Songklanakarin J. Sci. Technol.
41 (6), 1287-1293, Nov. - Dec. 2019

http://www.sjst.psu.ac.th

Original Article

Effect of omega-9 rich fish oil on antioxidant enzymes and relative immune gene expressions in Nile tilapia (*Oreochromis niloticus*)

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Received: 7 January 2018; Revised: 12 July 2018; Accepted: 22 July 2018

Abstract

Freshwater fish oil (FFO) was extracted from by-products of the fisheries industry. The amounts of omega-3, 6, and 9 fatty acids in the FFO were 1.38, 12.29, and 42.9 g/100 g, respectively. The diet of Nile tilapia was supplemented with FFO for 4 months to evaluate the effect of FFO on gene expression of antioxidant enzymes and growth-associated immune response. Nile tilapia supplemented with 1% and 1.5% FFO demonstrated growth. Gene expression of antioxidant enzymes including superoxide dismutase, catalase, and glutathione and cytokines of immunity, including TNF-α, IL-1β, and IgM were determined. Three antioxidant enzymes and immune cytokines in the liver tissue were not statistical different among the experimental groups, but expression of GPx and IgM increased in the kidneys of the FFO-supplemented Nile tilapia. IL-1β gene expression in the kidneys also significantly increased. This indicated that the proinflammatory cytokine IL-1β might be enhanced by omega-6 fatty acid. FFO supplemented in fish feed can improve growth, but the related immune gene expressions remain unclear.

Keywords: freshwater fish oil, omega-9 fatty acid, oxidative enzymes, immune cytokines, Nile tilapia

1. Introduction

Fish processing industries generate substantial amounts of solid waste that pose considerable disposal problems (Rai, Swapna, & Bijinu, 2012). Nowadays, the entire freshwater catfish family, such as striped catfish and Mekong giant catfish, is commercially produced throughout the Mekong river region and at inland freshwater aquaculture farms (Kriangsak, Niwooti, & Doungporn, 2010). Catfish has become one of the most popular freshwater fish species apart from wels catfish and channel catfish in European and US markets because its flesh is white and of mild flavour. Also, it is virtually boneless and its price is extremely competitive (Wiwat, Doungporn, & Kriangsak, 2015). Currently, in the preparation of fish for the consumer market, up to 50% of the whole fish is commonly discarded as waste, but this waste could be better utilized in several ways, such as recovery of the flesh, extraction of fish oil, and for use in animal feed products (Taylor & Alasalvar, 2002). Adipose tissue is a kind of fish waste that has not previously been properly or fully utilized. This tissue makes up 5 to 10% of the total body weight of the fish (Teerawat, Kriangsak, Sudaporn, Chutima, & Doungporn, 2018), and it may represent valuable alternative lipid sources for aquafeed.

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and their elimination by protective mechanisms, and it can lead to chronic inflammation and suppressed immune responses (Reuter, Gupta, Chatur vedi, & Aggarwal, 2010). ROS are involved in maintaining cell homeostasis and regulating functions such as gene expression, signal transduction, and activation of receptors (Kumar & Pandey, 2015). The antioxidant defence system is

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associated with health status and immune system in fish (Martínez, Morales, & Sanz, 2005). Fish immunity is also bound up with inflammation response, which is primarily mediated by cytokines and related to signalling molecules in the head, kidney, and spleen of fish (Biswas *et al.,* 2012). Various inflammations initiate the inflammatory process that results in the synthesis and secretion of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB/AP-1) (Hussain, Tan, Yin, & Blachier, 20 16). These proinflammatory cytokines are involved in the non-specific immunity in fish (Wang *et al.*, 2018).

