Thesis for Degree of Master of Fisheries Science

Evaluation of dietary fermented tuna by-product meal in juvenile Olive flounder, *Paralichthys olivaceus*

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성장기 넙치 사료 내 발효참치부산물의 평가

Advisors: Prof. Sungchul C. Bai

by

Oncul Fatma Ozgun

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A dissertation

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성장기 넙치 사료 내 발효참치부산물의 평가

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요약문

치어기 넙치(*Paralichthys olivaceus*) 사료 내 발효참치부산물(FTBM)의 어분 대체효과를 평가하고자 8 주간 사육실험을 진행하였다. 실험어는 평균 11.06 ± 0.11 g (mean ± SD)의 치어기 넙치를 20 마리씩 3 반복으로 무작위 배치하였으며, 실험사료는 참치부산물의 어분대체율에 따라 각각 0%, 12.5%, 25%, 37.5% or 50% 의 총 5 가지 ($FTBM_0$, $FTBM_{12.5}$, $FTBM_{25}$, $FTBM_{37.5}$, and $FTBM_{50}$)로 제작하였다. 모든 실험사료의 조단백질과 총에너지가는 동일하게 제작하였다. 사육실험 종료 후 증체율 (WG), 일간성장률 (SGR), 사료효율 (FE)에서 $FTBM_0$ 실험구와 $FTBM_{12.5}$ 실험구가 다른 실험구에 비해 유의적으로 높은 결과값을 나타내었다($P < 0.05$). 또한 *Edwarsiella tarda* 를 이용한 공격실험 결과 누적생존율에 있어서

$FTBM_0$ 실험구와 $FTBM_{12.5}$ 실험구가 다른 실험구에 비해 유의적으로 높은 결과값을 나타내었다. 하지만 전어체분석, glutamic pyruvic transaminase (GPT), total protein (TP), 비특이적 면역반응인 superoxide dismutase (SOD), lysozyme 활성 분석 결과 전 실험구에서 유의적인 차이를 보이지 않았다. 본 실험의 결과에 따르면 발효참치부산물은 치어기 넙치의 사료 내 어분의 12.5%까지 대체 할 수 있는 것으로 나타났다.

Evaluation of dietary fermented tuna by-product meal in juvenile olive flounder, *Paralichthys olivaceus*

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Abstract

An 8-week feeding trial was conducted to evaluate the effects of dietary fermented tuna by-product meal (FTBM) as a fishmeal (FM) replacer in juvenile olive flounder, *Paralichthys olivaceus*. Triplicate groups of 20 fish averaging 11.06 ± 0.11 g (mean ± SD) were fed one of the five experimental diets replacing FM with FTBM at 0 %, 12.5 %, 25 %, 37.5 % or 50 % (denoted as $FTBM_0$, $FTBM_{12.5}$, $FTBM_{25}$, $FTBM_{37.5}$, and $FTBM_{50}$, respectively). All experimental diets were prepared to be isonitrogenous and isocaloric. After the feeding trial, weight gain, specific growth rate and feed efficiency from fish fed $FTBM_0$ and $FTBM_{12.5}$ diets were significantly higher ($P < 0.05$) than those from fish fed the other diets. Also, fish fed $FTBM_0$ and $FTBM_{12.5}$ showed significantly higher ($P < 0.05$) resistance to *Edwarsiella tarda* infection compared to the other treatments. However, whole body proximate composition, glutamic pyruvic transaminase, total protein and non-specific immune parameters (superoxide dismutase and lysozyme activities) showed no significant differences among fish fed all the

experimental diets ($P > 0.05$). These results indicated that dietary fermented tuna by-product meal could replace up to 12.5 % of fishmeal in juvenile olive flounder, *Paralichthys olivaceus*.

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I. Introduction

Olive flounder, *Paralichthys olivaceus* is a carnivorous fish and is one of the most important fishes in East Asian countries such as Korea, Japan and China. Accordingly, many feeding trials have been performed to investigate the nutritional requirements of this species (Estevez et al. 1997; Lee et al. 2000a; Lee et al. 2000b; Alam et al. 2002; Kim et al. 2002; Lee et al. 2002). Commercial of Olive flounder aquaculture started from the late 1980s in Korea (Kim et al., 2002). In 2016, the production of Olive flounder in Korea is approximately 41,620 metric tons aquaculture production was nearly 55.000 tons in 2009 (KOSTAT, 2016).

Fishmeal (FM) has been regarded as the most important protein source in fish diets because of its palatability and high protein quality (Lee et al., 2001). The compound feed production for olive flounder was estimated at 27,865 tonnes while FM use in commercial diets is nearly 50-70 % (Tacon et al., 2008). Considering that FM is expensive (Jeon et al., 2014) and has limited supply, the continuous search for alternative sources such as animal and plant-derived products to replace conventional protein sources is highly needed (Naylor et al., 2009; Tacon et al., 2008).

In recent years, several studies have been conducted on fishmeal replacement by animal and plant protein sources such as poultry by-product meal (Markey et al., 2010), cottonseed and soybean meal (Park et al., 2005), rice distillers dried grain (Bae et al., 2015), corn gluten meal (Regost, 1999), canola meal (Luo et al., 2012) and meat and bone meal (Tan et al., 2005). Studies have also shown considerable success in incorporating by-products from fisheries processing such as shrimp waste, scallop by-product, squid liver meal (Cho et al., 2005), and tuna by-product

meals in practical feeds for various fish species (Hardy et al. 2005; Whiteman et al., 2005; Uyan et al., 2006; Tekinay et al., 2009). Therefore, animal protein sources such as fisheries by-products have great potential as substitute to fishmeal (Hardy et al., 2005).

Tuna processing industry in Korea generates a substantial amount of wastes and by-product at approximately 100 tonnes daily (Kim et al., 2014). These low-value tuna by-products can therefore be utilized as a potential fishmeal substitute because of its high nutrient content (55 % crude protein and >10 % crude lipid) (Kim et al., 2014). In addition to, use of tuna by-product meal do not pose danger of disease transfer or infection (Greger, 2004). Inclusion of tuna by-product meal at 20-30 % levels in olive flounder, *Paralichthys olivaceus* (Kim et al., 2014) and spotted rose napper, *Lutjanus guttatus* (Hernández et al., 2014) diets resulted in enhanced growth and feed utilization. High-level (30-40 %) inclusion of tuna by-product meal in juvenile Korean rockfish, *Sebastes schlegeli* diets showed positive effect on growth and feed utilization (Jeon et al., 2014). Furthermore, the nutritional quality of the fisheries by-products can be further improved by combining with plant-based protein during fermentation process (Fagbenro et al., 1995; Sun et al., 2007; Kader et al., 2011; Kim *et al.* 2014).

This study aims to evaluate the dietary inclusion of the fermented tuna by-product meal (FTBM) on growth, feed utilization, blood parameters, innate immune responses, and disease resistance of juvenile olive flounder, *Paralichthys olivaceus*.

II. Materials and Methods

Experimental diets

Garlic husks (1 kg in dry weight) were processed by adding 2 L of water and 5 % of corn steep liquor (CSL). The processed mixture was then inoculated with one percent each of cellulolytic and proteolytic bacteria, *Bacillus licheniformis* and *B. subtilis*, respectively. The mixture was incubated at 30°C for 5 days to produce a fermented garlic husk. Tuna by-product were crushed, dried and mixed with fermented garlic husk at 4:1 dry weight ratio. The mixture was incubated at 65 to 70 °C for 5 days to obtain a fermented tuna by-product meal (FTBM) with moisture content of 10 % or less.

