Thesis for Degree of Master of fisheries science

Evaluation of the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic (OTC) in juvenile olive flounder, *Paralichthys olivaceus*

by

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치어기 넙치에 있어 항생제 (OTC) 대체를 위한 법제유황 (Immuno-F)의 적정 수준평가

Advisors : Sungchul C. Bai

by

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Evaluation of the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic (OTC) in juvenile olive flounder, *Paralichthys olivaceus*

A dissertation

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요 약 문

치어기 넙치에 있어서 항생제 (OTC)를 대체하기 위한 법제유황 (Immuno-F)의 적정 수준을 평가하기 위하여 8주간 실험을 수행하였다. 실험어는 평균 무게 12.6 ± 0.17 g (mean±SD)인 넙치를 대상으로 3반복으로 한 수조당 20 마리씩 사육하였다. 실험사료는 항생제 및 항생제 대체물질인 법제유황의 첨가 수준(단위 ppm)에 따라 총 8개 실험구(Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, F_{2000}, OTC_{4000} ; Cont: 대조구, F: 법제유황, OTC: oxytetracycline)를 제작하였다. 8주간의 사육 실험 종료 후, 증체율 일간성장률에 있어서 OTC_{4000} 실험구는 Cont, F_{25}, F_{500}, F_{1000} 그리고 F_{2000} 실험구보다 유의하게 높은 값을 나타내었다 (P<0.05). 하지만, F_{50}, F_{250} 그리고 OTC_{4000} 실험구들간에는 유의하게 차이를 보이지 않았고 (P>0.05), Cont, F_{25}, F_{500}, F_{1000} 그리고 F_{2000} 실험구들간에도 유의하게 차이를 보이지 않았다 (P>0.05). 사료효율, 단백질전환효율에 있어서 OTC_{4000} 실험구는 F_{500}, F_{1000} 그리고 F_{2000} 실험구보다 유의하게 높은 값을 나타내었다 (P<0.05). 하지만, Cont, F_{25}, F_{50}, F_{250} 그리고 OTC_{4000} 실험구들간에는 유의하게 차이를 보이지 않았다 (P>0.05). 중성지방에 있어서 F_{25}, F_{50}, F_{250}, F_{500} 그리고 OTC_{4000} 실험구들간에는 유의하게 차이를 보이지 않았다 (P>0.05). 하지만, F_{1000} 실험구가 F_{25}, F_{50}, F_{250}, F_{500} 그리고 OTC_{4000} 실험구들보다 유의하게 높은 값을 나타내었다 (P<0.05). 공격실험으로 *Edwardsiella tarda* 를 복강주사한 후 누적 생존율을 조사해본 결과, 6 일째에 F_{50} 실험구가 F_{2000} 실험구보다 유의하게 높은 값을 나타내었다 (P<0.05). 하지만, Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000} 그리고 OTC_{4000} 실험구들간에는 유의하게 차이를 보이지 않았다 (P>0.05). 따라서, 치어기 넙치에 있어 50 ppm의 법제유황의 첨가가 항생제 (OTC)를 대체할 수 있을 것으로 판단되었다.

Evaluation of the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic (OTC) in juvenile olive flounder, *Paralichthys olivaceus*

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Abstract

An 8-week feeding trial was conducted to evaluate the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic (OTC) on growth performance, hematology, and disease resistance in juvenile olive flounder. Each of 20 fish averaging 12.6 ± 0.17 g (mean±SD) were randomly allocated into 8 groups of three tanks, and fed one of the eight isonitrogenous and isocaloric (crude protein 46.7%, 160 kJ g^{-1}) experimental diets formulated by supplementing Immuno-F at 0 ppm (Cont), 25 ppm (F_{25}), 50 ppm (F_{50}), 250 ppm (F_{250}), 500 ppm (F_{500}), 1000 ppm (F_{1000}) and 2000 ppm (F_{2000}), or oxytetracycline 4000 ppm (OTC_{4000}). Weight gain and specific growth rate of fish fed OTC_{4000} diet were significantly higher than those of fish fed Cont, F_{25}, F_{500}, F_{1000} and F_{2000} diets (P<0.05). However, there were no significant differences among fish fed F_{50}, F_{250} and OTC_{4000} diets (P>0.05), and among fish fed Cont, F_{25}, F_{500}, F_{1000} and F_{2000} diets (P>0.05). Feed efficiency and protein efficiency ratio of fish fed OTC_{4000} diet were significantly higher than those of fish fed F_{500}, F_{1000} and F_{2000} diets (P<0.05). However, there were no significant differences among fish fed Cont,

F_{25}, F_{50}, F_{250} and OTC_{4000} diets (P>0.05). There were no significant differences in serum triglyceride levels of fish fed F_{25}, F_{50}, F_{250}, F_{500} and OTC_{4000} diets (P>0.05). However, serum triglyceride levels of fish fed F_{1000} diet were significantly higher than those of fish fed F_{25}, F_{50}, F_{250}, F_{500} and OTC_{4000} diets (P<0.05). For challenge test with *Edwardsiella tarda* at the 6th day, cumulative survival rates of fish fed F_{50} diet were significantly higher than those of fish fed F_{2000} diet (P<0.05). However, there were no significant differences among fish fed Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000} and OTC_{4000} diets (P<0.05). Therefore, results may indicate that dietary processed sulfur level of 50 ppm could replace the antibiotic (OTC) in juvenile olive flounder.

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Minhye Park

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Ⅰ. Introduction

Olive flounder *Paralichthys olivaceus* is one of the most commercially important marine fish species for aquaculture in eastern Asia including Korea, Japan and China (Cho et al., 2006). Olive flounder is a popular food fish in Korea and its culture production has steadily increased since the late 1980s (Kim et al., 2002). In 2017, the production of olive flounder in Korea is approximately 41,207 metric tons and maximum aquaculture production is nearly 55,000 tons in 2009 (KOSTAT., 2018). However, the expanding and intensive aquaculture industry of olive flounder in Korea has suffered problems such as infectious diseases caused by viruses, bacteria and parasites. To resolve the problem, antibiotics have been used in fish farm as traditional strategy for fish diseases. Oxytetracycline (OTC) is one of the most commonly used antibiotics because of its broad range and low cost (Park et al., 2016). The potential hazards of using antibiotics in aquaculture are development of antibiotic-resistant microorganisms, antibiotic residuals in fish products, contamination of surrounding ecological system and reduced efficiency of antibiotics against the diseases caused by resistant pathogens (McPhearson et al., 1991; Hernandez Serrano, 2005). Moreover, consumer interest in the quality and safety of farmed fish has increased, along with interest in the absence of concomitant pollutants, antibiotics and carcinogens (Jahncke,

2007; Hwang et al., 2013). For these reasons, administration of antibiotics in aquaculture is strictly restricted or banned. This encouraged researchers to investigate for an alternative to antibiotics. Many studies have been carried out on eco-friendly feed additives to improve the growth, immune systems and disease resistance of aquaculture species (Francis, Makkar & Becker, 2005; Sahu, Das, Mishra, Pradhan & Sarangi, 2007; Ahmad & Abdel-Tawwab, 2011).