In a previous study, crude fish oil from freshwater hybrid catfish displayed high levels of antioxidants in an ABTS radical scavenging assay and no sign of acute toxicity in albino rats after receiving a dose of crude fish oil at 5000 mg/kg (Wiwat *et al.,* 2105). The catfish treated with 5% crude fish oil showed a significant increase in body weight ($P<0.05$) and the fish flesh showed a high content of omega fatty acids (Sittikorn, Kriangsak, Chutima, & Doungporn, 2015). Moreover, adipose tissue in the visceral abdomen was extracted and partially purified which resulted in freshwater fish oil (FFO) at a yield of 300 mL per 1 kg of adipose tissue. FFO had a clear yellow colour and met the standards of the Food and Drug Administration, Thailand, including acid value, peroxide value, iodine value, and free fatty acid. The omega-9 acid (oleic acid) content in the FFO was found to be four times higher than in commercial fish oil and exhibited oxidative defence via a decrease of lipid peroxidation and an increase in endogenous antioxidants such as glutathione (GSH) (Teerawat *et al.,* 2018). A deficiency of GSH causes oxidative stress which results in an imbalanced immune response, inflammation, and heightened susceptibility to infection (Ghezzi, 2011). In addition, it has been reported that oleic acid is able to reduce inflammation associated with saturated fatty acid-induced inflammation in human subjects (Harvey *et al.*, 2010). However, there are no reports regarding omega-9 fatty acids in fish feed. Therefore, this study aimed to evaluate the effect of omega-9 rich FFO dietary supplementation on oxidative enzymes and immune gene expression associated with the health of Nile tilapia (*Oreochromis niloticus*).

2. Materials and Methods

2.1 Preparation of freshwater fish oil

FFO was extracted from the adipose tissue of freshwater hybrid catfish (*Pangasius* sp.) obtained from Thai Panga Farm, Kalasin, Thailand. The extraction process of FFO was according to Teerawat *et al.* (2018) which showed high omega-9 content and associated anti-oxidant activity. Briefly, the adipose tissue from the visceral abdomen was cleaned and steamed at 90 °C for 30 min. The liquid oil was then filtered through a filter sack and squeezed by a screw compressor. To separate the solid particles from the oil, the squeezed liquid was subsequently centrifuged at 4500 rpm/ min at 25 °C for 10 min. The supernatant FFO was separated and the fatty acid composition was analysed at the Central Laboratory (Thailand) Co. Ltd., Chiang Mai Branch, following the in-house methods based on AOAC 996.06 (Association of Official Analytical Chemists [AOAC], 2016).

2.2 Formulation of fish feeds

Four iso-nitrogenous (30% crude protein) and iso-energetic (400 Kcal/g) diets were formulated that contained different levels of freshwater fish oil (Table 1). A previous study showed that supplementation of feed with 1% and 1.5% FFO resulted in significant increases in growth performance (Teerawat *et al.,* 2018). All ingredients were ground and blended in a food mixer, extruded, and sun-dried. The dried feed was stored at ambient temperature.

Table 1. Ingredients of the dietary treatments in Nile tilapia (*Oreochromis niloticus*).

Ingredients	Dietary treatments			
	0%	0.5%	1%	1.5%
Fish meal	15	15	15	15
Soybean meal	37	39	37	37
Broken rice	26	31	26	23
Rice bran	20	13	20	22
Freshwater fish oil	0	0.5	1	1.5
Soybean oil	1	0	0	0
Multi-vitamin (Premix)	1	1	1	1
Energy (Kcal/g)	400	400	400	400
Protein (%)	30	30	30	30
Proximate composition (%)				
Protein	30.06	30.92	30.65	30.90
lipid	8.80	7.89	7.86	7.74

2.3 Fish experiment in cages

Nile tilapia were supplied by the Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai, Thailand. In total, 360 fish (50.0±3.0 g) were selected and divided equally into 12 net cages (1.2×1.2× 1.2 m) fixed to a platform in the middle of an earthen pond. Thirty Nile tilapia juveniles were randomly stocked into each net cage in three replicates. The general health of the fish was observed twice a day (08:00 h and 16:00 h) over the course of the experiment (120 days). The growth performance of all fish, i.e. body weight and weight gain, was measured and recorded every month. The animal facilities and protocols were approved by the Maejo University Animal Care and Use Committee, Chiang Mai, Thailand.

