A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Evaluation of a mixture of essential oil and prebiotic in diets for olive flounder, *Paralichthys olivaceus.*

Min-Gi Kim

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2016. 02.

Evaluation of a mixture of essential oil and prebiotic in diets for olive flounder, *Paralichthys olivaceus.*

Min-Gi Kim

(Supervised by Professor Kyeong-Jun Lee)

A dissertation submitted in partial fulfillment of the requirement for the degree of

2016. 02.

This dissertation has been examined and approved.

.....
Suk-Geun Jung, Prof. of Marine Life Science

.....
Sang-Rul Park, Prof. of Marine Life Science

.....
Kyeong-Jun Lee, Prof. of Marine Life Science

2016. 02

Date

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

국문 초록

이 연구는 사료 내 essential oils와 prebioics 혼합물(EOP)의 첨가가 넙치 치어의 성장, 사료효율, 비특이적면역력, 소화율, 질병저항성에 미치는 영향을 평가하기 위하여 수행되었다. 4개의 실험사료는 동일한 조단백질(50%)과 에너지(17.2 MJ kg^{-1})를 포함하도록 제작되었다. 어분을 기초로 하여 대조구 사료와 EOP를 각각 0.1%, 0.2%, 0.4% 첨가한 3개의 실험사료를 제작하였다. 실험 I 에서는 평균 27.5 g 의 넙치 치어를 대상으로 10주간 만복급이 하였다. 사양실험 기간동안 성장률은 전 실험구에서 유의적인 차이가 없었으나 사료효율에 있어서는 0.2% 실험구가 대조구에 비하여 유의적으로 높게 나타났다. 전어체 분석 결과, 모든 그룹에서 유의적인 차이는 없었다. 혈액학적 분석에서는 혈중 triglyceride 분석에서 0.2% 실험구가 가장 높은 수치를 나타내었다. 비특이적 면역분석결과, immunoglobulin, anti-protease, myeloperoxidase 활성에서 EOP-0.1 실험구가 타 실험구에 비해 가장 높은 수치를 나타내었다. 외관상소화율 실험결과, EOP를 첨가한 모든 실험구가 대조구에 비하여 높은 수치의 소화율을 보였다.

실험 II 에서는 8.92 g 의 넙치를 이용하여 실험 I 과 동일한 사료를 7주간 만복급이 하였다. 성장율과 사료효율에서는 유의적인 차이가 나타나지 않았다. 혈액학적 분석에서 hemoglobin, hematocrit, cholesterol 의 수치가 EOP-0.1 실험구에서 유의적으로 가장 높았다. 비특이적 면역분석결과 anti-protease 와 myeloperoxidase 활성이 EOP의 첨가량이 증가함에 따라 유의적으로 증가하는 경향을 보였다. 사양실험 종료 후 15 마리의 넙치치어를 대상으로 *Edwadsiella tarda* 5×10^5 cell/ml 를 복강 내 0.1 ml 씩 주사하여 공격실험을 진행하였다. 공격실험 결과 EOP-0.1 실험구

가 생존율이 가장 낮았으며 EOP-0.4 실험구가 가장 높은 생존율을 나타내었다.

실험 I, II의 결과, 사료 내 EOP의 첨가는 30~150 g 크기 넙치의 사료 효율, 소화율, 그리고 비특이적 면역력을 증가 시킬 수 있을 것으로 사료된다. 하지만 30 g 이하 치어의 경우에는 사료 내 EOP의 첨가 효과가 미비할 것으로 사료된다. 따라서 넙치 사료 내 EOP의 적정 첨가량은 0.1 - 0.2%인 것으로 판단된다.

