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Published in: Animal Feed Science and Technology
DOI: 10.1016/j.anifeedsci.2021.115035

Available online: 19 Jul 2021

Citation:
Park, Y., Park, M., Hamidoghli, A., Kim, C.-H. & Bai, S. C. (2021). Optimum dietary processed sulfur (Immuno-F) level has antibiotic effects on the growth, hematology and disease resistance of juvenile olive flounder, Paralichthys olivaceus. Animal Feed Science and Technology, 279: 115035. doi: 10.1016/j.anifeedsci.2021.115035

Optimum dietary processed sulfur (Immuno-F) level has antibiotic effects on the growth, hematology and disease resistance of juvenile olive flounder, *Paralichthys olivaceus*

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Running title: Evaluation of the optimum dietary processed sulfur level in juvenile olive flounder

Abstract

A great deal of research has been focused on feed additives that can boost the immune system of cultured fish since the food and drug administration (FDA) banned the use of antibiotics as feed supplementations in 2017. Sulfur is generally an essential element for the growth of animals, and sulfur-containing compounds are known to have anti-oxidant and anti-bactericidal effects. Although dietary sulfur has many potentials as a functional additive as well as an antibiotic replacer, it can also be toxic when supplemented at the high levels. Thus, it is necessary to determine the optimum amount in fish diet to exert positive effects on immune responses. Hence, an 8-week feeding trial was conducted to evaluate the optimum dietary processed sulfur (Immuno-F) level to replace antibiotic oxytetracycline (OTC) in juvenile olive flounder based on growth performance, hematology, and disease resistance. 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 F_{25}, F_{500}, and OTC_{4000} diets were significantly higher than those of fish fed F_{1000} and F_{2000} diets. In addition, superoxide dismutase, lysozyme and myeloperoxidase activities were not significantly different between sulfur and OTC supplemented groups. 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. In challenge test against *Edwardsiella tarda* at the 6^{th} day, cumulative survival rates of fish fed F_{50} diet were slightly higher than those of fish fed F_{250}-F_{2000} diets. Therefore, these results indicate that dietary processed sulfur at 50 ppm supplementation could replace antibiotic (OTC) in juvenile olive flounder.

Keywords: sulfur; oxytetracycline; growth; immune responses; olive flounder

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is a temperate marine fish species with high commercial value for aquaculture in eastern Asian countries including China, Korea and Japan. Total production of cultured flounder in the Republic of Korea has continuously increased from the late 1980s due to fast growing and established seedling production (Kim et al., 2002; FAO, 2020). However, to meet the increasing market needs, intensive flounder farming becomes inevitable. Thus, this has caused various problems including infectious diseases caused by viruses, bacteria and parasites and subsequently resulted in significant economic losses.

Antibiotics have been used as a traditional strategy to overcome disease outbreaks. Oxytetracycline (OTC) is one of the antibiotics commonly employed to animal production and aquaculture due to broad range of effects and low cost (Nazaret and Aminov, 2014). However, the possible adverse effects of continues use of antibiotics in aquaculture are (1) development of antibiotic-resistant microorganisms, (2) antibiotic residuals in the fish intestine, (3) contamination of natural habitats as well as decreasing efficiency of the antibiotics against the resistant pathogens (McPhearson et al., 1991; Serrano, 2005). Furthermore, with growing issues in concomitant pollutants, antibiotics and carcinogens in fish culture, customer's interest in the seafood safety and quality has increased (Jahncke, 2007; Hwang et al., 2013). Thus, administration of antibiotics in aquaculture has been strictly restricted or banned and this has encouraged researchers to investigate for alternatives to these substances. Over the last decade, many study groups in this field have focused on screening eco-friendly bio-active food components which could improve the growth, immune systems and disease resistance of commercial species in aquaculture (Francis et al., 2005; Sahu et al., 2007; Ahmad and Abdel-Tawwab, 2011).