Five isonitrogenous and isocaloric diets were formulated and prepared to the five diets replaced fishmeal (FM) with the fermented tuna by-product meal (FTBM) at 0 %, 12.5 %, 25 %, 37.5 %, and 50 % (denoted as $FTBM_0$, $FTBM_{12.5}$, $FTBM_{25}$, $FTBM_{37.5}$ and $FTBM_{50}$, respectively). Fishmeal, FTBM and wheat gluten meal were used as protein sources, fish oil as lipid source, and wheat flour and cornstarch as carbohydrates sources. Tables 1 and 2 shows the proximate and amino acid composition of fishmeal (FM) and fermented tuna by-product meal (FTBM).

Diet preparation and storage were performed according to Bai and Kim (1997). Briefly, dry ingredients were mixed with an electric mixer, and fish oil and tap water were added to make it dough which was then passed through a screw-type pelleting machine (Baokyong Commercial Co., Busan, Korea) for pelleting. Pellets were air-dried for about 48 h at room temperature, sieved to achieve the desired particle size, sealed and stored at -20 °C until use.

Table 1. Proximate composition of fishmeal (FM) and the fermented tuna by-product (FTBM)[1]

Ingredients	FTBM (%)	FM (%)
Moisture (%)	14.06 ± 0.08	3.17 ± 0.02
Crude protein (%)	57.07 ± 0.08	67.72 ± 0.05
Crude lipid (%)	8.06 ± 0.22	8.92 ± 0.12
Ash (%)	12.24 ± 0.55	17.10 ± 0.19

[1] Values are mean (± SD) of duplicate samples.

Table 2. Amino acid composition of fishmeal (FM) and the fermented tuna by-product meal (FTBM)[1]

Amino acids	FTBM (%)	FM (%)
Threonine	2.62 ± 0.05	3.09 ± 0.08
Valine	3.33 ± 0.12	3.78 ± 0.16
Isoleucine	2.85 ± 0.06	3.33 ± 0.21
Leucine	4.59 ± 0.22	5.30 ± 0.01
Tryptophan	0.63 ± 0.02	0.70 ± 0.09
Phenylalanine	2.56 ± 0.02	2.87 ± 0.02
Histidine	2.52 ± 0.06	2.76 ± 0.11
Arginine	3.72 ± 0.15	4.08 ± 0.13
Lysine	4.85 ± 0.08	5.77 ± 0.31
Methionine	1.42 ± 0.11	1.92 ± 0.06
Cystine	1.03 ± 0.02	1.46 ± 0.08

[1] Values are mean (± SD) of duplicate samples.

Table 3. Ingredients and proximate composition (% dry matter) of the experimental diets

Ingredients	Diets[1]				
	FTBM $_0$	FTBM $_{12.5}$	FTBM $_{25}$	FTBM $_{37.5}$	FTBM $_{50}$
Fishmeal (Chile)[2]	63.80	55.83	47.85	39.88	31.90
FTBM[3]		9.46	18.93	28.39	37.85
Wheat gluten meal[4]	6.5	6.5	6.5	6.5	6.5
Corn starch	8.70	6.83	5.19	3.31	1.68
Wheat flour	12	12	12	12	12
Fish oil	5.0	5.0	4.9	4.9	4.8
Vitamin mix[5]	2.0	2.0	2.0	2.0	2.0
Mineral mix[6]	2.0	2.0	2.0	2.0	2.0
Lysine		0.002	0.005	0.007	0.01
Methionine		0.023	0.045	0.068	0.09
Cellulose		0.36	0.58	0.95	1.17
Proximate analysis					
Moisture	13.17	12.40	12.51	12.85	12.74
Crude protein	56.64	55.51	55.58	55.46	55.06
Crude lipid	11.07	10.35	10.78	11.60	11.94
Ash	13.51	12.75	12.75	12.99	13.14
Nitrogen-Free Extract[7]	5.61	8.99	8.37	7.10	7.12
Gross energy (kJ g^{-1})[8]	14.57	14.67	14.75	14.82	14.89

[1]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

[2]The Feed Co Ltd., Seoul, Rep. of Korea; (crude protein: 67.7 %, crude lipid: 8.90 %, ash: 17.1 %) .

[3]Fermented tuna by-product meal (FTBM) Tuna by-products + fermented garlic husk (4:1) (crude protein: 57.1 %, crude lipid: 8.10 %, ash: 12.2 %)

[4]The Feed Co Ltd., Seoul, Rep. of Korea; (crude protein: 80.9 %, crude lipid: 1.70 %, ash: 0.80 %) .

[5]Contains (as mg/kg 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

[6]Contains (as mg/kg 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.

[7]Nitrogen-free extract, estimated by difference [NFE = 100 − (crude protein + crude lipid + ash)]

[8]Gross energy was calculated using standard physiological values of 16.7, 16.7 and 37.7 kJ g-1 for protein, carbohydrate and lipid, respectively (Garling and Wilson, 1976)

Experimental fish and feeding trial

Juvenile olive flounder were obtained from a private hatchery (Woojung Aquafarm, Pohang, Republic of Korea) and acclimated for 2 weeks during which they were fed with formulated control diet prior to the start of the feeding trial. Triplicate groups of 20 fish with an average weight of 11.06 ± 0.11 g (mean ± SD) were randomly distributed in 15, 50-L capacity aquaria connected to a semi-recirculation system with tanks receiving filtered seawater at a flow rate of 0.8~1.0 L/min and fish were fed twice (09:00 and 18:00 hours) daily for 8 weeks at 2~3 % of estimated biomass. Water quality parameters were monitored throughout the experiment. Water temperature remained between 22.5 °C and 23.1 °C, supplemental aeration was provided to maintain the dissolved oxygen near saturation, and pH was 7.5 ± 0.3.

Sample collection and analysis

At the end of the experiment, juvenile olive flounder were unfed for 24 hours prior to sampling for individual body weight and total number of surviving fish per tank. Parameters are percent weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), hepatosomatic index (HSI), visceralsomatic index (VSI) and condition factor (CF) and survival were determined and also calculated following these formula (Yoo et al. 2007; Kim et al. 2014):

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

Survival rate (%) = (total fish - dead fish) × 100 / total fish

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

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

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

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

Three fish from each tank were used for analyze whole body proximate composition. Proximate composition analyses of the experimental diets and whole body were performed using the standard methods of AOAC (1995). Diet and fish samples were dried at 105 °C to constant weight to estimate their moisture content. Crude ash was determined by incineration at 550 °C 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). Gross energy of experimental diets was calculated from the physiological values of 16.7, 16.7, and 37.7 kJ g^{-1} respectively, for protein, carbohydrate, and lipid (Garling and Wilson, 1976). The amino acid composition of the experimental diets was analyzed with an automatic amino acid analyzer (SykamS4330, Erasing, Germany).