ABSTRACT

This study was conducted to evaluate the effects of dietary supplementation of mixture of essential oils and prebiotics (EOP) on growth performance, feed utilization, innate immunity, digestibility and disease resistance of juvenile olive flounder. Four experimental diets were formulated to be isonitrogenous (50% crude protein) and isocaloric (17.2 MJ kg^{-1}). A fish meal based diet was considered as a control and three other experimental diets were formulated to contain EOP at levels of 0.1, 0.2 and 0.4%. In Exp I, Quadruplicate groups of fish (initial body weight 27.5 g) were fed each of the experimental diets to apparent satiation twice daily for 10 weeks. At the end of the feeding trial, weight gain was numerically increased by the supplementation of EOP but not significant. However, significantly lower feed conversion ratio and higher protein efficiency ratio were observed in fish fed 0.2% diet compared to the control group. Hematological, biochemical and whole body proximate composition were not affected significantly by the dietary treatment except that triglyceride level was elevated significantly in EOP-0.1 fed group. However, immunoglobulin level, anti-protease and myeloperoxidase activities were significantly increased with the dietary increment of EOP showing the highest values in EOP-0.1 group.

In Exp II, smaller fish (initial fish weight: 8.92 ± 0.01g) were fed the same experimental diets used for the Exp I for 7 weeks. Growth and feed utilization were not affected by the dietary treatment but plasma hemoglobin, hematocrit and cholesterol levels were significantly elevated by EOP while EOP-0.1 group showed the highest values among all the dietary groups. Anti-protease and myeloperoxidase activities were significantly increased with increasing levels of EOP. Digestibility test results showed that both protein and dry matter digestibility were significantly increased by EOP. Challenge test with *E. tarda* showed that

EOP-0.1 group had higher disease resistance compared to the control group. Results of this study indicate that dietary supplementation of EOP can enhance protein efficiency, innate immunity and disease resistance of juvenile olive flounder and optimum supplementation level is approximately 0.1-0.2%.

LIST OF FIGURES

LIST OF TABLES

Ⅰ. INTRODUCTION

It has been reported that antibioitcs may enhance growth and feed efficiency of a host by killing intestinal micro-flora and thus increasing amino acid utilization by the host in some animal species (Rawles et al., 1997).

Antibiotics and chemical disinfectants have been used as traditional means of controlling fish disease. However, their utilization in aquaculture practices needs to be restricted due to the occurrence of antibiotic-resistant bacterial strains, water pollution in the aquaculture environment and accumulation of residual antibiotics in aquaculture products (Lunden et al., 2002; Petersen et al., 2002; Alcaide et al., 2005; Cabello, 2006). Moreover, the use of antibiotics can lead to alteration of gastrointestinal microbiota and induction of resistant bacterial populations with lasting adverse effects on public health (Verschuere et al., 2000). To rectify such undesirable impacts, several alternatives have been developed for prevention of fish disease of which probiotics have shown promising results (Gatesoupe et al., 2010; Merrifield et al., 2010; Dimitroglou et al., 2011; De et al., 2014; Hoseinifar et al., 2014).

As an environment-friendly measure of controlling fish disease, probiotics have been recently received great attention in aquaculture industry (Wang et al., 2008; Nayak et al., 2010). Gastrointestinal tract (GI) is generally identified as an organ of nutrient digestion/absorption, but recently studies are being conducted focusing on its critical role in immune function (Llewellyn et al., 2014). Furthermore, GI tract is characterized as one of the most important sites of interaction with surrounding environment along with skin and gills, and is considered as one of the major routes of infection by pathogenic agents (Groff and Lapatra, 2000; Birkbeck and Ringø, 2005; Ringø et al., 2007). Probiotics are viable cell preparations that exert beneficial effects on the health of a host through improvement of

intestinal balance (Merrifield et al., 2010). They exclude pathogens from the GI tract through the production of inhibitory compounds, competing for nutrients, space and adhesion sites, stimulation of immune function and contribution in enzymatic digestion (Verschuere et al., 2000; Gatesoupe et al., 2010).

A wide range of probiotics have been examined in aquaculture that most of them belong to *Saccharomyces, Clostridium, Lactobacillus, Bacillus, Enterococcus, Shewanella, Leuconostoc, Lactococcus, Carnobacterium* and *Aeromonas* species (Kesarcodi-Watson et al., 2008; De Rodriganez et al.,2009). The selection of an appropriate probiotic is a very critical criterion that should be taken into account (Sun et al., 2010). Beneficial probiotic strains should be able to be colonized and established in the host gut (Verschuere et al., 2000; Nayak, 2010). *Bacillus spp.* has extensively been examined as one of the most beneficial probiotic in aquafeed due to their higher resistance against harsh gastric conditions resulting from their spore-forming ability (Hyronimus et al., 1998; Casula and Cutting, 2002). The beneficial effects of *Bacillus spp.* on fishes have been reported in terms of non-specific immune responses (El-Dakar et al., 2007; Nayak et al., 2007; Cha et al., 2013).