2.4 RNA extraction and gene expression by semi-quantitative reverse transcriptase-polymerase chain reaction

Four fish from each cage were randomly selected and anesthetized with 1% clove oil. Liver and kidney tissues were quickly dissected and kept in an ice box for RNA extraction. Total RNA was extracted from the liver and kidney tissues at the end of the 120 days of feeding using an RNA extraction kit (Amresco™, Dallas, TX, USA), following the manufacturers protocol. The quality and concentration of RNA were estimated by spectrophotometry. Approximately 1 µg of total RNA from each tissue sample was reverse trans-

cribed (RT) to produce cDNA using the 5× iScript mastermix^{TM} cDNA synthesis (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Initially, polymerase chain reaction (PCR) was performed using primers for β-actin which served as a positive control for the RT-PCR. The expressions of oxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) primers are shown in Table 2. The PCR conditions were as follows: initial denaturation at 94 ºC for 2 min, followed by PCR cycling at 94 ºC for 2 s, 58 ºC for 30 s, and 72 ºC for 30 s, for 25 to 35 cycles, to fully denature the template, and then extension at 72 ºC for 7 min.

The expressions of TNF-α, interleukin-1β (IL-1β), and immunoglobulin M (IgM) were examined using the primers presented in Table 3. The PCR conditions were similar to those mentioned above, except that PCR cycling was increased to 36 to 40 cycles. A single major band of each PCR product was visualized using 1.5% agarose/GelRed (1 µL/10 mL). Semi-quantitative assessments of the mRNA levels were determined by quantifying the intensity of each band using ImageJ software (National Institutes of Health, Bethesda, MD, USA) compared to the β-actin gene.

Table 2. Primer sequence used for examination of the expressions of antioxidant genes in the liver and kidney tissues of Nile tilapia.

Gene	Primer sequence	Product size (base pair)
SOD	F: 5´-TGGAGGCGGCCACATTA R: 5´- AGCCACCGTAACAGCAGACAT	151
CAT	F: 5´-AGATCCATCAGGAAAGGCTCAA R: 5´-GCAAAACGCAAGTGCTGACA	121
GPx	F: 5´-CGACCTGACAGCTAAGCTGTTG R: 5´-GGCGGAGTAGCGAGAATGAA	151
β-actin	F: 5´-TCTGGTCGTACCACTGGTATCG R: 5´-AGGAGTAGCCACGCTCTGTCA	166

SOD=superoxide dismutase, CAT=catalase, GPx=glutathione peroxidase

Table 3. Primer sequences used for examination of the expressions of immunological genes in the liver and kidney tissues of Nile tilapia.

Gene	Primer sequence	Product size (base pair)
TNF-α	F: 5´CAAAACAGAGCCCCACTCCA R: 5´GAGTAGCGCCAGATCCTGTG	288
IL-1β	F: 5´GACAATGAAGCGTGTGGTCAA R: 5´CTGCTGGTGAACTGAGGTGG	180
IgM	F: 5´TGGTTTTCGGGTGAAGAGGG R: 5´GGTCAGCCT-CTCTGCCATTT	424
β-actin	F: 5´-TCTGGTCGTACCACTGGTATCG R: 5´-AGGAGTAGCCACGCTCTGTCA	166

TNF-α=tumour necrosis factor-alpha, IL-1β=interleukin-1β, IgM= immunoglobulin M.

2.5 Statistical analysis

The data were expressed as mean±standard error (SE) analysed via one-way analysis of variance (ANOVA). In the case of significant differences ($P<0.05$), the Tukey HSD post hoc test was followed.

3. Results

3.1 Fatty acid composition of FFO

The amounts of saturated fatty acid and unsaturated fatty acid in FFO were similar to the amounts in marine fish oil (MFO) from a commercial brand. The quantity of polyunsaturated fatty acid in FFO, especially omega-3 fatty acid, was lower than in MFO. Interestingly, the quantity of omega-9 (oleic acid) was four times greater than in MFO. A comparison of the fatty acid compositions of FFO and MFO is presented in Table 4. The amounts of omega-3, 6, and 9 fatty acids, which affect growth, were 1.38, 12.29, and 42.9 g/100g and 34.15, 6.24, and 11.94 g/100g in FFO and in MFO, respectively.