Sulfur (S) is an essential element for the growth of most animals including humans and sulfur-containing compounds are generally found in the animal cells. Battin and Brumaghim (2009) reported that sulfur-containing compounds in mice and humans have antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, indicating their potential effects on immune responses. Several studies (Leustek et al., 2000; Stipanuk, 2004; Yang et al., 2015) have proved that sulfur biomolecules in plants and animals play important roles in improving their immunes system. Those sulfur-containing biomolecules are associated with free radical scavenging, DNA methylation and repair, regulation of gene expression, remodelling of extracellular matrix components, lipid metabolism, and detoxification. In addition, sulfur compounds in animal diets have many beneficial effects on immune function, anticancer,

antithrombotic and antioxidant effect (Ariga and Seki, 2006; Grimble, 2006). For instance, in blood plasma of pigs and cattle fed sulfur-containing diets there were increases in the ratio of polyunsaturated fatty acid to saturated fatty acid, as well as sulfur-containing antioxidants contents like methionine and cysteine (Lee et al., 2009; Richter et al., 2012; Shin et al., 2013; Song et al., 2013). Besides, sulfur-containing organic compounds in garlic and onion extracts have been widely used as medicines for diseases such as typhus, cholera, and dysentery before the emerging of modern antibiotics (Block, 1992), and inhibited the growth of pathogenic microorganisms (Elnima et al., 1983). However, sulfur is highly toxic, as it can cause side effects when supplemented in humans or animals diet (Bouchard and Conrad, 1973; Kim et al., 2006). Thus, it is necessary to process sulfur to remove the toxic property for use as a medicine and their optimum amount in diet should be determined to exert positive effects on host immunity. Generally, sulfur is processed through heat melting of mineral sulfur followed by separation of liquid and subsequent cooling (Lee et al., 2010). Processed sulfur is evaluated not only to supply nutrients but also to prevent arthritis and diabetes (Kim et al., 2015). In this way, processed sulfur has been recognized for its function and potential, however, more studies are required to fully understand its role in aquaculture.

Therefore, the present experiment was designed to evaluate the optimum dietary processed sulfur (Immuno-F) in juvenile olive flounder based on growth performance, hematology, and disease resistance, and to investigate the potential of this component as an antibiotic replacer.

2. Materials and methods

This experiment was carried out under the guidelines of Animal Ethics Committee Regulations, No. 554 issued by the Pukyong National University, Busan, Rep. Korea.

2.1. 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 (Samyang anipharm Co. LTD. Seoul, Korea) 4000 (OTC_{4000}). The concentration of OTC in the experimental diets was determined based on our previous studies (Park et al., 2011; Won et al., 2020) and manufacturer's instructions. In addition, a study on mullet (Hamed and Elias, 1971) indicated that the 4000 ppm supplementation showed the highest effect on controlling parasite,

Heterophyes sp. in fish culture. 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. Sulfur was processed (Immuno-F, EF-Biotech Company, Bucheon, Rep. of Korea) employing heat melting method as shown by Lee et al. (2010). 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.

The experimental diets were prepared and stored as previously described by Hardy and Barrows (2003). The dry ingredients were mixed thoroughly and fish oil with 30 % filtered tap water were added. The diets were pelleted using a laboratory pelleting machine using a 2-mm diameter die plate (Baokyong Commercial Co., Busan, Korea). Then, all experimental diets were kept at -20 °C in the refrigerator until use.

Preferred Table 1.

Preferred Table 2.

2.2. Experimental fish and feeding trial

The present experiment was conducted at the Institute of Fisheries Sciences, Pukyong National University, Busan, Republic of Korea. Olive flounder juveniles 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. After the accumulation period, 540 fish averaging 12.6 ± 0.17 g (mean ± SD) were weighed and randomly distributed into 24 indoor tanks (20 fish/tank) with a 35-L volume receiving a constant flow (0.8~1.0 L min^{-1}) of seawater. The eight dietary treatments were assigned to each tank with three replicates in a completely randomized design. Fish were fed twice daily (09:00 and 18:00 hours) for eight weeks at 2~3 % of wet BW/day. The water temperature and pH were maintained at 18 ± 1 °C and 7.5 ± 0.3, respectively, throughout the experimental period. Supplemental aeration was provided, using air pumps and air stones, to maintain the dissolved oxygen near saturation.

2.3. Sample collection

After the feeding trial, fish were starved for 24 hours, and the total number and weight of juvenile olive flounder in each tank were determined to calculate weight gain (WG),

specific growth rate (SGR), feed efficiency (FE), and protein efficiency ratio (PER). Three fish per tank were selected randomly for weight and length analysis to calculate condition factor (CF) and then dissected to obtain liver and viscera samples to determine hepatosomatic index (HSI) and viscerosomatic index (VSI), respectively (Yoo et al., 2007; Kim et al., 2014).