Sulfur is an essential element for the growth of human, animal and S-containing compounds found in all body cells (Stipanuk, 2004). Sulfur compounds have antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (Battin & Brumaghim, 2009). Leustek et al. (2000) and Stipanuk (2004) reported that sulfur biomolecules exert important functions in all living organisms, including free radical scavenging, enzyme function, DNA methylation and repair, regulation of gene expression, protein synthesis, remodeling of extracellular matrix components, lipid metabolism, and detoxification in plants and animals (Yang et al., 2015). Studies have been reported sulfur compounds have many beneficial effects on animals, such as immune function, anticancer, antithrombotic and antioxidant effect (Ariga & Seki, 2005; Grimble, 2006). For example, the supplementation of dietary sulfur to pigs and cattle displayed increase in the ratio of polyunsaturated fatty acid to saturated fatty acid, as well as sulfur-containing antioxidants contents such as methionine and cysteine (Lee et al., 2009; Lee et al., 2013; Richter et al., 2012; Song et al., 2013). In addition,

sulfur-containing organic compounds in garlic and onion extracts have been widely used as medicines for diseases such as typhus, cholera, and dysentery before modern antibiotics are emerging (Block, 1986; Block, 1992), and inhibit the growth of pathogenic microorganisms (Kumar et al., 1998). However, sulfur is highly toxic, as it can cause side effects if ingested by humans or animals (Barrenrine & Ruffin, 1958; Bouchard & Conrad, 1973; Choi & Kim, 2002; Park et al., 2010), and therefore, it is necessary to process sulfur to remove the toxic property for use as a medicine (Kim, 2015). Sulfur is processed through heat melting of mineral sulfur followed by separation of liquid and subsequent cooling (Lee et al. 2010). Processed sulfur is proved not only to supply nutrients, to relieve muscle convulsion, but also to prevent arthritis and diabetes (Kim et al., 2014b). In this way, processed sulfur has been recognized for its function and potential, however, there is still lack of research on aquaculture.

Therefore, the present experiment was designed to evaluate the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic on growth performance, hematology, and disease resistance in juvenile olive flounder.

II. Materials and Methods

Experimental Diets

Ingredients and proximate composition of the basal diet is shown in Table 1. Eight isonitrogenous and isocaloric (crude protein 46.7%, 16.0 kJ g^{-1}) diets were formulated by supplementing Immuno-F at 0 ppm (Cont), 25 ppm (F_{25}), 50 ppm (F_{50}), 250 ppm (F_{250}), 500 ppm (F_{500}), 1000 ppm (F_{1000}) and 2000 ppm (F_{2000}), and oxytetracycline 4000 (OTC_{4000}). Defatted fish meal, flounder muscle powder (FMP), soybean meal and wheat gluten meal were used as the major protein sources; fish oil as the lipid source; corn starch and wheat flour as the carbohydrate sources in the experimental diets. Processed sulfur (Immuno-F) was provided by EF-Biotech Company, Bucheon, Rep. of Korea. Proximate composition of processed sulfur is shown in Table 2. The graded levels of processed sulfur and OTC were supplemented at the expense of cellulose in the experimental diets.

Procedures for diet preparation and storage were as previously described by Bai & Kim (1997). Briefly, after thoroughly mixing the dry ingredients and fish oil with 30 % filtered tap water, experimental diets were pelleted using a laboratory pelleting machine without heating using a 2-mm diameter module (Baokyong Commercial Co., Busan, Korea). After processing, all diets were kept at -20 °C in the refrigerator until use.

Table 1. Composition and proximate analysis of the basal diet

Ingredients	%
Defatted fish meal [1]	25.0
FMP[2]	25.0
Wheat flour	15.0
Soybean meal	3.00
Corn starch	12.8
Wheat gluten meal	7.08
Fish oil	9.50
Vitamin mix[3]	1.00
Mineral mix[4]	1.00
Processed sulfur (Immuno-F)	0.00
OTC[5]	0.00
Cellulose[6]	0.62
Proximate analysis (% of DM basis)	
Moisture	11.4
Crude protein	46.7
Crude lipid	10.6
Crude ash	6.30

[1]Defatted Fish meal (Chile) by the process described, Hong et al., (2017)
[2]Flounder muscle powder; (crude protein: 88.74 %, crude lipid: 6.33 %, ash: 6.64 %).
[3]Contains (as mg kg^{-1} in diets) : Ascorbic acid, 300; dl-Calcium panthothenate, 150; Choline bitatrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine·HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl-α-tocopherol acetate, 201; Retinyl acetate,

6; Biotin, 1.5; Folic acid, 5.4; B_{12}, 0.06

[4]Contains (as mg kg^{-1} in diets) : NaCl, 437; $MgSO_4 \cdot 7H_2O$, 1,380; $NaH_2P_4 \cdot 2H_2O$, 878; $Ca(H_2PO_4) \cdot 2H_2O$, 1,367; KH_2PO_4, 2,414; $ZnSO_4 \cdot 7H_2O$, 226; Fe-Citrate, 299; Ca-lactate, 3,004; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

[5]Oxytetracycline

[6]Cellulose, Sigma-Aldrich Korea Yongin, Republic of Korea; Cellulose was replaced by the graded level of processed sulfur (Immuno-F) and OTC for the experimental diets

Table 2. Proximate composition of processed sulfur (% of dry matter basis)

Ingredients	%
Moisture	14.5
Crude protein	11.5
Crude lipid	42.7
Crude ash	4.75

Experimental fish and feeding trial

This experiment was conducted at the Institute of Fisheries Sciences, Pukyong National University, Busan, South Korea. Juvenile olive flounder were obtained from a private hatchery (Haebaragi Aquafarm, Geoje, Republic of Korea) and fed a commercial diet for 2 weeks to be acclimated to the experimental conditions and facilities prior to the start of the feeding trial. Five hundred forty fish averaging 12.6 ± 0.17 g (mean ± SD) were weighed and randomly distributed into 27 indoor tanks (20 fish/tank) with a 35-L volume receiving a constant flow (0.8~1.0 L min^{-1}) of seawater. Each tank was then randomly assigned to one of the three replicates of the eight dietary treatments. Fish were fed twice daily (09:00 and 18:00 hours) for eight weeks at 2~3 % of wet BW/day. Throughout the experimental period, the water temperature and pH were maintained at 18 ± 1 °C and 7.5 ± 0.3, respectively. Supplemental aeration was provided to maintain the dissolved oxygen near saturation.

Sample collection and biochemical analysis

At the end of the feeding trial, juvenile olive flounder were starved for 24 hours, and the total number and weight of fish in each tank were determined for calculation of weight gain (WG), specific growth rate (SGR), feed efficiency (FE), and protein efficiency ratio (PER). Three fish per tank were randomly selected, individually weighed, and then dissected to obtain

liver and viscera samples for determination of hepatosomatic index (HSI), viscerosomatic index (VSI), and condition factor (CF), respectively (Yoo et al., 2007; Kim et al., 2014a).

Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

Specific growth rate (SGR, %/day) = ($\log_e$ final wt. - $\log_e$ initial wt.) × 100 / days

Feed efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

Protein efficiency ratio (PER) = (wet weight gain / protein intake)

Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt.

Viscerosomatic index (VSI, %) = viscera wt. x 100 / body wt.

Condition factor (CF) = (wet weight / total length^3) × 100

Three fish from each tank were used for the analysis of whole-body proximate composition. Proximate composition analysis of the experimental diets and fish whole-body were performed by the standard methods of AOAC (1995). Samples of fish whole-body were dried at 105 ℃ to constant weight to estimate their moisture content. Crude ash was determined by incineration at 550 ℃ for 3 h. Crude protein was determined using the Kjeldahl method (N × 6.25) after acid digestion, and crude lipid was measured by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Hoganas, Sweden). Total sulfur was analyzed by a sulfur analyzer (SC-432DR, LECO Co., USA).

Three additional fish per tank were randomly captured and anesthetized with ethylene glycol phenyl ether (200 mg L^{-1}) and blood samples were obtained from the caudal vein using 1 mL disposable syringe without anticoagulant. The blood sample was separated by centrifugation (5000 x g) for 10 min. Then, the serum was stored at -70 °C for blood biochemical parameters including plasma triglyceride (TG) and total cholesterol (TCHO) and glutamic pyruvic transaminase (GPT) activities with a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan). Another set of blood samples of the same fish were allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 x *g* for 10 min and stored at −70 °C for the analysis of non-specific immune responses including superoxide dismutase (SOD), lysozyme, and myeloperoxidase (MPO) activities.