3.2 Effect of FFO on growth performance

The growth performance of Nile tilapia treated with various levels of FFO over 4 months is provided in Tables 5 and 6. The fish body weight (BW) at the second through fourth months and the weight gain (WG) at the first through fourth months showed significant increases ($P<0.05$) in the group supplemented with 1% FFO compared to the control group. Furthermore, there was a statistical difference between the BW and WG of Nile tilapia treated with 1.5% FFO and the control group at the third and fourth months.

3.3 Effects of FFO on gene expressions of antioxidant enzymes in the liver and kidney tissues

Figures 1 and 2 indicate no significant differences in the gene expressions of oxidant enzymes ($P>0.05$), including SOD, CAT, and GPx, in the liver and kidney tissues of Nile tilapia fed with 0.5% to 1.5% FFO for 120 days. However, GPx gene expression tended to increase in the kidney tissue of Nile tilapia fed with FFO-supplement diets.

3.4 Effects of FFO on gene expressions of immune cytokines in the liver and kidney tissues

Gene expressions of proinflammatory cytokines and the non-specific genes TNF-α and IL-1β and specific gene IgM in the liver and kidney tissues of the Nile tilapia fed with 0.5% to 1.5% FFO for 120 days are shown in Figures 3 and 4. The gene expressions of the three immunity cytokines in the liver tissue were not statistically different in any of the groups compared with the control group. However, expression of the non-specific immunity gene IL-1β in the kidney tissue was significantly different in the Nile tilapia fed with 1% and 1.5% FFO in comparison with the control group ($P<0.05$) (Figure 4B). IgM gene expression tended to increase in the kidney tissue of the Nile tilapia fed with the 1.5% FFO-supplemented diet (Figure 4C).

Table 4. Fatty acid composition of freshwater fish oil.

Fatty acid composition	Amount (g/100 g)	
	Freshwater fish oil (FFO)	Marine fish oil (MFO)
Saturated fatty acids	38.28	35.27
Monounsaturated fatty acids	47.19	23.78
- Oleic acid (C18:1 n-9)	42.12	11.24
Polyunsaturated fatty acids	14.53	40.97
- Linolenic acid (C18:2 n-6)	10.83	4.47
- α-Linolenic acid (C18:3 n-3)	0.87	0.83
- Eicosapentaenoic acid (C20:5 n-3)	0.12	20.98
- Docosahexaenoic acid (C22:6 n-3)	0.29	12.25
Unsaturated fatty acid	61.72	64.75
- omega-3	1.38	34.15
- omega-6	12.29	6.24
- omega-9	42.29	11.94

Table 5. Effect of freshwater fish oil on body weight in Nile tilapia.

% freshwater fish oil	Month			
	February 1st month	March 2nd month	April 3rd month	May 4th month
0	171±7.79	196±9.71[a]	200±7.14[a]	211±10.57[a]
0.5	178±7.34	190±9.44[a]	199±7.91[a]	209±9.46[a]
1.0	195±9.61	240±13.31[b]	259±12.14[b]	273±10.25[b]
1.5	195±8.82	215±11.11[a]	252±12.22[b]	271±9.36[b]

Data are presented as mean±SE.
Note: [a-b] means in the same column with different superscript letters are statistical difference comparing with control (P<0.05).

4. Discussion

The aim of this study was to determine the fatty acid composition of freshwater fish oil (FFO) extracted from a by-product of freshwater hybrid catfish processing and determine its effect on fish growth. The FFO contained high amounts of omega-9 fatty acids which exhibit biological activity and may affect antioxidant and immune responses in Nile tilapia. The results provided evidence that FFO increased the growth rate of Nile tilapia and tended to involve GPx, antioxidant enzymes, and IgM which is a specific-immune cytokine.