From three fish randomly selected from each tank, blood samples were obtained from the caudal vein using 1 mL disposable syringe without anticoagulant. The blood samples were kept until coagulation in room temperature for 30 minutes and the serum was separated by centrifugation (5000 × g) for 10 min. Then, the serum was stored at −70 °C for later analysis of plasma total protein (TP), glucose levels (GLU), glutamic oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) activities with a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

Superoxide dismutase (SOD) activity was measured by the superoxide radical based on reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) in accordance with the procedure of products. Each endpoint assay was observed by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 effect with superoxide) and after 20 minutes of reaction time at 37 °C. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg.

Lysozyme activity was analyzed in 0.1 mL test serum which was added to 2 mL suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in a 0.05 M sodium phosphate buffer (pH 6.2). The reactions were conducted at 20 °C and absorbance at 530 nm was measured between 0.5 min and 4.5 min on a spectrophotometer (Infinite® m200 PRO, Tecan Trading AG, Switzerland).

Challenge test

A bacterial pathogen, *Edwardsiella tarda*, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Fish (n = 5 per tank) were distributed according to their dietary treatment groups into 50 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/mL. Fish mortality was recorded daily for 9 days.

Statistical analysis

All data were analyzed by one-way ANOVA using SAS version 9.1 (SAS Institute, Cary, NC, USA). Duncan's multiple range test (Duncan, 1955) was used to compare significant differences among the treatments diets at $P < 0.05$ significance.

III. Results

Growth performance

Table 4 shows growth performance and survival of juvenile olive flounder fed the experimental diets for 8 weeks. Weight gain (WG), specific growth rate (SGR) and feed efficiency (FE) of fish fed $FTBM_0$ and $FTBM_{12.5}$ were significantly higher than those of fish fed $FTBM_{25}$, $FTBM_{37.5}$, $FTBM_{50}$ diets ($P < 0.05$). No significant difference ($P > 0.05$) was observed in WG, SGR and FE of fish fed $FTBM_0$ and $FTBM_{12.5}$ diets. Protein efficiency ratio (PER) in fish fed $FTBM_0$ and $FTBM_{12.5}$ diets were significantly higher than those fed $FTBM_{37.5}$ and $FTBM_{50}$ ($P < 0.05$), but did not significantly differ from that of $FTBM_{25}$ and $FTBM_{37.5}$ diets. Also, no significant difference in PER between fish fed $FTBM_{37.5}$ and $FTBM_{50}$ diets ($P > 0.05$). Survival rates ranged from 94.12% to 100%, and did not show significant differences among treatments ($P > 0.05$). Although, the highest survival rate was found in fish fed $FTBM_{12.5}$ and $FTBM_{25}$ while the lowest was seen in those fed $FTBM_0$.

Hepatosomatic index (HSI), visceral somatic index (VSI), and condition factor (CF) in juvenile olive flounder fed experimental diets are presented in Table 5. No significant differences were found in HSI among treatment groups ($P > 0.05$). VSI in fish fed $FTBM_{37.5}$ and $FTBM_{50}$ diets were significantly higher than those fed $FTBM_{12.5}$ ($P < 0.05$). CF values of olive flounder did not indicate significant differences among all dietary treatments ($P > 0.05$).

Whole body proximate composition

Whole body proximate composition is provided in Table 6. No significant difference ($P > 0.05$) was observed in body protein, lipid, moisture and ash contents among fish fed all the experimental diets after 8 weeks of feeding.

Hematological parameters

Table 8 shows the results of hematological parameters such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total protein (TP) and glucose (GLU). GOT levels were significantly higher in $FTBM_0$, $FTBM_{12.5}$ and $FTBM_{50}$ than those fed $FTBM_{25}$ diet ($P < 0.05$). However, no significant difference ($P > 0.05$) was observed in GOT levels of fish fed $FTBM_{25}$ and $FTBM_{37.5}$ diets. Also, GOT level was significantly higher in $FTBM_0$ than those fed $FTBM_{37.5}$ diet ($P < 0.05$). GPT and total protein did not indicate significant differences ($P > 0.05$) among the dietary treatments. Glucose levels were significantly elevated in fish fed $FTBM_0$. Fish fed $FTBM_{50}$ showed significantly lower glucose values compared to other dietary treatments ($P < 0.05$).

Non-specific immune responses

The results of SOD and lysozyme activities are shown Table 9. No significant difference in SOD activity was observed among all dietary treatments ($P > 0.05$). Also, lysozyme activity did not show significant differences in all dietary treatments ($P > 0.05$).

Challenge test with *E. tarda*

Dramatic change in cumulative survival rate of juvenile olive flounder infected with *E. tarda* occurred on days 7 to 9 post-challenge (Fig. 4). During the challenge test, the first mortalities

occurred on the fifth day and it was pronounced after the sixth day post-injection. At day 9, fish fed $FTBM_0$ and $FTBM_{12.5}$ showed significantly higher ($P < 0.05$) resistance to *E. tarda* compared to fish fed $FTBM_{50}$, but it did not differ from those fed $FTBM_{25}$ and $FTBM_{37.5}$ diets.

Table 4. Growth performance and survival of juvenile olive flounder *Paralichthys olivaceus* fed the experimental diets for 8 weeks[1]

	Diets[2]				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
WG (%)[3]	152 ± 1.54^a	150 ± 2.53^a	135 ± 2.59^b	128 ± 5.33^c	121 ± 4.61^c
SGR (%/day)[4]	2.20 ± 0.01^a	2.18 ± 0.02^a	2.04 ± 0.03^b	1.96 ± 0.06^c	1.89 ± 0.05^d
FE (%)[5]	98.8 ± 0.01^a	96.3 ± 0.46^a	88.8 ± 2.61^b	86.2 ± 3.45^b	78.9 ± 1.14^c
PER[6]	1.89 ± 0.15^a	1.90 ± 0.05^a	1.79 ± 0.05^{ab}	1.72 ± 0.07^{bc}	1.58 ± 0.02^c
Survival (%)[7]	94.1 ± 10.19	100 ± 0.00	100 ± 0.00	96.6 ± 2.97	98.2 ± 3.04