Superoxide dismutase (SOD) activity was measured using an SOD assay kit (K335-100, BioVision, USA) in accordance with the manufacturer's instructions. Twenty microliters of serum was used to inhibit superoxide anion produced by xanthine and xanthine oxidase reaction in the presence of WST-1(Water Soluble Tetrazolium dye) substrate. Each endpoint assay was measured by absorbance at 450 nm after 20 min reaction at 37 °C. Percent inhibition was calculated using specific equation stated in the kit protocol.

The turbidometric assay previously described by Hultmark et al. (1980), with slight variations, was used to determine serum lysozyme activity. Briefly,

200 µl of a suspension of 0.75 mg ml^{-1} lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in PBS (pH 6.4, 0.1 M) was added to wells of a 96-well plate and 20 µl of serum was then added to each well. Absorbances were read at 570 nm by a microplate reader (Infinite M200 nanoquant, Tecan, Zurich, Switzerland) after incubation at room temperature for 0 and 30 min. One unit of LSZ activity corresponded to a reduction in absorbance of 0.001 min^{-1}.

Serum myeloperoxidase activity (MPO) was estimated using the method of Quade and Roth (1997), with slight modifications. Twenty microliter of serum samples were diluted with 80 µl of Hanks' balanced salt solution (without Ca^{2+} or Mg^{2+}) in wells of a 96-well plate, and 35 µl of 20mM 3,3′,5,5′ tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich, USA) and 35 µl of 5mMH_2O_2 were added to each well. After 2-min incubation, 35 µl of 4mH_2SO_4 was added to stop the color change and absorbance at 450 nm was read.

Challenge test

A bacterial pathogen, *Edwardsiella tarda*, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Fish (n = 4 per tank) were distributed according to their dietary treatment groups into 11 L aquarium for the challenge test with no water exchange. Fish were injected intraperitoneally with 0.1 mL of culture suspension of pathogenic *E. tarda* containing 1×10^8 CFU m L^{-1}. Fish mortality was recorded daily for 6 days.

Statistical Analysis

All data were analyzed by one-way ANOVA (Statistic 3.1; Analytical Software, St. Paul, MN, USA) to test the effects of the dietary treatments. When a significant treatment effect was observed, a Scheffe test was used to compare means. Treatment effects were considered at $P<0.05$ level of significance.

III. Results

Growth performance

Table 3 and Figures 1-4 show the growth performance of juvenile olive flounder fed different experimental diets for 8 weeks. Weight gain (WG) and specific growth rate (SGR) of fish fed OTC_{4000} diet were significantly higher than those of fish fed Cont, F_{25}, F_{500}, F_{1000} and F_{2000} diets ($P<0.05$). However, there were no significant differences among fish fed F_{50}, F_{250} and OTC_{4000} diets ($P>0.05$), and among fish fed Cont, F_{25}, F_{500}, F_{1000} and F_{2000} diets ($P>0.05$). Feed efficiency (FE) and protein efficiency ratio (PER) of fish fed OTC_{4000} diet were significantly higher than those of fish fed F_{500}, F_{1000} and F_{2000} diets ($P<0.05$). However, there were no significant differences among fish fed Cont, F_{25}, F_{50}, F_{250} and OTC_{4000} diets ($P>0.05$). There were no significant differences in hematosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF) among the treatments ($P>0.05$).

Proximate composition and total sulfur in fish whole body

Proximate composition and total sulfur in fish whole body are provided in Table 4. There were no significant differences in proximate composition among fish fed all the experimental diets ($P>0.05$). Also, There were no significant differences in total sulfur among fish fed all the experimental diets ($P>0.05$).

Hematological parameters

Table 5 and Figures 5-7 show hematological parameters such as pyruvic transaminase (GPT), triglyceride (TG), and total cholesterol (TCHO). Serum GPT levels of fish fed F_{250} and F_{1000} diets were significantly lower than those of fish fed F_{25} diet ($P<0.05$). However, there were no significant differences among fish fed Cont, F_{50}, F_{250}, F_{500}, F_{1000}, F_{2000} and OTC_{4000} diets ($P>0.05$), and among fish fed Cont, F_{25}, F_{50}, F_{500}, F_{2000} and OTC_{4000} diets ($P>0.05$). There were no significant differences in serum TG levels of fish fed F_{25}, F_{50}, F_{250}, F_{500} and OTC_{4000} diets ($P>0.05$). However, serum TG levels of fish fed F_{1000} diet were significantly higher than those of fish fed F_{25}, F_{50}, F_{250}, F_{500} and OTC_{4000} diets ($P<0.05$). Serum TCHO levels of fish fed OTC_{4000} diet were significantly lower than those of fish fed F_{1000} diet ($P<0.05$). However, there were no significant differences among fish fed Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{2000} and OTC_{4000} diets ($P>0.05$).

Non-specific immune responses

Non-specific immune responses are shown in Table 6 and Figures 8-10. There were no significant differences in superoxide dismutase (SOD), lysozyme, and myeloperoxidase (MPO) activities of fish fed the experimental diets ($P>0.05$).

Challenge test

Cumulative survival rate in juvenile olive flounder challenged *E. tarda* for 6 days is shown in Figure 11. During the challenge test, the first mortality occurred on day 2. After the 6 days challenge test, cumulative survival rate of fish fed F_{50} diet was significantly higher than those of fish fed F_{2000} diet ($P<0.05$). However, there were no significant differences among fish fed Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000} and OTC_{4000} diets ($P>0.05$).

Table 3. Growth performance and organosomatic indices of juvenile olive flounder fed the experimental diets for 8 weeks[1]

	Diets							
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}
WG (%)[2]	80.4 ± 7.77^{bc}	75.5 ± 1.38^{bc}	87.1 ± 0.73^{ab}	87.3 ± 5.02^{ab}	64.8 ± 2.75^{c}	70.2 ± 1.45^{c}	65.2 ± 5.90^{c}	101 ± 7.21^{a}
SGR (% day^{-1})[3]	1.40 ± 0.10^{bc}	1.34 ± 0.02^{bc}	1.49 ± 0.01^{ab}	1.49 ± 0.06^{ab}	1.19 ± 0.04^{c}	1.27 ± 0.02^{c}	1.19 ± 0.09^{c}	1.66 ± 0.09^{a}
FE (%)[4]	79.2 ± 7.55^{abc}	78.4 ± 7.74^{abcd}	84.2 ± 2.21^{ab}	82.7 ± 2.64^{ab}	63.9 ± 2.28^{cd}	69.0 ± 2.22^{bcd}	62.4 ± 3.37^{d}	92.8 ± 4.06^{a}
PER[5]	1.70 ± 0.16^{abc}	1.70 ± 0.17^{abc}	1.81 ± 0.05^{ab}	1.80 ± 0.06^{ab}	1.37 ± 0.05^{cd}	1.50 ± 0.05^{bcd}	1.34 ± 0.07^{d}	2.01 ± 0.09^{a}
HSI (%)[6]	1.32 ± 0.14	0.91 ± 0.05	1.04 ± 0.06	1.26 ± 0.16	1.08 ± 0.29	1.12 ± 0.11	0.90 ± 0.25	0.99 ± 0.18
VSI (%)[7]	4.19 ± 0.42	3.83 ± 0.15	3.73 ± 0.02	3.66 ± 0.05	4.11 ± 0.14	4.14 ± 0.04	4.11 ± 0.28	4.14 ± 0.12
CF[8]	0.77 ± 0.06	0.83 ± 0.04	0.77 ± 0.01	0.76 ± 0.01	0.72 ± 0.01	0.80 ± 0.00	0.80 ± 0.05	0.74 ± 0.03

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.05). Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

[2]Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

[3]Specific growth rate (SGR, % day^{-1}) = (log_e final wt. - log_e initial wt.) × 100 / days

[4]Feed efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

[5]Protein efficiency ratio (PER) = (wet weight gain / protein intake)

[6]Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt.