Many studies have reported that the essential omega-3 fatty acids are typically associated with initiating anti-inflammatory responses, while omega-6 fatty acids are associated with pro-inflammatory responses (Stacie, Megan, & Robert, 2015; Tortosa, Navas, Marín, & Orenes, 2017). In addition, non-essential omega-9 fatty acids serve as necessary components of pro- and anti-inflammatory processes via their ability to stimulate enzymes and produce cytokines, which may subsequently affect disease risk (Johnson & Bradford, 2014). The extracted FFO exhibited high amounts of oleic acid (omega-9 fatty acids) and its activity on antioxidant activity was determined from a decrease in lipid peroxidation and an increase in glutathione was shown in our previous study (Teerawat *et al.*, 2018).

Free radical production in cells is relatively low in normal conditions due to various and very active defence systems, including antioxidant compounds and enzymes such as SOD, CAT, and GSH-Px (Deleve & Kaplowitz, 1991; Remacle *et al*., 1992). Antioxidant defence systems include a series of antioxidant enzymes and low-molecular non-enzymatic antioxidants that protect the biological systems from environmental stress and maintain cell homeostasis and functions (Pandey *et al.*, 2003; Van der Oost, Beyer & Vermeulen, 20 03). In the present study, the gene expression of three antioxidant enzymes did not increase in the liver and kidney tissues in Nile tilapia supplemented with FFO for four months. However, gene expression of GPx tended to increase in Nile tilapia fed with 1% and 1.5% FFO. This result was similar to the study by Venkatraman *et al.* (1994), who reported that a fish diet containing 10% fish oil plus vitamin E (400 IU/kg diet) fed to autoimmune-prone mice for 6.5 months stimulated endogenous antioxidant defences by increasing the activity and expression of hepatic and kidney anti-oxidant enzymes, such as GPx, SOD, and CAT. Moreover, a study by Erdogan *et al.* (2004) demonstrated that administration of omega-3 fatty acids led to decreased lipid peroxidation and increased SOD activity. FFO is mainly composed of omega-9 fatty acids, such as oleic acid, whereas MFO contains high levels of omega-3 fatty acids such as eicosapentaenoic acid and docosahexaenoic acid. MFO, therefore, displayed the expression of antioxidant genes in hepatic and kidney tissue more so than FFO.

TNF-α and interleukin-1b (IL-1β) are two well-known cytokines involved in non-specific immunity in fish that enhance various cellular responses such as phagocytosis, chemotaxis, macrophage proliferation, and lysozyme synthesis (Dinarello, 1997; Goetz et *al.*, 2004). IL-1β has been detected in teleost fish species and is involved in the regulation of immunity through the stimulation of T cells. In ad-

Table 6. Effect of freshwater fish oil on weight gain in Nile tilapia.

% freshwater fish oil	Month			
	February 1st month	March 2nd month	April 3rd month	May 4th month
0	47.21±0.59[a]	72.2±2.58[a]	76.17±1.12[a]	86.96±4.66[a]
0.5	49.92±1.42[a]	62.5±1.93[a]	71.08±0.69[a]	81.13±3.27[a]
1.0	61.83±0.69[b]	106.88±3.24[b]	125.29±6.97[b]	139.50±7.15[b]
1.5	56.17±2.08[b]	76.04±3.84[a]	112.96±4.27[b]	131.42±2.73[b]

Data are presented as mean±SE.
Note: [a-b] means in the same column with different superscript letters are statistical difference comparing with control (P<0.05).