Three fish from each tank were used to perform 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 °C to constant weight to estimate the moisture content. Crude ash was determined by incineration at 550 °C for 3 h in a muffle furnace. Crude protein (N × 6.25) was determined using the Kjeldahl method following three main steps of strong acid digestion, distillation, and titration. Crude lipid was measured by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Hoganas, Sweden). Furthermore, total sulfur in fish whole-body was analysed by a sulfur analyser (SC-432DR, LECO Co., USA).

For biochemical and non-specific immune response analysis, three additional fish per tank were randomly removed from tanks, 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 samples were separated by centrifugation at 5000 × *g* for 10 min and the serum was stored at -70 °C in 1.5 eppendorf tubes for further analysis.

2.4. Biochemical analysis

Blood biochemical parameters such as plasma triglyceride (TG), total cholesterol (TCHO) and glutamic pyruvic transaminase (GPT) activities were measured using a chemical analyser (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

2.5. Non-specific immune response analysis

SOD activity of blood serum collected from the experimental fish was measured employing an SOD assay kit (K335-100, BioVision, USA) in accordance with the instructions provided by the manufacturer. Fish serum (20 µl) was mixed with Water Soluble Tetrazolium dye (WST-1) solution to inhibit superoxide anion produced by xanthine and the xanthine oxidase reaction.

The reaction was measured with a microplate reader (TECAN M200 Plate Reader, Tecan Trading AG, Switzerland) at 450 nm after 20 min reaction at 37 °C. Values were presented as percent inhibition as measured by the equation in the kit protocol.

Serum lysozyme activity was determined by the turbidometric assay previously described by Hultmark et al. (1980), with small modifications. The procedures we followed by making a 200-µl suspension including 0.75 mg ml^{-1} lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in PBS (pH 6.4, 0.1 M) that was added to a 96-well plate with 20 µl of serum. After incubation at room temperature for 0 and 30 min, the absorbance of mixtures was read at 570 nm by a microplate reader (TECAN M200 Plate Reader, Tecan Trading AG, Switzerland). One unit of lysozyme activity corresponded to a reduction in absorbance of 0.001 min^{-1}.

Serum myeloperoxidase activity (MPO) was measured as previously described by Quade and Roth (1997) with minor modifications. Olive flounder serum samples (20 µl) were mixed with 80 µl of Hanks' balanced salt solution (without Ca^{2+} or Mg^{2+}) in a 96-well plate, then, 35 µl of 20mM 3,3′,5,5′ tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich, USA) was added to the mixture. After this, 35 µl of 5mMH_2O_2 was added to each well of the plate. Samples were incubated for 2-min and 35 µl of 4MH_2SO_4 was added before reading the absorbance at 450 nm in the microplate reader.

2.6. Challenge test against Edwardsiella tarda

The common bacterial pathogen, *Edwardsiella tarda*, of olive flounder farms was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Four fish per tank were distributed according to their dietary treatment groups (each with three replicates) into 11 L aquarium for the challenge test with no water exchange. Challenge test was conducted as described previously by Park et al. (2016). Injection of fish was performed intraperitoneal with 0.1 mL of culture suspension of *E. tarda* containing 1×10^8 CFU m L^{-1}. Fish mortality was recorded daily for six days. To confirm virulence of *E. tarda*, swabs from skin, gill, liver and kidney of dead fish were checked on modified selective agar to determine the mortalities caused by *E. tarda*.

2.7. Calculations and statistical analysis

Fish growth performance was investigated using the following equations:

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

Specific growth rate (SGR, %/day) = (ln final wt. - ln 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

As for the challenge test against *E. tarda*, survival rate of fish during the six days was calculated by:

Survivability = ((initial number of fish – number of dead fish) /initial number of fish) × 100

To test the effects of the dietary treatments, all data were analysed by one-way ANOVA (Statistic 3.1; Analytical Software, St. Paul, MN, USA). When a significant treatment effect was observed between the dietary groups, a Scheffe test was used to compare the means. Treatment effects were considered at $P<0.01$ level of significance. Results of cumulative survival rate after challenge test were analysed by GraphPad Prism 8.4.0 (GraphPad Software Inc., San Diego, CA, USA) and presented by a Kaplan-Meier plot. Log-rank (Mantel-Cox), Logrank test for trend and Gehan-Breslow-Wilcoxon tests were performed to compare survival curves.