[7]Viscerosomatic index (VSI, %) = viscera wt. x 100 / body wt.

[8]Condition factor (CF) = (wet weight / total $length^3$) × 100

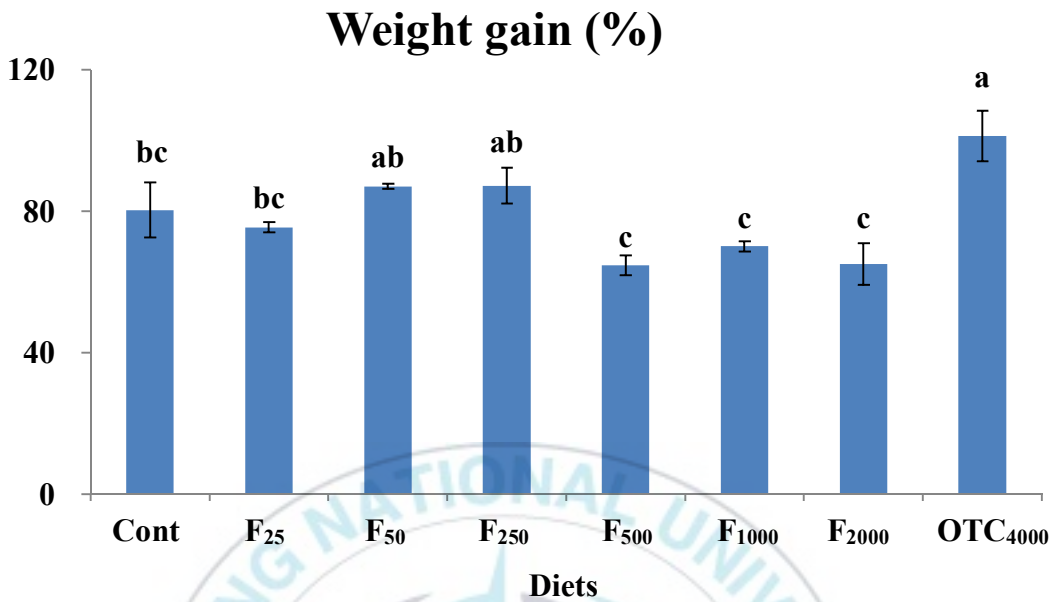


Fig. 1. Weight gain of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

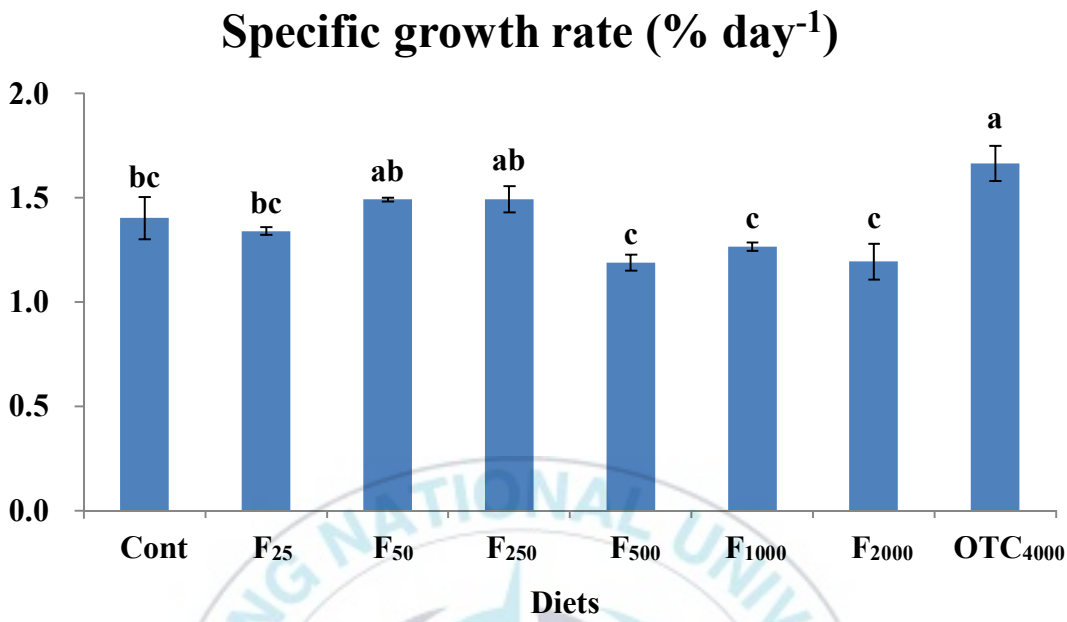


Fig. 2. Specific growth rate of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

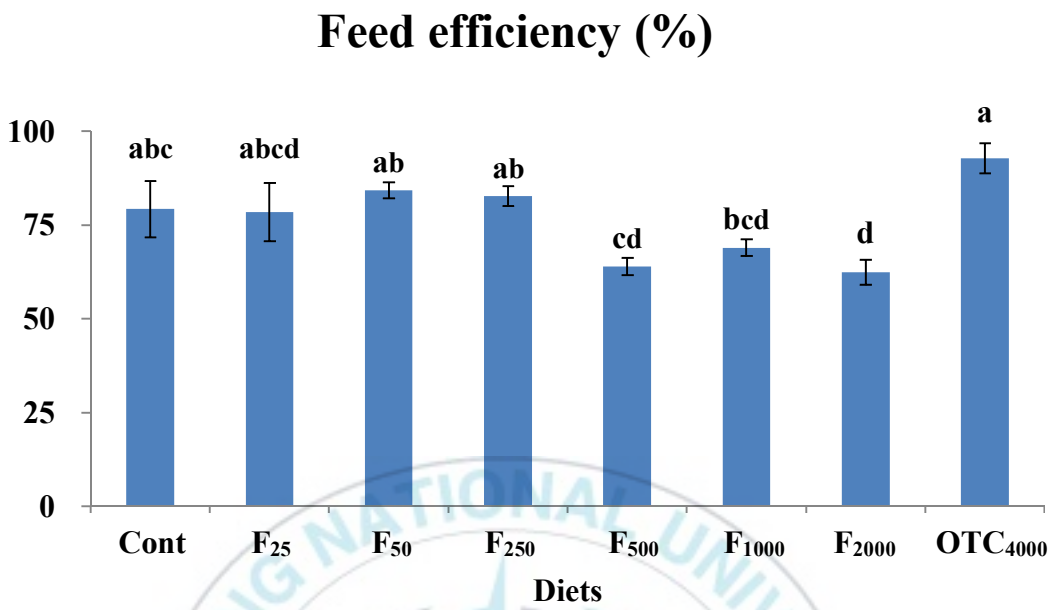


Fig. 3. Feed efficiency of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

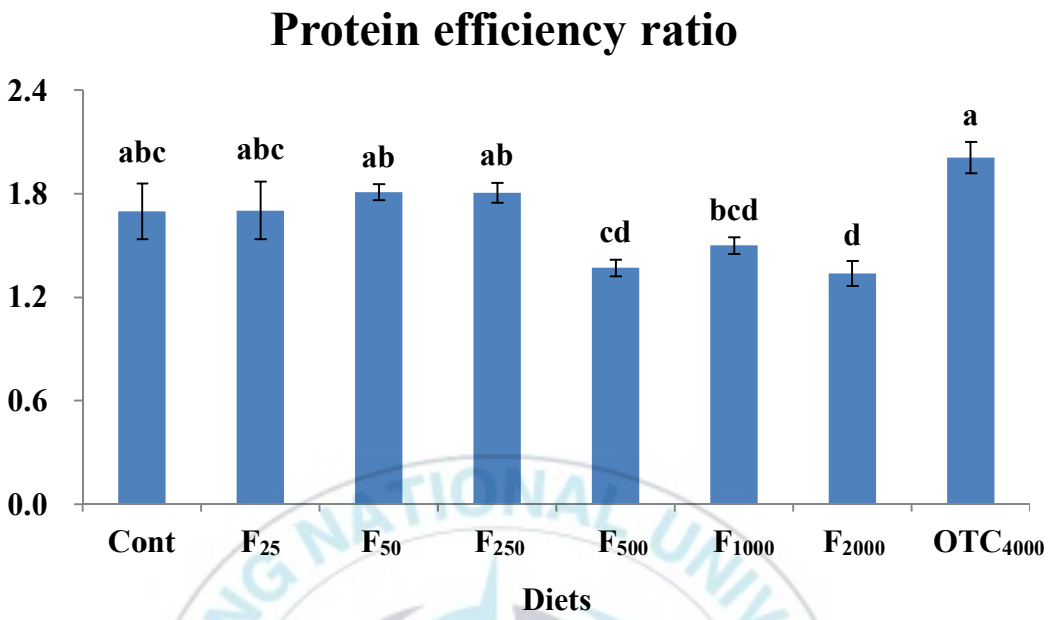


Fig. 4. Protein efficiency ratio of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