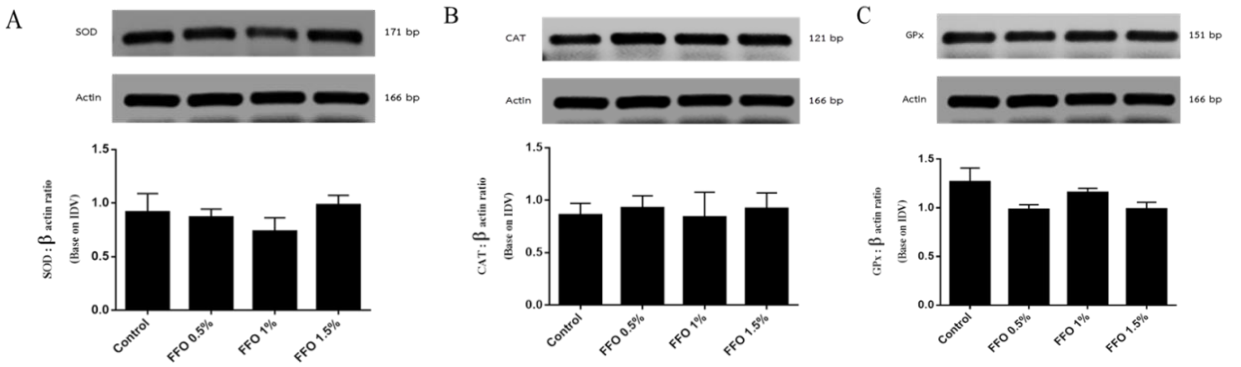

Figure 1. Effect of freshwater fish oil supplemental diet on antioxidant gene expression in the liver tissue of Nile tilapia. A: Superoxide dismutase (SOD), B: Catalase (CAT), C: Glutathione peroxidase (GPx).

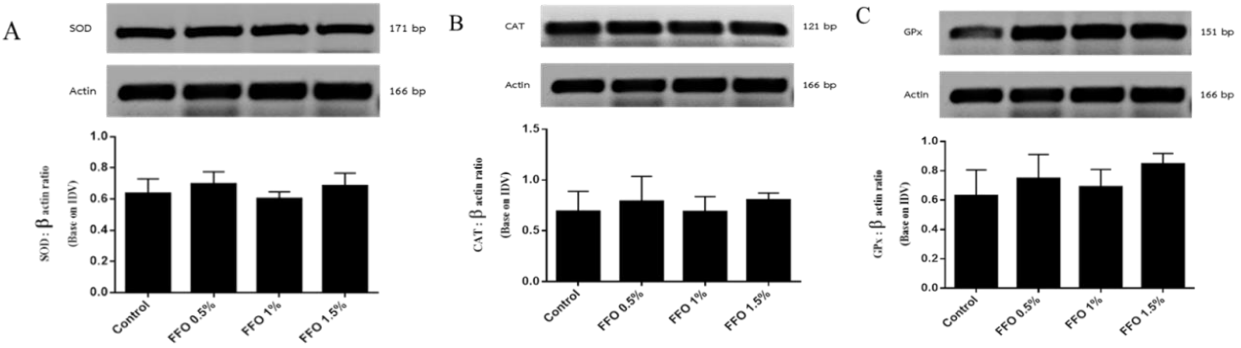

Figure 2. Effect of freshwater fish oil supplemental diet on antioxidant gene expression in kidney tissue of Nile tilapia. A: Superoxide dismutase (SOD), B: Catalase (CAT), C: Glutathione peroxidase (GPx).

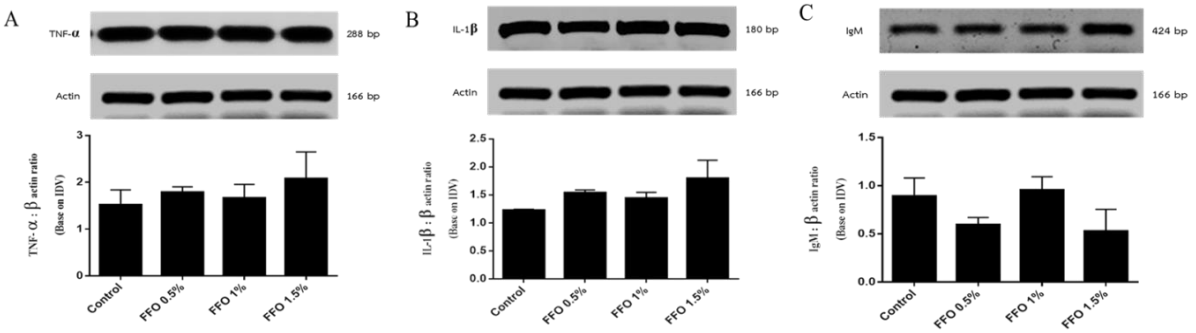

Figure 3. Effect of freshwater fish oil supplemental diet on gene expression of immunity in the liver tissue of Nile tilapia. A: Tumor necrosis factor alpha (TNF-α), B: Interleukin-1β (IL-1β), C: Immunoglobulin M (IgM).