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

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

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

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

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

[7]Survival rate = (total fish - dead fish) × 100 / total fish

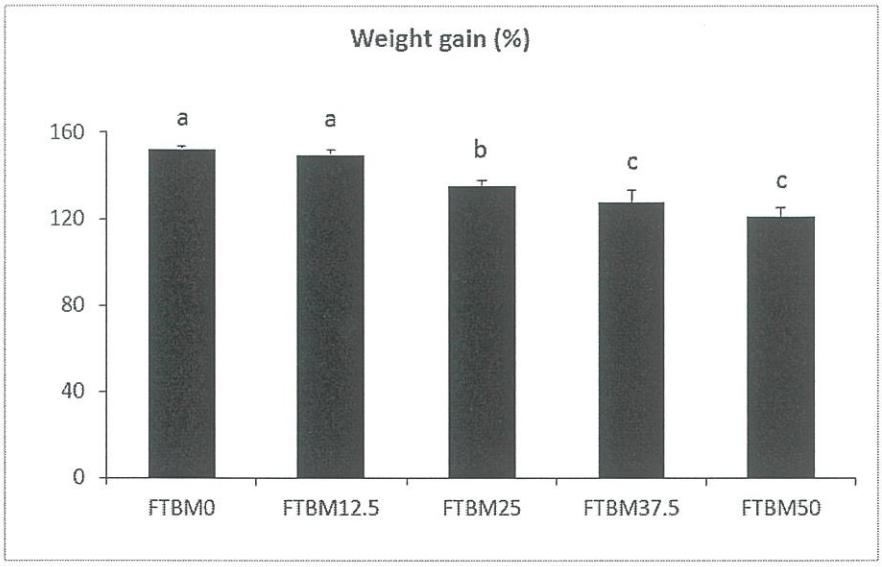

Fig.1-a. Weight gain of juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

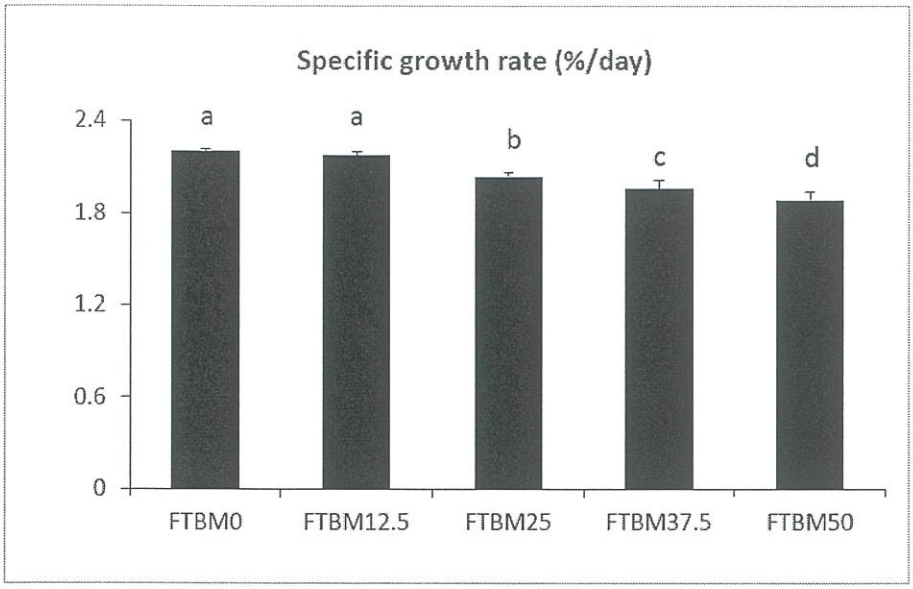

Fig.1-b. Specific growth rate of juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

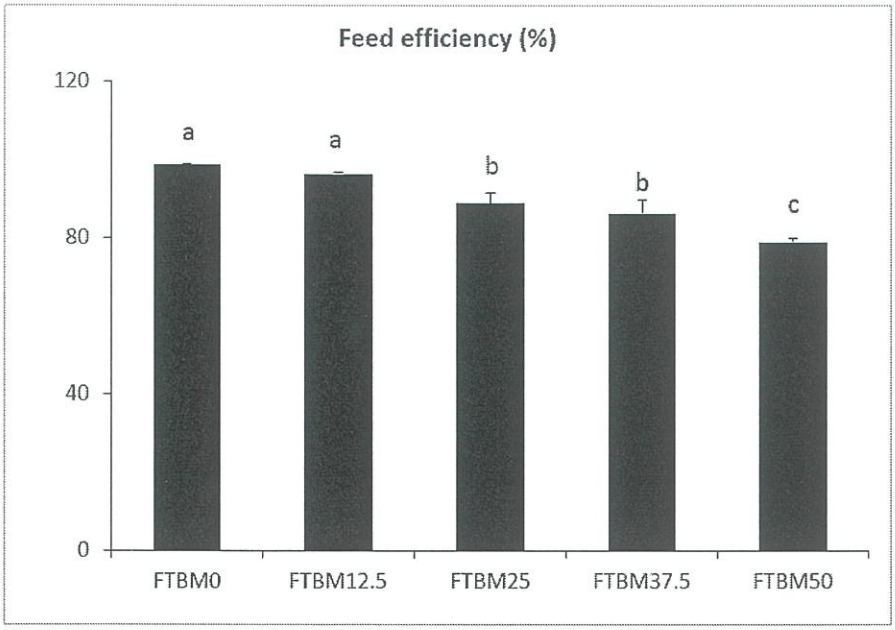

Fig. 1-c. Feed efficiency of juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

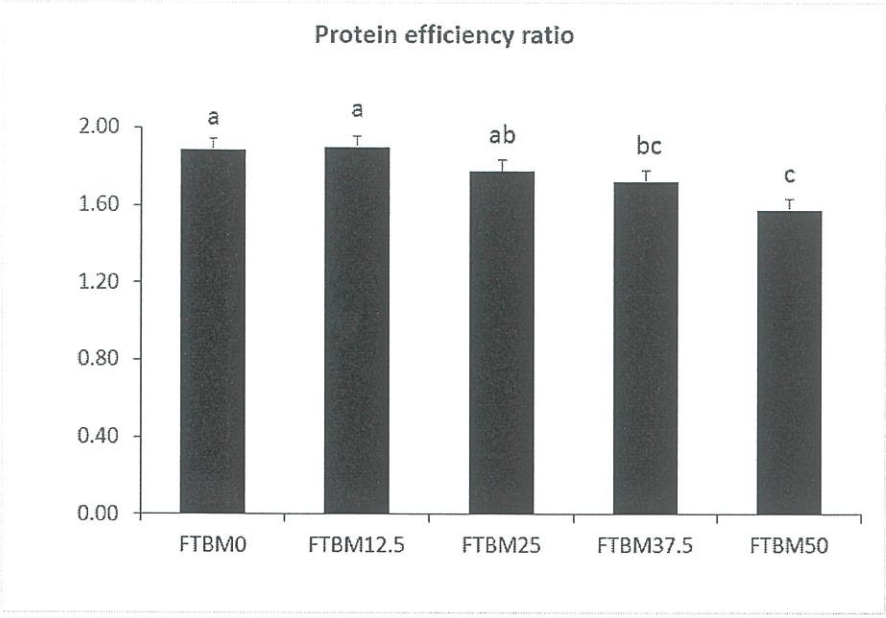

Fig. 1-d. Protein efficiency ratio of juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

Table 5. Fish body composition of juvenile olive flounder *Paralichthys olivaceus* fed the experimental diets for 8 weeks[1]

	Diets[2]				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
HSI (%)[3]	0.89 ± 0.19	0.79 ± 0.10	0.83 ± 0.33	0.80 ± 0.20	0.79 ± 0.26
VSI (%)[4]	3.06^{ab} ± 0.45	2.83^{b} ± 0.33	3.11^{ab} ± 0.40	3.48^{a} ± 0.40	3.48^{a} ± 0.59
CF[5]	0.93 ± 0.04	0.88 ± 0.07	0.84 ± 0.10	0.83 ± 0.09	0.84± 0.07

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

[3]Hepatosomatic index (HSI) = liver wt. × 100 / body wt

[4]Visceralsomatic index (VSI, %) = viscera wt. × 100 / body wt

[5]Condition factor = (wet weight / total length3) × 100

Table 6. Amino acid composition (% of dry matter) of the experimental diets[1]