Table 4. Proximate composition and total sulfur in whole-body of juvenile olive flounder fed the experimental diets for 8 weeks (%, wet matter basis)[1]

	Diets							
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}
Moisture (%)	77.1 ±0.73	77.5 ±0.40	77.4 ±0.34	77.7 ±0.42	77.8 ±0.29	78.0 ±0.38	77.8 ±0.41	77.5 ±0.82
Crude Protein (%)	17.6 ±0.44	17.3 ±0.66	17.2 ±0.22	17.0 ±0.43	17.2 ±0.61	17.3 ±0.46	17.1 ±0.38	17.2 ±0.50
Crude Lipid (%)	1.53 ±0.49	1.26 ±0.15	1.47 ±0.32	1.45 ±0.28	0.86 ±0.20	1.06 ±0.31	1.04 ±0.02	1.41 ±0.64
Crude Ash (%)	4.11 ±0.20	4.22 ±0.04	4.22 ±0.53	4.09 ±0.11	4.55 ±0.22	4.11 ±0.09	4.11 ±0.14	4.03 ±0.08
Total Sulfur (%)	0.85 ±0.04	0.85 ±0.00	0.76 ±0.03	0.87 ±0.03	0.84 ±0.04	0.84 ±0.04	0.80 ±0.00	0.82 ±0.02

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.05). Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

Table 5. Hematological parameters of juvenile olive flounder fed the experimental diets for 8 weeks[1]

	Diets							
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}
GPT (U L^{-1})[2]	5.00 ±1.00^{ab}	7.00 ±1.00^{a}	5.00 ±0.00^{ab}	4.00 ±0.00^{b}	5.00 ±1.00^{ab}	4.50 ±0.50^{b}	5.00 ±0.00^{ab}	5.00 ±0.00^{ab}
TG (mg dL^{-1})[3]	303 ±39.0^{ab}	272 ±22.5^{bc}	262 ±35.5^{bc}	266 ±1.50^{bc}	155 ±3.50^{d}	378 ±47.0^{a}	309 ±13.0^{ab}	190 ±5.00^{cd}
TCHO (mg dL^{-1})[4]	98.0 ±20.0^{ab}	103 ±12.5^{ab}	104 ±9.00^{ab}	84.0 ±3.00^{b}	72.0 ±6.00^{b}	164 ±52.5^{a}	117 ±10.5^{ab}	81.5 ±0.50^{b}

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.05). Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

[2] GPT (U L^{-1}): Glutamic pyruvic transaminase activity

[3] TG (mg dL^{-1}): Triglyceride

[4] TCHO (mg dL^{-1} : Total cholesterol

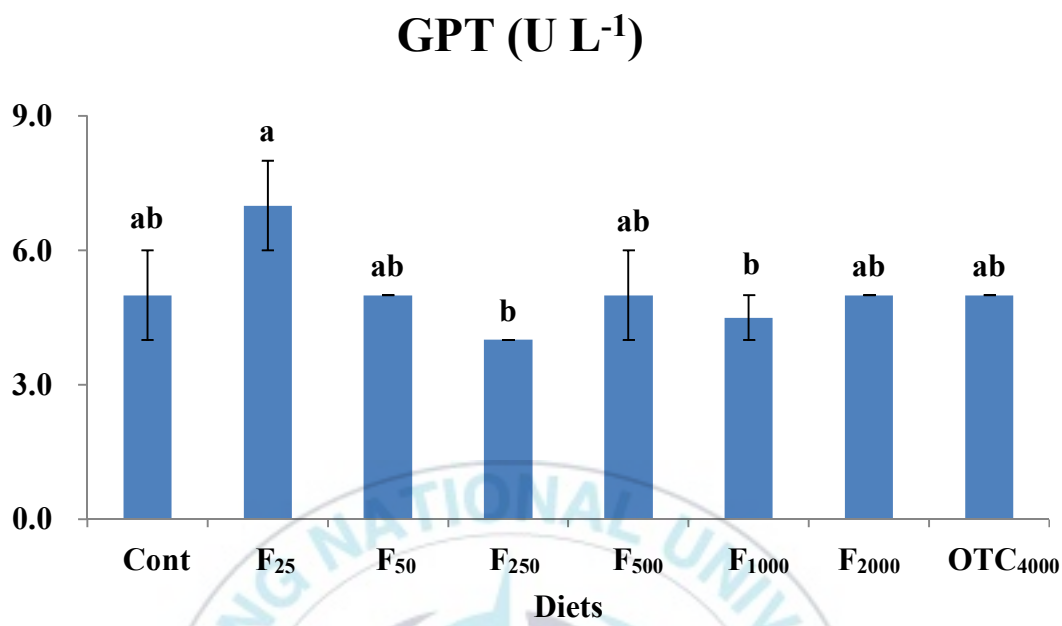
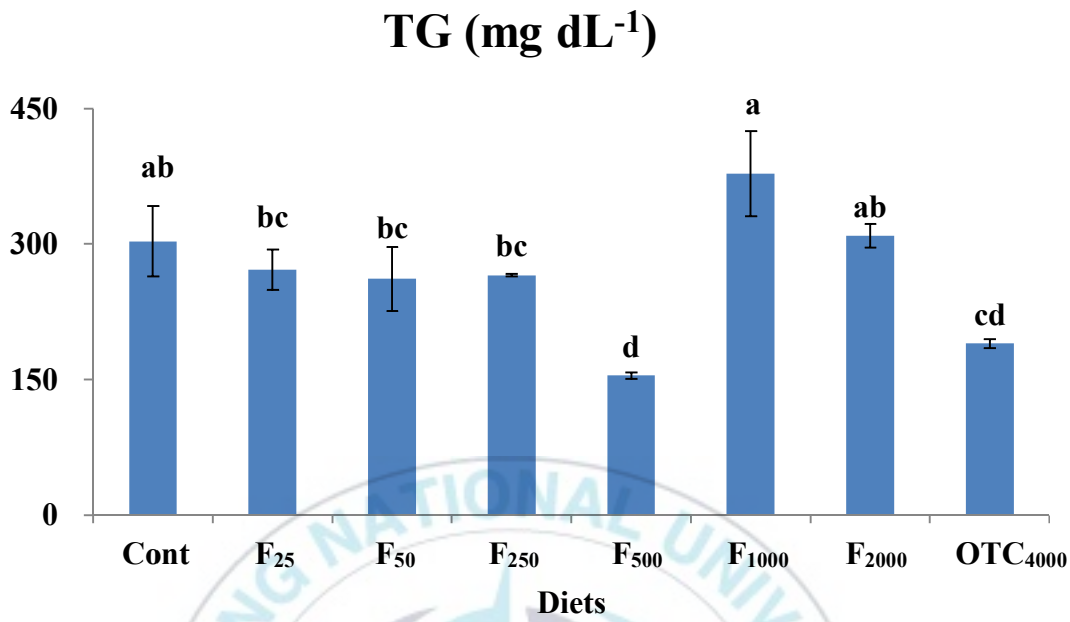
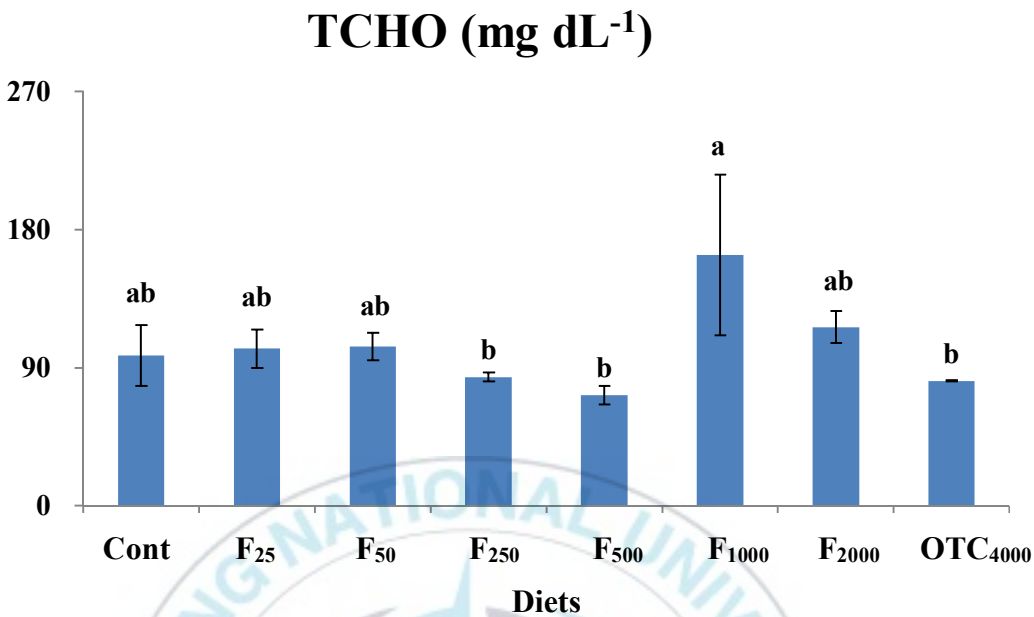