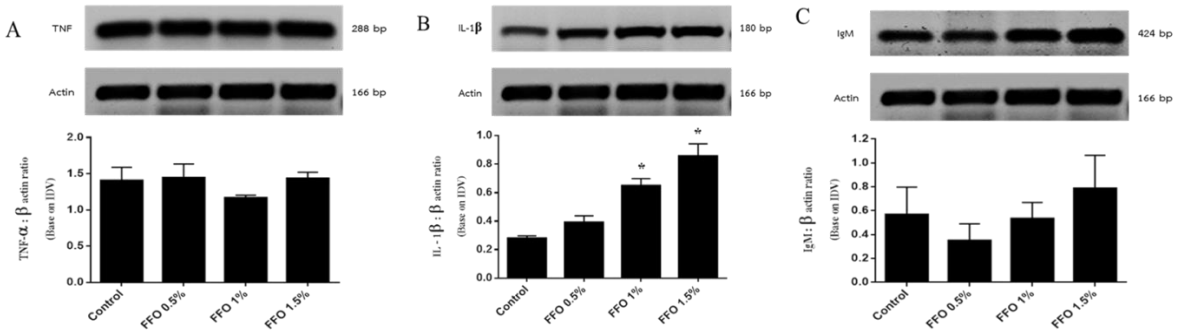

Figure 4. Effect of freshwater fish oil supplemental diet on gene expression of immunity in kidney tissue of Nile tilapia. A: Tumor necrosis factor alpha (TNF-α), B: Interleukin-1β (IL-1β), C: Immunoglobulin M (IgM).

dition, the transcripts of TNF-α and IL-1β significantly increased in the liver of fish fed vegetable oil diets (Peng *et al.*, 2017). However, gene expressions of TNF-α in the liver and kidney tissues were not significantly different in Nile tilapia supplemented with FFO in this study. The IL-1β gene expression in kidney was significant higher in Nile tilapia fed with 1% and 1.5% FFO which indicated that proinflammatory cytokines such as IL-1β might be enhanced by the high amounts of omega-6 fatty acid or arachidonic acid in FFO. However, the expression of IL-1β in this study did not affect the growth of Nile tilapia.

IgM is classified as the primordial immunoglobulin of the adaptive immune response or specific immunity (Acton *et al.*, 1971; Wilson & War, 1992). The kidney is the key organ in fish that responds to stress and it also interacts with the nervous, endocrine, and immune systems. The results of this study showed that the expression of IgM tended to increase in the kidney tissue of Nile tilapia fed a diet supplemented with 1.5% FFO. According to the results, IL-1β and IgM in kidney tissue showed higher expression than in the liver tissue. In most cases, studying the immune response requires the animal to have previously been subjected or challenged to a stress situation, such as infection by pathogens or stressful physical environments (Tort, 2011). This oxidative stress might stimulate greater immune response more efficiently than normal physiological conditions, similar to those in this study. Thus, gene expressions of the proinflammatory cytokines in this study were not significant and the mechanism of this response remains unclear. Such responses may depend on the intensity and duration of stress factors to enhance certain immune response pathways. Challenging fish with stress factors, such as pathogens or external factors, should be considered to stimulate a greater immune response in future studies.

5. Conclusions

The results of this study showed that FFO can increase the growth performance of tilapia. The mechanism of FFO might involve the antioxidant and anti-inflammatory properties of omega-9 fatty acids in FFO, but no significant effect on the increase of immune gene expression was found. However, FFO can be supplemented in fish feed or FFO can replace marine fish oil to improve growth and decrease the cost of aquaculture.

Acknowledgements

This study was financially supported by the National Research Council of Thailand (NRCT). The authors are grateful to the graduate school, Faculty of Fisheries Technology and Aquatic Resources at Maejo University for providing the necessary facilities.

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