	Diets				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
Aspartic acid	5.51 ± 0.07	5.36 ± 0.05	5.13 ± 0.20	4.95 ± 0.00	4.60 ± 0.06
Threonine	2.38 ± 0.03	2.33 ± 0.02	2.25 ± 0.05	2.14 ± 0.02	2.06 ± 0.08
Serine	2.24 ± 0.04	2.19 ± 0.02	2.13 ± 0.05	2.03 ± 0.03	1.95 ± 0.08
Glutamic acid	9.56 ± 0.11	9.47 ± 0.10	9.18 ± 0.14	8.76 ± 0.06	8.47 ± 0.30
Proline	3.60 ± 0.18	3.29 ± 0.13	2.93 ± 0.12	2.81 ± 0.02	2.81 ± 0.06
Glycine	3.34 ± 0.08	3.29 ± 0.05	3.33 ± 0.12	3.33 ± 0.02	3.23 ± 0.09
Alanine	3.50 ± 0.04	3.40 ± 0.04	3.32 ± 0.10	3.18 ± 0.03	3.05 ± 0.10
Valine	3.07 ± 0.03	2.94 ± 0.03	2.87 ± 0.04	2.67 ± 0.02	2.56 ± 0.06
Isoleucine	2.66 ± 0.01	2.60 ± 0.02	2.50 ± 0.03	2.35 ± 0.01	2.25 ± 0.07
Leucine	4.37 ± 0.03	4.26 ± 0.00	4.12 ± 0.07	3.89 ± 0.01	3.73 ± 0.14
Tyrosine	1.54 ± 0.07	1.40 ± 0.05	1.48 ± 0.08	1.32 ± 0.08	1.21 ± 0.06
Phenylalanine	2.50 ± 0.04	2.44 ± 0.00	2.35 ± 0.03	2.20 ± 0.02	2.09 ± 0.08
Histidine	2.13 ± 0.06	2.19 ± 0.03	2.07 ± 0.01	2.08 ± 0.04	2.05 ± 0.12
Lysine	4.37 ± 0.02	4.19 ± 0.00	3.98 ± 0.07	3.73 ± 0.02	3.52 ± 0.15
Arginine	3.50 ± 0.09	3.33 ± 0.00	3.19 ± 0.04	3.11 ± 0.02	3.02 ± 0.13
Cystine	1.12 ± 0.03	1.08 ± 0.03	1.05 ± 0.06	0.97 ± 0.06	0.96 ± 0.08
Methionine	1.46 ± 0.20	1.40 ± 0.24	1.43 ± 0.17	1.40 ± 0.08	1.40 ± 0.14

[1]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

Table 7. Proximate body composition (% wet matter basis) of olive flounder *Paralichthys olivaceus* juvenile fed the experimental diets for 8 weeks[1]

	Diets[2]				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
Moisture	75.8 ± 0.56	76.1 ± 0.62	76.4 ± 1.13	76.7 ± 0.85	76.2 ± 0.20
Crude protein	17.4 ± 0.45	17.4 ± 0.47	17.3 ± 0.62	17.0 ± 0.66	17.6 ± 0.24
Crude lipid	2.50 ± 0.29	2.04 ± 0.34	2.13 ± 0.46	2.17 ± 0.28	2.71 ± 0.32
Ash	4.38 ± 0.21	4.22 ± 0.13	3.93 ± 0.38	3.97 ± 0.21	3.94 ± 0.27

[1]Values are mean ± SD from triplicate groups. Absence of superscripts indicates no significant difference between treatments ($P > 0.05$).
[2]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

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

	Diets[2]				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
GOT(U L^{-1})[3]	28.3 ± 3.21^{a}	26.0 ± 1.00^{ab}	20.7 ± 2.52^{c}	23.0 ± 1.73^{bc}	27.3 ± 3.21^{ab}
GPT (U L^{-1})[4]	8.33 ± 0.58	7.00 ± 1.00	8.67 ± 1.15	8.67 ± 2.08	7.33 ± 1.53
TP (g dL^{-1})[5]	3.00 ± 0.10	2.87 ± 0.31	3.00 ± 0.26	2.97 ± 0.23	3.00 ± 0.40
Glucose (mg dL^{-1})	33.7± 1.53^{a}	24.3 ± 0.58^{bc}	27.0 ± 1.73^{b}	25.7 ± 1.15^{bc}	23.0 ± 2.65^{c}

[1]Values are mean ± SD from triplicate groups. Values in each column sharing the same superscripts are not significantly different ($P > 0.05$). Absence of superscripts indicates no significant difference between treatments.

[2]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

[3]Glutamic oxaloacetic transaminase

[4]Glutamic pyruvic transaminase

[5]Total protein

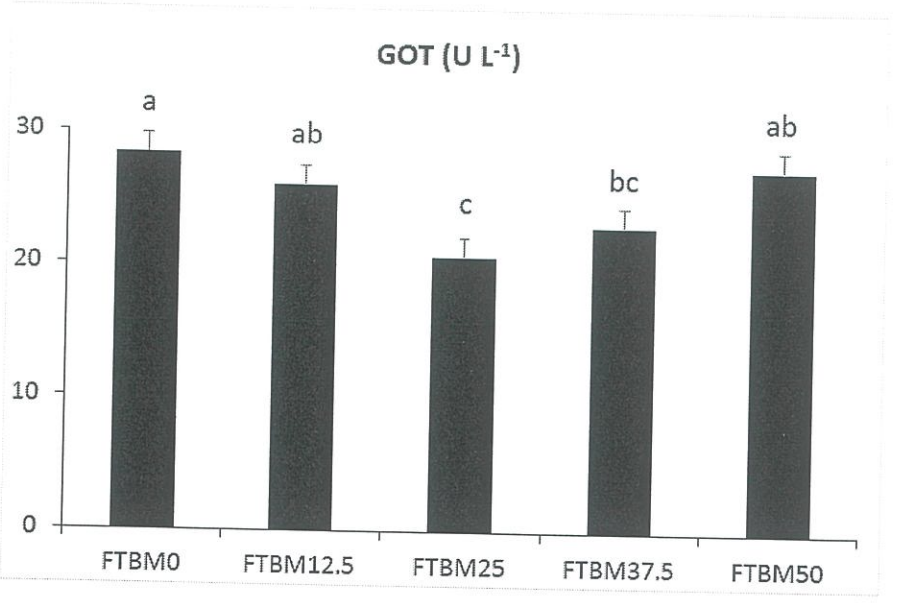

Fig.2-a. Glutamic oxaloacetic transaminase (GOT) in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

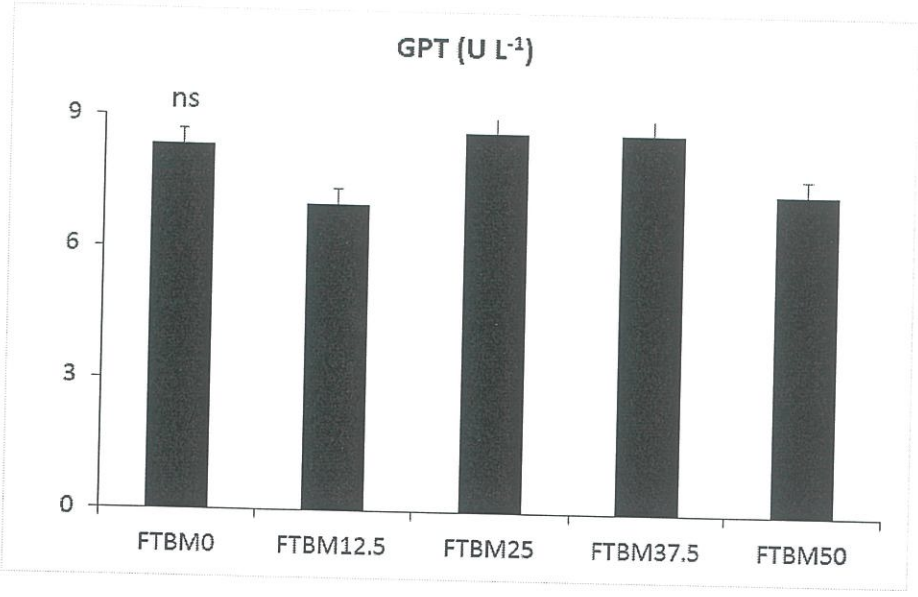

Fig.2-b. Glutamic pyruvic transaminase (GPT) in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