Fig. 5. Glutamic pyruvic transaminase activity (GPT) in juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet



Fig, 6. Triglyceride (TG) of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet



Fig, 7. Total cholesterol (TCHO) of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

Table 6. Non-specific immune responses of juvenile olive flounder fed the experimental diets for 8 weeks[1]

	Diets							
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}
SOD[2] (% superoxide inhibition)	46.6 ±2.33	38.0 ±4.77	49.2 ±1.49	42.5 ±5.62	42.9 ±6.17	51.0 ±4.54	43.6 ±3.82	39.8 ±2.18
LYZ[3] (Units ml^{-1})	1.18 ±0.02	1.88 ±0.59	2.07 ±0.47	1.10 ±0.16	1.98 ±0.37	2.07 ±0.55	1.73 ±0.42	1.28 ±0.13
MPO[4] (absorbance at 450 nm)	1.25 ±0.08	1.36 ±0.17	1.23 ±0.11	1.25 ±0.11	1.39 ±0.43	1.33 ±0.14	1.28 ±0.03	1.08 ±0.00

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$). Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

[2]SOD (% superoxide inhibition): Superoxide dismutase activity

[3]LYZ (Units ml^{-1}): Lysozyme activity

[4]MPO (absorbance at 450 nm): Myeloperoxidase activity

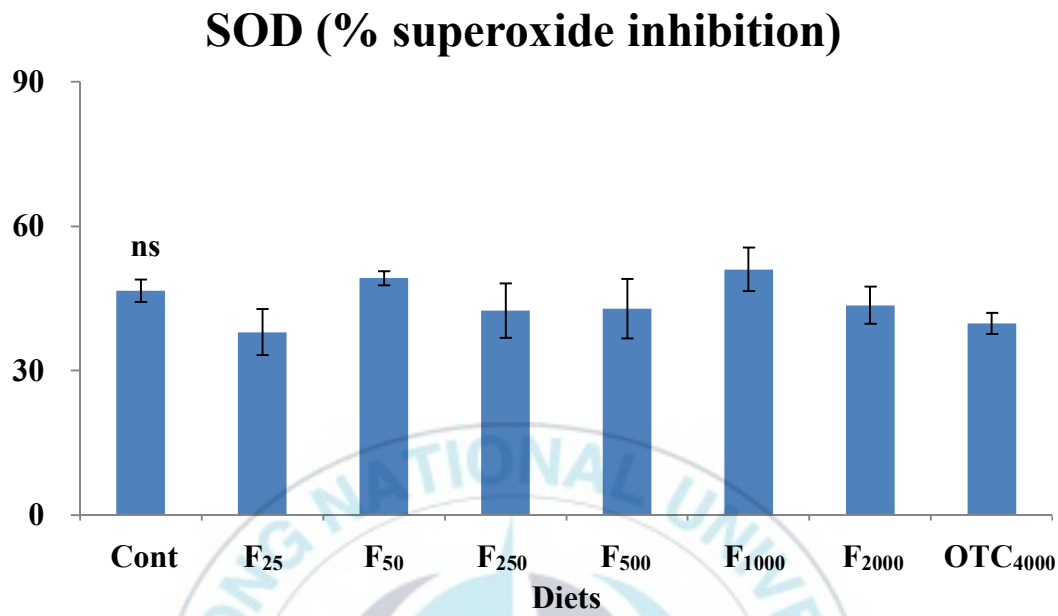


Fig. 8. Superoxide dismutase (SOD) activity of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

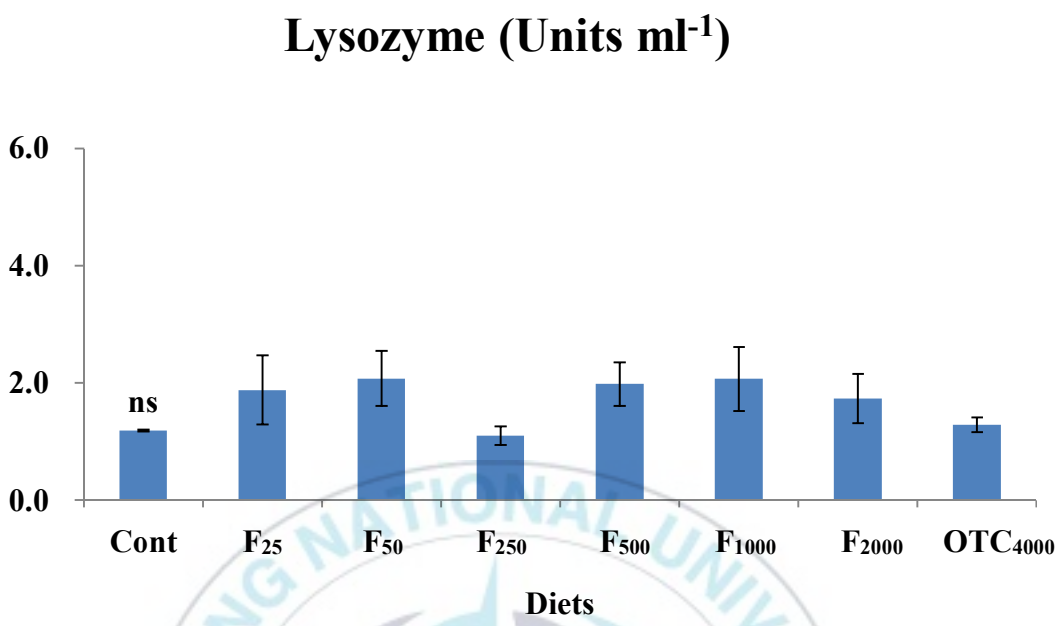


Fig. 9. Lysozyme activity of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