3. Results

3.1. Growth performance

Table 3 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 ($P<0.01$). However, there were no significant differences among fish fed F_{50}, F_{250} and OTC_{4000} diets ($P>0.01$), and among fish fed Cont, F_{25}, F_{500}, F_{1000} and F_{2000} diets ($P>0.01$). 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 ($P<0.01$). However, there were no significant differences among fish fed Cont, F_{25}, F_{50}, F_{250} and OTC_{4000} diets ($P>0.01$). There were no significant differences in hematosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF) among the treatments ($P>0.01$).

Preferred Table 3.

3.2. 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>0.01). Also, there were no significant differences in total sulfur among fish fed all the experimental diets (P>0.01).

Preferred Table 4.

3.3. Hematological parameters

Figures 1-3 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<0.01). 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>0.01), and among fish fed Cont, F_{25}, F_{50}, F_{500}, F_{2000} and OTC_{4000} diets (P>0.01). 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>0.01). 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<0.01). Serum TCHO levels of fish fed OTC_{4000} diet were significantly lower than those of fish fed F_{1000} diet (P<0.01). 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>0.01).

Preferred Fig. 1.

Preferred Fig. 2.

Preferred Fig. 3.

3.4. Non-specific immune responses

Non-specific immune responses are shown in Table 5. There were no significant differences in superoxide dismutase (SOD), lysozyme, and myeloperoxidase (MPO) activities of fish fed the experimental diets (P>0.01).

Preferred Table 5.

3.5. Challenge test against Edwardsiella tarda

Cumulative survival rate in juvenile olive flounder challenged *E. tarda* for 6 days is shown in Figure 4. The first mortality occurred on day 2 during the period of challenge test. After the six-day challenge test, cumulative survival rate of fish fed the F_{50} diet was slightly higher than those of fish fed the F_{250}-F_{2000} diets, but there were no significant differences among all dietary groups (P>0.01).

Preferred Fig. 4.

4. Discussion

With growing interest of culturing healthy and disease-resistant fish in an antibiotic-free environment many countries have banned to import the fishery products treated with antibiotics and/or chemicals (Lee et al., 2016). Hence, a number of studies have been carried out to develop feed additives as alternatives for antibiotics in different fish speciese such as rainbow trout (Park et al., 2017) and starry flounder (Park et al., 2016). In the present study, we investigate effects of dietary processed sulfur to study whether they can replace oxytetracycline.

Dietary sulfur could have beneficial effects in growth of juvenile olive flounder

Our results indicated that dietary processed sulfur at the inclusion level of 50-250 ppm (F_{50} and F_{250}) could be equally effective compared to oxytetracycline antibiotic at 4,000 ppm by significantly promoting the growth performance in olive flounder. This result is in agreement with the findings from a previous study (Cho, 2011), in which organic sulfur was shown to be an effective dietary additive for improving the growth performance of juvenile olive flounder. Likewise, in broiler chickens, dietary processed sulfur improved the growth while replacing dietary antibiotic (Park et al., 2010). This could be attributed to the fact that sulfur is a key component of amino acids such as methionine and cysteine, vitamins such as thiamine and biotin, and insulin like hormones in fish (Tacon, 1987). In our previous study of rock bream, dietary sulfur amino acids including methionine and cysteine showed higher growth and feed utilization as well as taurine sparing effects compared to control diet, suggesting their potential roles in fish growth (Ferreira et al., 2015). To prove that, future studies should be carried out to evaluate changes in the composition of amino acids and vitamins in response to sulfur-containing diets. Our results

also indicated that the processed sulfur at 500-2000 ppm of dietary inclusion (F_{500}, F_{1000} and F_{2000}) resulted in depressed growth performance as compared with those of olive flounder fed the 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 have negative effects on growth performance of olive flounder (Kim et al., 2013). Therefore, care should be taken to maintain the optimum supplementation level. Meanwhile, we also found that there were no adverse effects of processed sulfur supplementations on organosomatic indices of fish in terms of HSI, VSI and CF.

Higher sulfur-containing diet negatively influences blood composition of olive flounder

The evaluation of hematological parameters might be useful for the diagnosis of fish pathology and physiological status (Stoskopf, 1993) and it is known that amino acid containing diets alter blood composition in human (Schmidt et al., 2016; Teymoori et al., 2017). In the present study, serum glutamic pyruvic transaminase (GPT) levels ranged from 4 to 8, which were within acceptable ranges in olive flounder (Kim et al., 2013). Blood enzymes such as GPT are known to be sensitive indicators for tissue damage (Torre et al., 2000). Hence, we could suggest that the experimental fish were free of liver damage. It has been reported that increasing blood lipid levels could indicate declining health condition of the cultured fish (Kikuchi et al., 2009). The high blood lipid 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, which could lead to hyperlipidemia (Kritchevsky, 1995; Ye et al., 2011).