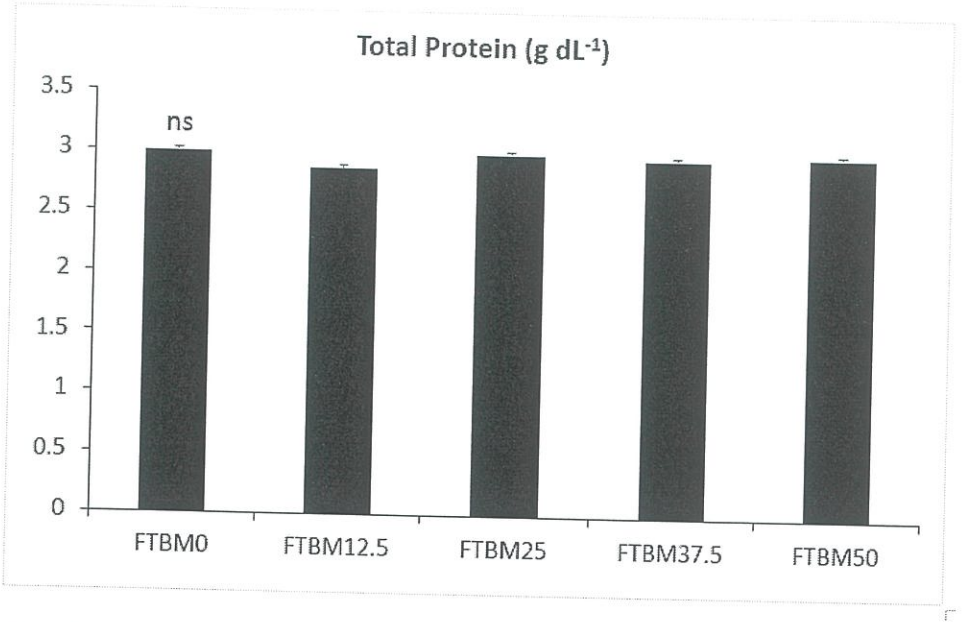

Fig.2-c. Total protein in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

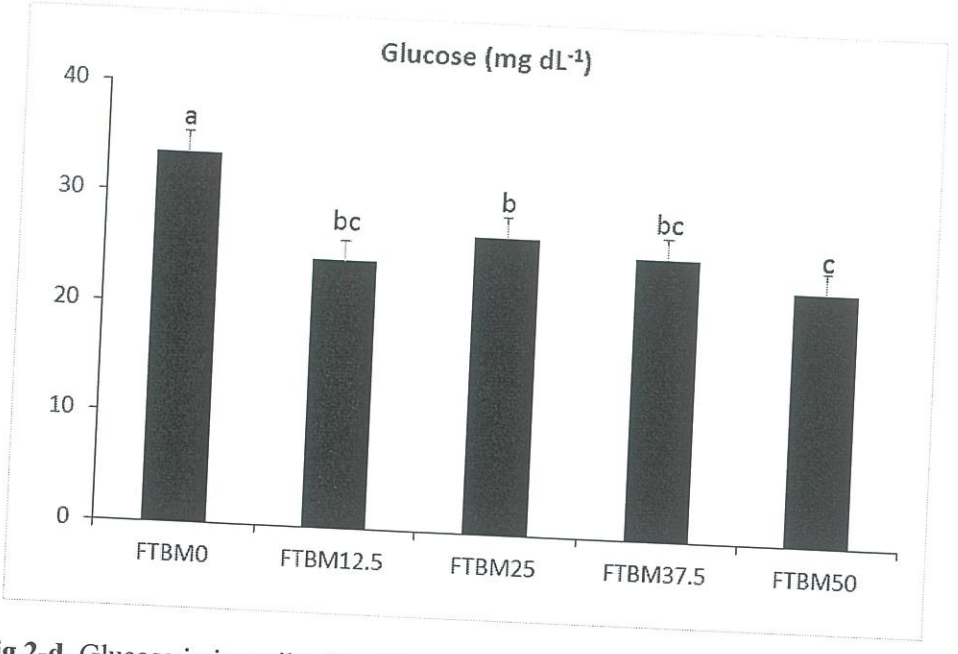

Fig.2-d. Glucose in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

Table 9. Non-specific immune response of olive flounder *Paralichthys olivaceus* juvenile fed the experimental diets for 8 weeks[1]

	Diets[2]				
	$FTBM_0$	$FTBM_{12.5}$	$FTBM_{25}$	$FTBM_{37.5}$	$FTBM_{50}$
SOD[3]	73.6 ± 9.09	65.2 ± 13.0	70.2 ± 12.3	68.4 ± 3.91	67.7 ± 18.3
Lysozyme (U/ml)	1.85 ± 0.07	1.80 ± 0.09	1.73 ± 0.26	1.87 ± 0.15	1.70 ± 0.32

[1]Values are mean ± SD from triplicate groups. Absence of superscripts indicates no significant difference ($P > 0.05$).

[2]Diets: $FTBM_0$-Basal diet (0 % of FTBM), $FTBM_{12.5}$-12.5 % of fishmeal (replaced by 9.40 % FTBM), $FTBM_{25}$-25.0 % of fishmeal (replaced by 18.9 % FTBM), $FTBM_{37.5}$-37.5 % of fishmeal (replaced by 28.4 % FTBM), $FTBM_{50}$-50.0 % of fishmeal (replaced by 37.9 % FTBM).

[3]SOD (% inhibition): Superoxide dismutase

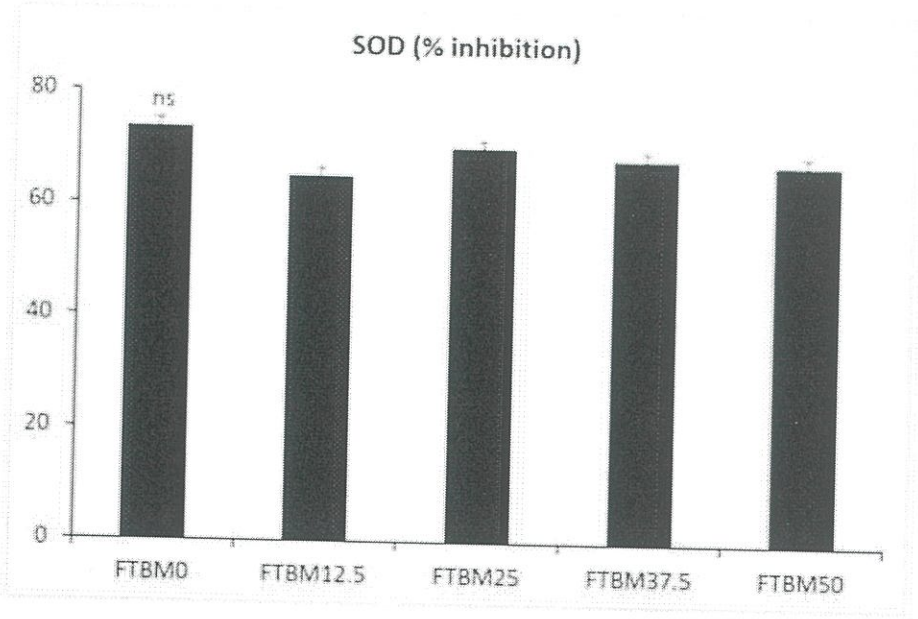

Fig. 3-a. Superoxide dismutase inhibition in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