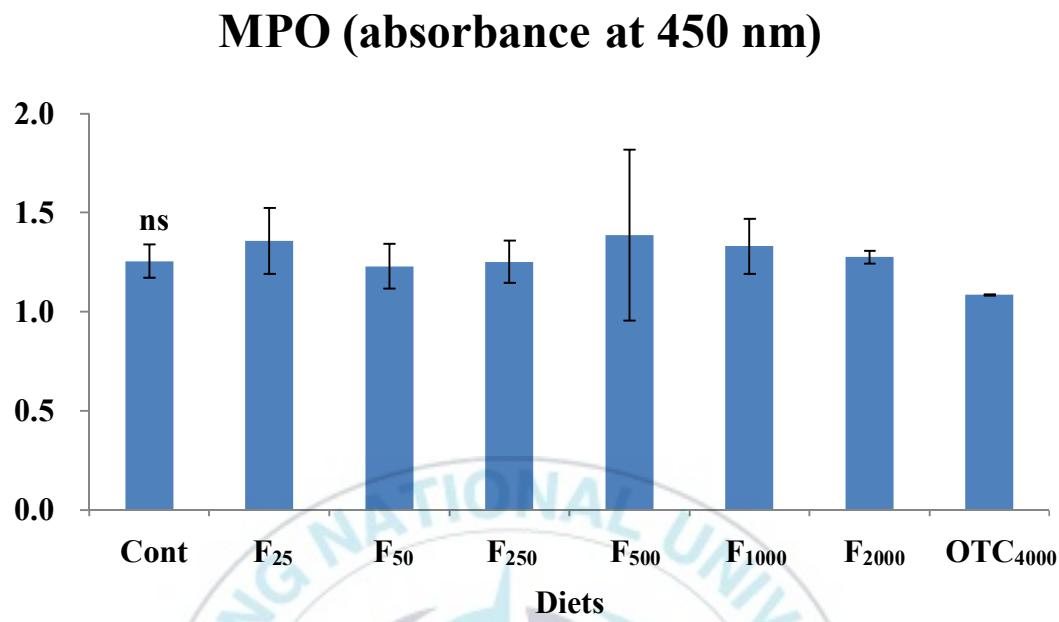


Fig. 10. Myeloperoxidase (MPO) activity of juvenile olive flounder fed the experimental diets for 8 weeks. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

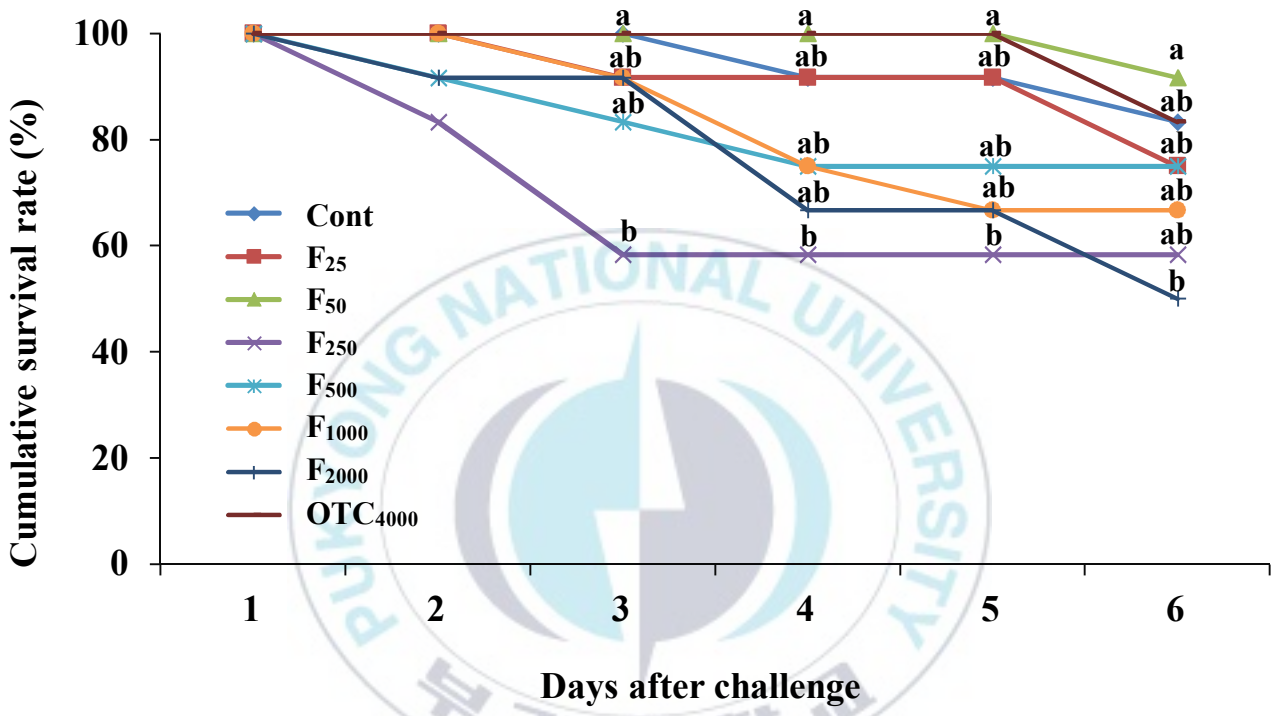


Fig. 11. Cumulative survival rate of juvenile olive flounder fed the experimental diets for 8 weeks and then experimental challenged with *E. tarda* for 6 day. Diets represent; Cont, F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} were supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 (ppm); OTC_{4000} = 4000 ppm at Oxytetracycline was supplemented into Cont diet

IV. Discussion and Conclusion

Growing healthy and disease-resistant fish in antibiotic-free environment have got momentum among fish farmer, as many countries refuse to import the fishery products treated with antibiotics and/or chemicals (Lee et al., 2017). As a result, extensive studies have been carried out to demonstrate the additives for the alternative development of antibiotics. The present study indicate that dietary processed sulfur at the inclusion level of 50-250 ppm (F_{50} and F_{250}) could be equally effective compared to antibiotic 4000 ppm of oxytetracycline to promote significant improvement in the growth performance in olive flounder. This result is in agreement with the findings from previous investigation (Cho, 2011), in which sulfur could be effective dietary additive for improving the growth performance of juvenile olive flounder. Likewise, in broiler chickens, dietary processed sulfur as antibiotic replacer could improve the growth (Park et al., 2010). Other results in this study show that processed sulfur at 500-2000 ppm of dietary inclusion (F_{500}, F_{1000} and F_{2000}) depressed the growth performance, comparing with those of olive flounder fed antibiotic (OTC) diet. This may suggest that the appropriate level of processed sulfur in the diet is beneficial for the growth of olive flounder, but high quantities of processed sulfur will weaken its good effect on growth performance of olive flounder (Kim et al., 2013). Therefore, care should be taken to maintain the optimum supplementation level. In this

study, we found that there were no adverse effects of processed sulfur supplementations on organosomatic indices of fish in terms of HSI, VSI and CF.

The evaluation of hematological parameters might be useful for the diagnosis of fish pathologies and physiological status (Stoskopf, 1993). In the present experiment, serum glutamic pyruvic transaminase (GPT) levels ranged from 4 to 8, which were within acceptable ranges in olive flounder (Kim et al., 2014). Blood enzymes such as GPT are known to be sensitive indicators for tissue damage (De la Tore et al., 2000). Hence, we could suggest that the experimental fish were free of liver damage. It is well known that increasing blood lipid levels is always considered to be signs of declining health condition of the cultured fish (Kikuchi et al., 2009). The high blood lipids levels might cause accumulation of hepatic triglyceride (TG) and total cholesterol (TCHO), and form the fatty liver disease (Quesada et al., 2009). In this study, serum TG and TCHO levels of fish fed F_{1000} diet were significantly higher than those of fish fed OTC_{4000} diet. Our observations imply that excessive supplementation of processed sulfur might cause some disorder in lipid metabolism, leading to hyperlipidemia (Kritchevsky, 1995; Ye et al., 2011).