Sulfur and antibiotic-containing diets could have similar effects on non-specific immune responses of olive flounder

Non-specific immune parameters that were employed in the present study have been recognized as the indicators of animal health, as they play critical roles in regulating the balance of releasing and clearing reactive oxygen species in immune cells (Holmblad and Söderhäll,

1999; Campa-Córdova et al., 2002; Xu et al., 2010). It is well documented that dietary sulfur enhances non-specific immune response and antioxidant activities in humans (Grimble, 2006; Bin et al., 2017). However, in the current experiment, in superoxide dismutase (SOD), lysozyme (LYZ) and myeloperoxidase (MPO) activities there were no significant differences between sulfur and OTC groups. These findings suggest that sulfur-containing diets could have a similar role as antibiotics in terms of immune responses. It should be also noted that the highest values in SOD and LYZ activities was found in F_{50} and F_{100} groups although the treads of the results are not consistent with other sulfur groups. Thus, further studies are needed to clarify immunological effects of dietary sulfur.

Optimum dietary sulfur level could improve disease resistance of olive flounder

Pathogenic bacteria *Edwardsiella tarda* is known as one of the major disease pathogens challenging several aquaculture farms (Katya et al., 2017). Serious economic losses have been reported for 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 has been reported that dietary sulfur could have an antibiotic effect against anaerobic bacteria by modulating intestinal H_2S levels (Viscomi et al., 2010), which result in increase in the oxygenation in colonocytes by the inhibition of β-oxidation (Babidge et al., 1998). In addition, 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).

5. Conclusions

Since the FDA banned the use of antibiotics in animal feed in 2017, many researchers have been looking for feed additives that can replace antibiotics and boost the host immune system without adverse effects, for instance, probiotic or prebiotic diets (Park et al., 2020). Our present study demonstrated that 50 ppm of dietary processed sulfur (Immuno-F) could replace the antibiotic (OTC), characterized by the growth performance, hematological parameters and disease resistance against the bacteria *E. tarda* in juvenile olive flounder. It should be noted that recent studies have shown that dietary sulfur may interact with intestinal microbiota by

increasing the abundance of sulphate reducing bacteria, and subsequently affecting mucosal immune system (Teigen et al., 2019). Thus, further studies on the interaction among dietary processed sulfur, host microbiota and intestinal immune system should be taken into consideration.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgments

This research was supported by the National Research Foundation of Korea (Grant No. NRF-2015R1D1A3A01017663) and the Feeds & Foods Nutrition Research Center (FFNRC), Pukyong National University (PKNU), Busan, Rep. of Korea.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Fig. 1. Glutamic pyruvic transaminase activity (GPT) in juvenile olive flounder fed the experimental diets for 8 weeks. Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

Fig. 2. Triglyceride (TG) of juvenile olive flounder fed the experimental diets for 8 weeks. Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

Fig. 3. Total cholesterol (TCHO) of juvenile olive flounder fed the experimental diets for 8 weeks. Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

Fig. 4. Cumulative survival rate of juvenile olive flounder fed the experimental diets for 8 weeks and then experimental challenged with *E. tarda* for 6 days. Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