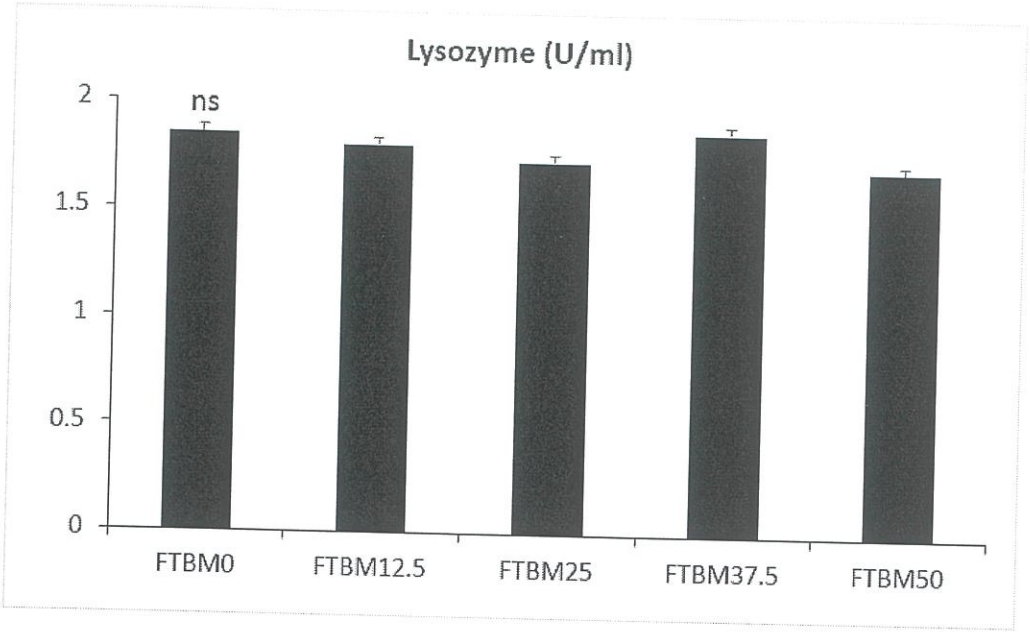

Fig.3-b. Lysozyme activity in juvenile olive flounder fed the experimental diets for 8 weeks.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

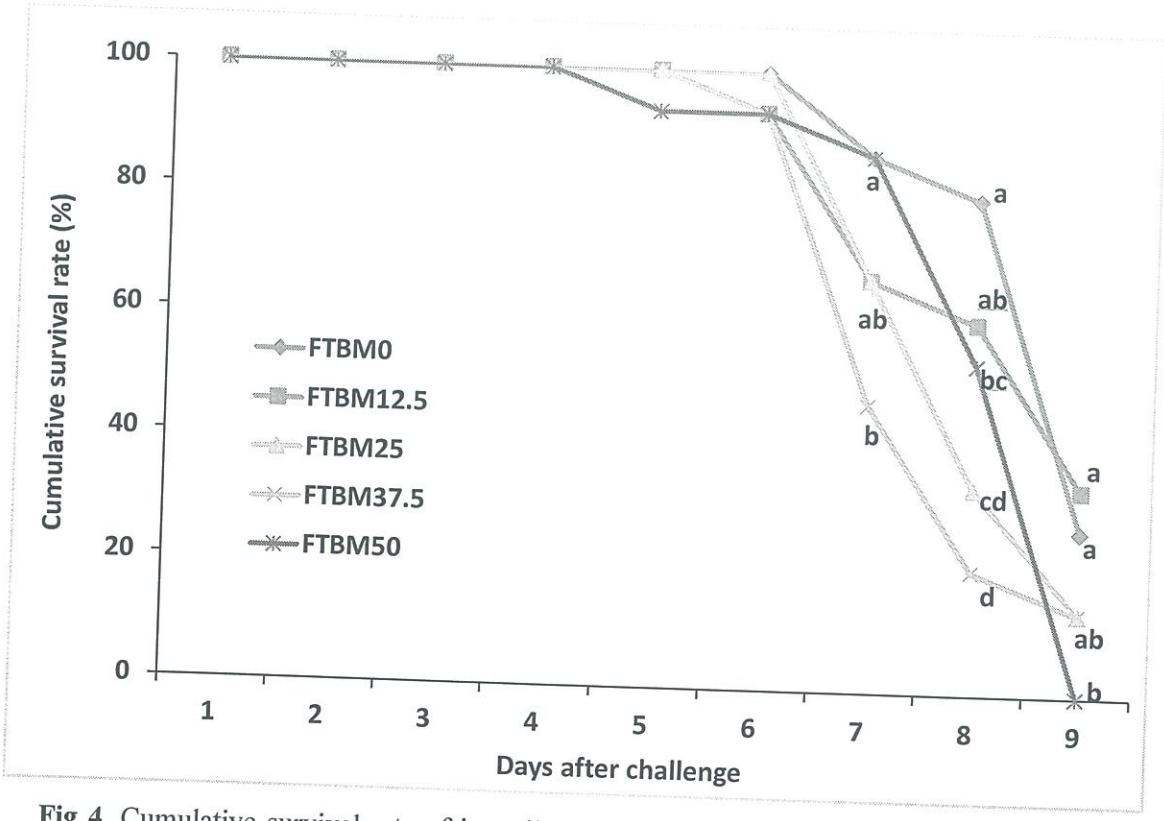

Fig 4. Cumulative survival rate of juvenile olive flounder, *Paralichthys olivaceus* fed the experimental diets for 8 weeks and then experimental challenged with *E. tarda* for 9 days.

$FTBM_0$ (control) – Basal diet (0 % of FTBM)

$FTBM_{12.5}$ – 12.5 % of fishmeal (replaced by 9.40 % FTBM)

$FTBM_{25}$ – 25.0 % of fishmeal (replaced by 18.9 % FTBM)

$FTBM_{37.5}$ – 37.5 % of fishmeal (replaced by 28.4 % FTBM)

$FTBM_{50}$ – 50.0 % of fishmeal (replaced by 37.9 % FTBM)

IV. Discussion

This study demonstrated that FTBM inclusion up to 12.5 % was considered as the level without adverse effects on growth performance of olive flounder. The optimum FTBM inclusion level in this study was lower than those from the study by Kim et al. (2014), who reported a 20 to 30 % inclusion level of tuna by-product meal in olive flounder diets. Uyan et al. (2006) found that about 50 % of fishmeal protein could be replaced by tuna muscle by-product protein in diets for Japanese olive flounder. In addition, about 30-40 % of fishmeal could be replaced by tuna by-product meal in the diet of Korean rockfish *Sebastes schlegeli* (Jeon et al., 2014), whereas 25-30 % of FM could be substituted with tuna by-product meal in spotted rose snapper *Lutjanus guttatus* diets (Hernández et al., 2014). It has been reported that fermentation of fishery by-product and plant protein mixture could improve its nutritional profile (Vijayan et al., 2009), thereby allowing higher inclusion level in fish diets. Kader et al. (2011) found that 36 % of fishmeal could be substituted with the blend of fermented soybean meal and scallop by-product for red sea bream, *Pagrus major,* diet. However, in this study, fermentation process did not promote higher level of FM replacement by FTBM due to lower protein content and amino acid composition of FTBM compared to that of FM.