Bacteria, *Edwardsiella tarda* has been identified as one of the major disease pathogen in the modern fish farming (Katya et al., 2016). Serious

economic losses have been reported from olive flounder farms because of notorious bacterial disease most commonly caused by this species. Interestingly, in the current experiment, higher cumulative survival rate was observed in the group of fish fed F_{50} diet up to 6-day after infection against *E. tarda*. It is well documented that sulfur-containing compounds found in garlic are antimicrobial components (Ponce et al., 2008). Likewise, our result is in agreement with previous study, which was reported the enhanced disease resistance against *E. tarda* after the immersion in 0.25 g/L (250 ppm) garlic juice in olive flounder (Woo et al., 2010).

Non-specific immune parameters have been recognized as the potential indicators of health, as they play a role in controlling the balance of release and clearing reactive oxygen species in immune cells (Campa-Córdova et al., 2002; Holmblad and Söderhäll, 1999; Xu et al., 2010). However, in the current experiment, superoxide dismutase (SOD), lysozyme (LYZ) and myeloperoxidase (MPO) activity were not much affected by dietary processed sulfur supplementation. Also, observations for the fish whole-body proximate composition followed the similar trend. These findings may suggest that although processed sulfur and/or antibiotic (OTC) could improve the growth performance, hematological characteristic and disease resistance in olive flounder, non-specific immune and whole-body proximate composition may not be much affected by such inclusion.

In conclusion, the present study demonstrate that dietary processed sulfur (Immuno-F) level of 50 ppm could replace the antibiotic (OTC), characterized by the growth performance, hematological characteristic and disease resistance against the bacteria *E. tarda* in juvenile olive flounder, *Paralichthys olivaceus*.

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VI. Appendix

Growth performance of olive flounder fed the experimental diets for 8 weeks

Diets	Reps	WG (%)	SGR (% day^{-1})	FE (%)	PER
Cont	1	88.13	1.50	86.78	1.86
	2	72.60	1.30	71.68	1.54
	3	80.36	1.40	79.23	1.70
F_{25}	1	75.52	1.34	78.44	1.70
	2	76.90	1.36	86.18	1.87
	3	74.14	1.32	70.70	1.54
F_{50}	1	87.83	1.50	86.43	1.85
	2	87.10	1.49	84.22	1.81
	3	86.37	1.48	82.02	1.76
F_{250}	1	82.25	1.43	85.33	1.85
	2	87.27	1.49	82.69	1.80
	3	92.29	1.56	80.06	1.74
F_{500}	1	67.52	1.23	66.21	1.42
	2	64.77	1.19	63.94	1.37
	3	62.02	1.15	61.66	1.32
F_{1000}	1	70.17	1.27	68.95	1.50
	2	71.62	1.29	71.17	1.55
	3	68.72	1.25	66.73	1.45
F_{2000}	1	71.07	1.28	65.78	1.41
	2	65.17	1.19	62.41	1.34
	3	59.27	1.11	59.04	1.27
OTC_{4000}	1	101.29	1.66	92.79	2.01
	2	94.08	1.58	88.73	1.93
	3	108.50	1.75	96.85	2.10

Organosomatic indices of olive flounder fed the experimental diets for 8 weeks

Diets	Reps	HSI (%)	VSI (%)	CF
Cont	1	1.18	3.77	0.71
	2	1.45	4.61	0.83
	3	1.32	4.19	0.77
F_{25}	1	0.91	3.83	0.83
	2	0.86	3.68	0.87
	3	0.96	3.98	0.79
F_{50}	1	1.10	3.75	0.79
	2	1.04	3.73	0.77
	3	0.98	3.72	0.76
F_{250}	1	1.42	3.61	0.76
	2	1.26	3.66	0.76
	3	1.10	3.71	0.75
F_{500}	1	0.79	3.97	0.71
	2	1.08	4.11	0.72
	3	1.37	4.25	0.73
F_{1000}	1	1.12	4.14	0.80
	2	1.01	4.18	0.79
	3	1.24	4.10	0.80
F_{2000}	1	0.66	3.84	0.75
	2	0.90	4.11	0.80
	3	1.15	4.39	0.85
OTC_{4000}	1	1.16	4.26	0.72
	2	0.81	4.02	0.77
	3	0.99	4.14	0.74

Whole-body proximate composition of olive flounder fed the experimental diets for 8 weeks

Diets	Reps	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)
Cont	1	76.5	18.1	1.60	4.13
	2	76.8	17.5	1.98	3.90
	3	77.9	17.2	1.00	4.29
F_{25}	1	77.2	18.0	1.19	4.23
	2	77.3	17.2	1.42	4.18
	3	77.9	16.7	1.15	4.25
F_{50}	1	77.0	17.1	1.85	3.98
	2	77.4	17.5	1.28	4.83
	3	77.7	17.1	1.29	3.85
F_{250}	1	77.3	17.4	1.70	4.11
	2	78.2	16.6	1.15	4.19
	3	77.7	17.0	1.50	3.98
F_{500}	1	77.5	17.9	0.72	4.58
	2	78.1	16.9	1.10	4.32
	3	77.8	16.8	0.77	4.76
F_{1000}	1	77.7	17.5	1.09	4.14
	2	77.8	17.6	0.73	4.19
	3	78.4	16.8	1.35	4.01
F_{2000}	1	77.8	17.1	1.01	3.95
	2	78.3	16.7	1.06	4.22
	3	77.4	17.5	1.04	4.15
OTC_{4000}	1	78.3	16.9	0.75	4.11
	2	77.6	16.9	1.46	3.95
	3	76.7	17.7	2.02	4.02

Hematological parameters and non-specific immune responses of olive flounder fed the experimental diets for 8 weeks

Diets	Reps	GPT (U L^{-1})	TG (mg dL^{-1})	TCHO (mg dL^{-1})	SOD (% superoxide inhibition)	LYZ (Units ml^{-1})	MPO (absorbance at 450 nm)
Cont	1	4.00	342.0	118.0	44.3	1.20	1.35
	2	6.00	264.0	78.0	48.9	1.16	1.21
	3	5.00	303.0	98.0	46.6	1.18	1.21
F_{25}	1	7.00	271.5	102.5	38.0	1.88	1.52
	2	6.00	249.0	115.0	33.2	2.47	1.37
	3	8.00	294.0	90.0	42.8	1.29	1.19
F_{50}	1	5.00	297.0	113.0	47.7	1.60	1.32
	2	5.00	261.5	104.0	49.2	2.07	1.10
	3	5.00	226.0	95.0	50.7	2.54	1.26
F_{250}	1	4.00	267.0	87.0	48.2	0.94	1.20
	2	4.00	265.5	84.0	42.5	1.10	1.38
	3	4.00	264.0	81.0	36.9	1.25	1.18
F_{500}	1	4.00	151.0	78.0	36.7	2.35	0.96
	2	5.00	154.5	72.0	42.9	1.98	1.39
	3	6.00	158.0	66.0	49.0	1.61	1.82
F_{1000}	1	4.50	378.0	163.5	51.0	2.07	1.30
	2	4.00	331.0	111.0	55.6	2.61	1.21
	3	5.00	425.0	216.0	46.5	1.52	1.48
F_{2000}	1	5.00	296.0	127.0	39.8	1.31	1.25
	2	5.00	309.0	116.5	43.6	1.73	1.26
	3	5.00	322.0	106.0	47.4	2.16	1.31
OTC_{4000}	1	5.00	185.0	82.0	42.0	1.16	1.08
	2	5.00	195.0	81.0	37.6	1.41	1.09
	3	5.00	190.0	81.5	39.8	1.28	1.08