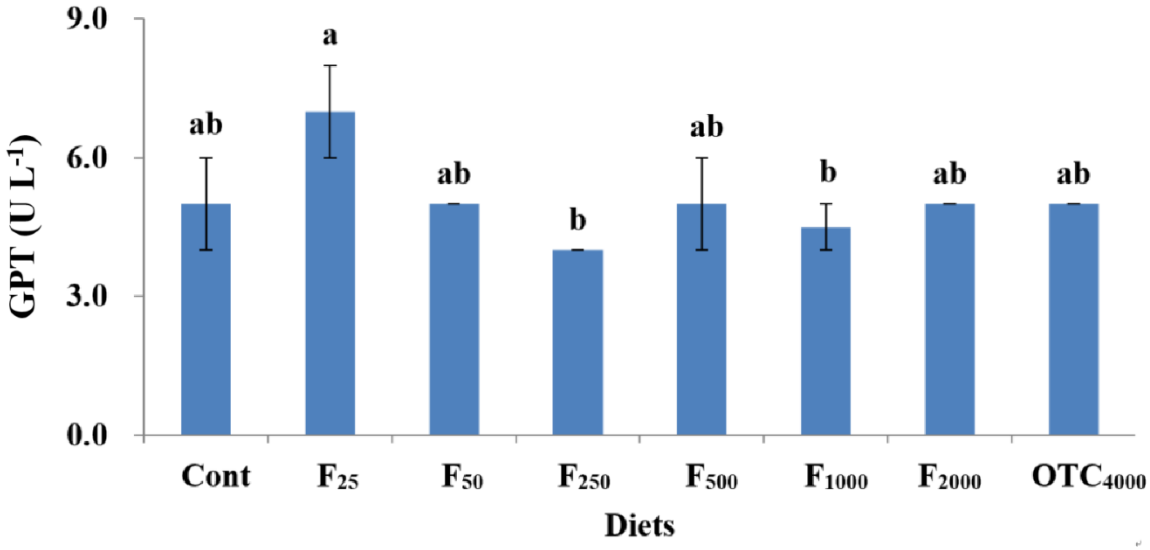
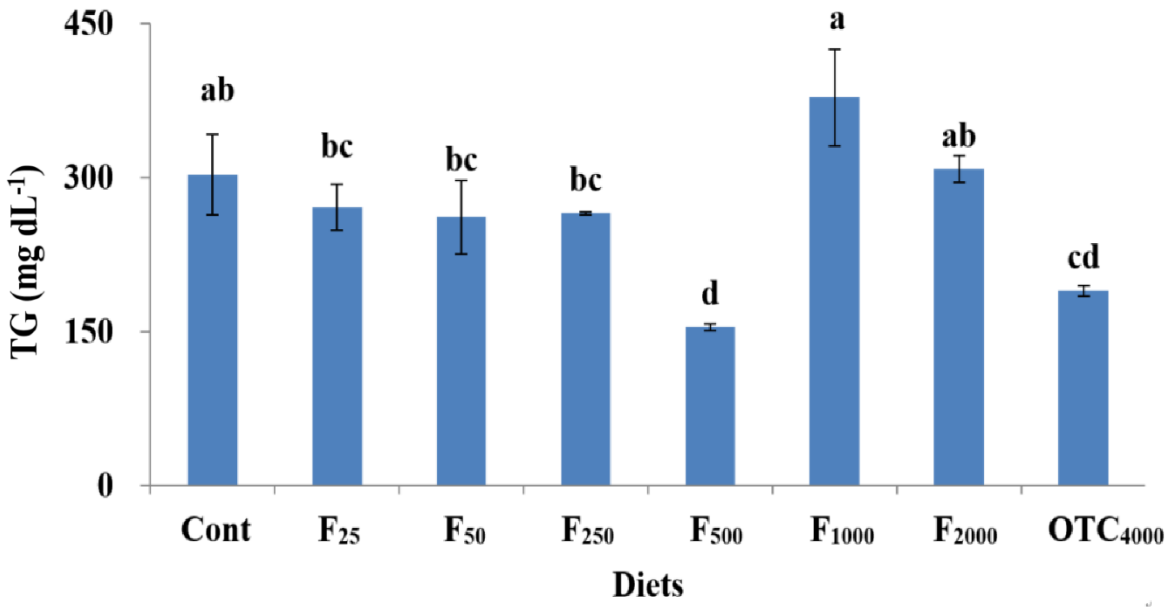


Fig. 1.