While no differences were observed in WG, SGR and FE among fish fed $FTBM_0$ and $FTBM_{12.5}$ diets, fish fed higher levels of FTBM showed lower growth and feed utilization. Essential amino acid requirements are important for the formulation of least cost and quality fish feeds (Alam et al., 2002). The essential amino acid requirements for olive flounder have been known, for lysine 1.5 to 2.1 % of the diet, for methionine 1.4 % to 1.5 % of the diet (in the presence of 0.06% of cysteine) (NRC 2011). The amino acid analysis in our study showed that FTBM does

not have sufficient amounts of lysine and methionine, matching the nutritional requirements of olive flounder. Therefore, we supplemented lysine and methionine in the experimental diets for meet the requirements of juvenile olive flounder. Poor feed efficiency was observed for fish fed $FTBM_{25}$, $FTBM_{37.5}$ and $FTBM_{50}$ diets, it is also possible that garlic husk which was combined with the tuna by-product meal during fermentation process did not improve the palatability and acceptability of the diets and therefore led to reduced growth and feed utilization of olive flounder particularly in FTBM levels higher than 12.5 %. On the other hand, results on protein efficiency ratio suggest that FTBM could still be supplied in the diet up to 25 %. Survival rates did not vary among treatments and were comparable to or higher than those from the other studies replacing FM with alternative animal and/or plant protein sources in the diet (Kader *et al.*, 2012; Kim *et al.*, 2014). In this study, no significant differences were observed in CF and HSI in all treatment groups. Kader et al. (2012) reported similar results on HSI of red sea bream, *Pagrus major* when fed diets with fermented soybean meal and scallop by-product blend. In addition, Kim et al. (2014) found no significant differences on CF of olive flounder when fed diets with tuna by-product meal.

In the present study, fish fed all test diets did not indicate differences in body proximate composition. The results could well agree with the results in olive flounder fed with diets containing soybean meal and squid by-product mixture (Kader et al., 2012). However, a number of studies utilizing many different types of tuna by-product meal in olive flounder and Korean rock fish reported the significant effects on body composition (Uyan *et al.* 2006; Jeon *et al.* 2014; Kim *et al.* 2014).

Blood parameters can be used as a measure of the physiological and health condition of fish (Kader et al., 2012). Supplementation of FTBM in the diets significantly affected blood parameters except for serum total protein and GPT levels. However, Kim et al. (2014) reported

that total protein was affected by dietary substitution of fish meal with tuna by-product meal. In our study, glucose levels and total cholesterol generally decreased with increasing FTBM inclusion levels. Elevated glucose levels in the control diet ($FTBM_0$) may suggest short-term stress due to handling (Melotti et al. 1992; Wendelaar-Bonga 1997; Lee et al., 2016). Kader and Koshio (2012) also reported that plasma constituents such as glucose and total cholesterol were affected by fermented soybean meal and scallop by-product blend for red sea bream (*Pargus major*). Additionally, blood enzymes such as GPT and GOT are known to be health markers of the animal's physiological condition (Ozgur et al., 2010). It is also known that these markers are sensitive indicators for tissue damage (De la Tore et al., 2000). In the present study, significantly lower GOT levels were found in diets containing $FTBM_{25}$ and $FTBM_{37.5}$ respectively, suggesting no severity of liver damage in these treatment groups. However, in contrast to our study, other researches have reported that the blood parameters of fish are not affected by the dietary substitution of alternative protein sources for fishmeal (Cho et al., 2005; Jeon et al., 2014; Lee et al., 2012).

Superoxide dismustase and lysozyme activities are indicators of non-specific immune responses in fish (Ellis 1999; Yonar 2012). In the present study, FTBM supplementation did not influence the activities of superoxide dismutase and lysozyme among treatment groups. This is while, Kader et al. (2012) reported that fermented soybean meal and squid by-product meal as substitutions of FM, increasing activities non-specific immune responses in olive flounder. Fermentation process of rendered animal and plant protein sources has been reported to increase the antioxidant properties in diets and non-specific immune responses of parrot fish, *Oplegnathus fasciatus* and olive flounder, *Paralichthys olivaceus* (Kim et al., 2009; 2010a). It is likely that inclusion of fermented garlic husk in diets have provided immunostimulant effects on olive

flounder since superoxide dismutase and lysozyme activities were not affected. Immunostimulatory effects on lysozyme activity have also been reported in various fish fed with Barodon (Shin et al., 2014), yeast glucan (Engstad et al., 1992), organic chromium (Gatta et al., 2001), and fermented garlic powder (Kim et al., 2010b).

The pathogenic bacterium, *E. tarda*, is one of the causative agents of bacterial diseases in olive flounder. It is responsible for septicemia, skin lesions, and diseases of the muscles and internal organs (Plumb 1993). In this study, at day 8, cumulative survival rates of fish fed $FTBM_0$ was significantly higher than those of fish fed $FTBM_{25}$, $FTBM_{37.5}$ and $FTBM_{50}$ diets. Although, there were no significant differences between $FTBM_0$ and $FTBM_{12.5}$ diets. Namely, this study shows that inclusion of $FTBM_0$ and $FTBM_{12.5}$ diets significantly improved the disease resistance of olive flounder 9-day post challenge with *E. tarda*. However, the results of previous studies show that challenges with *E. tarda* did not confirm such effects in olive flounder and red sea bream, *Pargus major* (Khosravi et al., 2015).

In conclusion, this study demonstrated that dietary inclusion of FTBM up to 12.5 % had no adverse effect on growth performance, proximate composition and non-specific immune responses and resistance to *E. tarda* infection of olive flounder.

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Oncul Fatma Ozgun

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Appendix

Raw data

	Rep	WG(%)	SGR(%/day)	FE(%)	Survival (%)
$FTBM_{0.0}$	1	153.25	2.21	98.76	100
	2	150.58	2.19	98.77	82
	3	153.25	2.21	98.78	100
$FTBM_{12.5}$	1	147.10	2.15	96.78	100
	2	149.63	2.18	96.32	100
	3	152.15	2.20	95.86	100
$FTBM_{25.0}$	1	137.22	2.06	90.60	100
	2	118.76	1.86	91.46	100
	3	133.56	2.02	86.91	100
$FTBM_{37.5}$	1	128.27	1.97	84.15	95.0
	2	122.38	1.90	84.25	95.0
	3	133.01	2.01	90.18	100
$FTBM_{50.0}$	1	116.02	1.83	78.61	100
	2	125.18	1.93	80.15	100
	3	121.37	1.89	77.92	95.0