets. These results suggested that the expression of these genes may be increased by NF-κB signaling activation in olive flounder fed SM.

PPARγ, a transcription factor, is activated by fatty acid binding, leading to transcription of *LXRα*, a transcription factor for *SREBP1*. SREBP1 is a transcription factor for *FASN*, which is a multi-enzyme protein that catalyzes fatty acid synthesis. Due to some changes in lipid metabolism-related gene expression, excess fatty acids may be synthesized from glucose and other substrates, resulting in triglyceride accumulation (TG). This is converted from the fatty acids in hepatocytes (Bradbury, 2006) [30]. The transcription of immune genes such as *TLR2, IRF3, IRF7, RELA*, and NF-κB target genes may be affected by lipid peroxidation and ROS. Therefore, SM intake may increase an immune response through lipid metabolism-related gene up-regulation.

Conclusion

This study revealed the molecular biological effects of SM on the liver of olive flounder, as compared with FM. This study will be helpful in the development of improved extruded pellets. In the future, research should investigate which component of soybean meal increases the expression of immune and lipid metabolism-related genes.

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