450
300
150
0
TG (mg dL-1)
ab
bc
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Cont
F25
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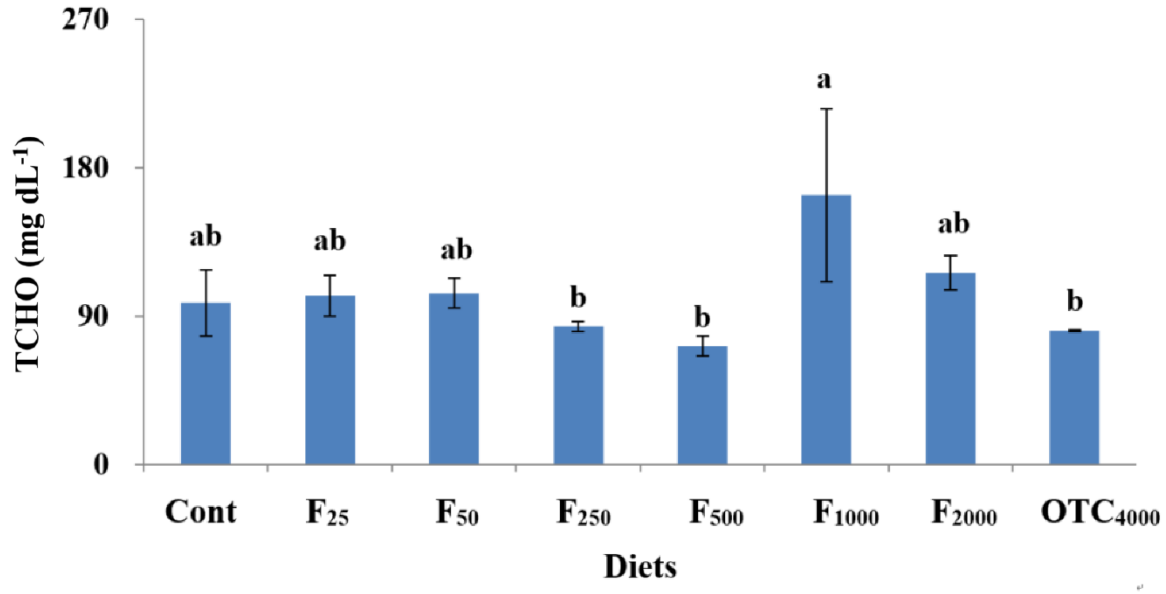
F250
F500
F1000
F2000
OTC4000
Diets

Fig. 2.


270
180
90
0
TCHO (mg dL-1)
ab
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Cont
F25
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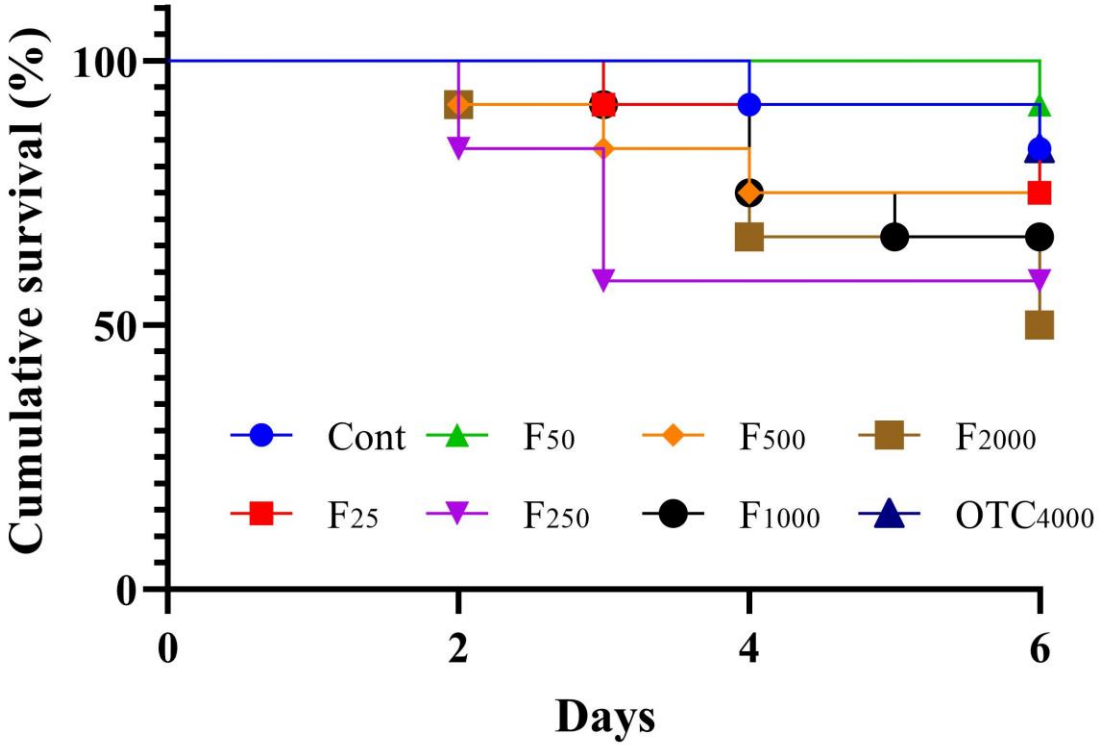
F250
F500
F1000
F2000
OTC4000
Diets

Fig. 3.


Cumulative survival (%)
100
50
0
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2
4
6
Days
Cont
F50
F500
F2000
F25
F250
F1000
OTC4000

Fig. 4.

Table 1

Composition and proximate analysis of the basal diet (g/kg, dry matter basis)

Ingredients	g/kg
Defatted fish meal [1]	250
FMP[2]	250
Wheat flour	150
Soybean meal	30.0
Corn starch	128
Wheat gluten meal	70.8
Fish oil	95.0
Vitamin mix[3]	10.0
Mineral mix[4]	10.0
Processed sulfur (Immuno-F)	0.0
OTC[5]	0.0
Cellulose[6]	6.2
Proximate analysis	
Moisture	114
Crude protein	467
Crude lipid	106
Crude ash	63

[1] Defatted Fish meal (Chile)

[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 (g/kg, dry matter basis)

Ingredients	g/kg
Moisture	145
Crude protein	115
Crude lipid	427
Crude ash	47.5

Table 3

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

	Diets[2]								SEM	*P* value
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}		
WG (%)[3]	80.4^{bc}	75.5^{bc}	87.1^{ab}	87.3^{ab}	64.8^{c}	70.2^{c}	65.2^{c}	101^{a}	2.54	<0.001
SGR (% day^{-1})[4]	1.40^{bc}	1.34^{bc}	1.49^{ab}	1.49^{ab}	1.19^{c}	1.27^{c}	1.19^{c}	1.66^{a}	0.03	<0.001
FE (%)[5]	79.2^{abc}	78.4^{abcd}	84.2^{ab}	82.7^{ab}	63.9^{cd}	69.0^{bcd}	62.4^{d}	92.8^{a}	2.16	<0.001
PER[6]	1.70^{abc}	1.70^{abc}	1.81^{ab}	1.80^{ab}	1.37^{cd}	1.50^{bcd}	1.34^{d}	2.01^{a}	0.04	<0.001
HSI (%)[7]	1.32	0.91	1.04	1.26	1.08	1.12	0.90	0.99	0.04	0.078
VSI (%)[8]	4.19	3.83	3.73	3.66	4.11	4.14	4.11	4.14	0.05	0.019
CF[9]	0.77	0.83	0.77	0.76	0.72	0.80	0.80	0.74	0.01	0.032

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.01).

[2] Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

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

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

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

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

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

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

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

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								SEM	*P* value
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}		
Moisture (%)	77.1	77.5	77.4	77.7	77.8	78.0	77.8	77.5	0.10	0.462
Crude Protein (%)	17.6	17.3	17.2	17.0	17.2	17.3	17.1	17.2	0.09	0.889
Crude Lipid (%)	1.53	1.26	1.47	1.45	0.86	1.06	1.04	1.41	0.08	0.243
Crude Ash (%)	4.11	4.22	4.22	4.09	4.55	4.11	4.11	4.03	0.05	0.227
Total Sulfur (%)	0.85	0.85	0.76	0.87	0.84	0.84	0.80	0.82	0.01	0.850

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.01$).

[2] Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

Table 5

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

	Diets								SEM	*P* value
	Cont	F_{25}	F_{50}	F_{250}	F_{500}	F_{1000}	F_{2000}	OTC_{4000}		
SOD[3] (% superoxide inhibition)	46.6	38.0	49.2	42.5	42.9	51.0	43.6	39.8	0.10	0.019
LYZ[4] (Units ml^{-1})	1.18	1.88	2.07	1.10	1.98	2.07	1.73	1.28	0.09	0.022
MPO[5] (absorbance at 450 nm)	1.25	1.36	1.23	1.25	1.39	1.33	1.28	1.08	0.08	0.604

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.01$).

[2] Diets were Cont (control diet), F_{25}, F_{50}, F_{250}, F_{500}, F_{1000}, and F_{2000} supplemented by processed sulfur (Immuno-F) at 0, 25, 50, 250, 500, 1000 and 2000 ppm, respectively, and OTC_{4000} supplemented by oxytetracycline at 4000 ppm in the Cont diet.

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

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

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

Conflict of Interest

The authors declare no competing financial interest.

Youngjin Park and **Minhye Park**: Data curation, Writing- Original draft preparation. **Ali Hamidoghli**: Visualization, Investigation, Writing - Review & Editing. **Chang-Hoon Kim**: Methodology, Writing - Review & Editing. **Sungchul C. Bai**: Supervision, Conceptualization, Methodology, Writing - Review & Editing.