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. They are also called as volatile oils, ethereal oils, aetherolea. Sometimes those are identified as the oil of the plant from which they were extracted, such as clove oil. An oil is "essential" in the sense that it contains the "essence of" the plant's fragrance—the characteristic fragrance of the plant from which it is derived. It does not mean indispensable as with the terms essential amino acid or essential fatty acid. Essential oils contain some bioactive properties due to the richness of its major chemical constituents, such as geranial, carvone, neral, linalool and limonene. Studies have proven that the essential oil of *L. alba* and its constituents have antibacterial (Fabri et al., 2011), antifungal (Nogueira et al., 2009), antiprotozoal (Oliveira et al., 2006), antiparasitic (Escobar et al., 2010) and anti-

inflammatory (Haldar et al., 2012) effects. Antimicrobial properties of essential oil are mainly due to phenolic compounds (Dormana et al., 2003; Burt, 2004; Lee et al., 2005).

Many studies have correlated or compared the functional properties of essential oils with those of their individual components and combinations of oils in order to find their possible synergistic effects. Essential oil is reported to express some resistant effects to viral, bacterial and fungal agents in animals including fish (Dorman and Deans, 2000; Isman and Machial, 2006; Sharif-Rohani et al., 2011; Soltani et al., 2012). Studies showed that the use of natural products derived from plants, for controlling parasites, has received great attention (Steverding et al., 2005; Ji et al., 2012; Huang et al., 2013). The results of previous studies clearly showed that essential oil is suitable anti-fungal agent for rainbow trout (*Oncorhynchus mykiss*) hatcheries (Soltani et al., 2009; Sharif-Rohani et al., 2011).

Recently antibiotic resistant strains have rapidly developed disease outbreak in the fish farming industry which cause a great difficulty in treating. Also, continuous chemotherapy will easily increase the antibiotic residual in the fish tissue. This phenomenon can develop bacterial resistance in the consumers also. Therefore, new treatment methods should be applied to suppress the growth of the bacterium with a minimum side effect. In recent years, use of medicinal plants has been considered in both medical and veterinary sciences. Plant essential oils with high efficiency against microorganisms of both gram negative and gram positive bacteria are some of the biological balances with no accumulation in the animal body. Thus, use of such environmentally natural products has a significant advantage compared to synthetic chemical drugs (Oussalah et al., 2006).

EOP consists of aromatic plant extracts, essential oils and prebiotics (fructo-oligosaccharides). The EOP can affect animal digestive tract to stimulate digestive functions by improving appetite and contributing to balance bacterial flora, and is able to induce a number of other benefits. Essential oils and extracts can be dose-dependent bacterioistatic

and bactericidal. Several studies have shown the effects of EOP on digestive physiology and digestion at weaning and on the microbiology of the gut poultry (Franz et al., 2009).

Recent studies have shown that essential oils have anti-microbial properties (Hammer et al., 1999; Dorman and Deans, 2000, Moreira et al., 2005; Sharma and Tripathi, 2006) as well as antifungal properties (Dambolena et al., 2008; Chee et al., 2009) .

Prebiotics are no digestible carbohydrates that trigger the growth of bacteria having favorable effects on the intestinal flora, probiotics. Probiotics can be defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO, 2001). Several studies indicate the probioitcs in aquaculture by enhancing the disease resistance by suppressing the pathogens (Tseng et al., 2009; Harikrishnan et al., 2011).

Evidence of the beneficial effects of probioitcs gave birth to the concept of prebiotics (Gibson and Roberfroid, 1995; Teitelbaum and Walker, 2002), which generally cannot be digested by the fish but are metabolized by helpful bacteria in the host gut. Correspondingly, the beneficial effects of some oligosaccharides have also been demonstrated on fish (Dimitroglou A et al., 2010).

Olive flounder is currently the most important marine fish cultured in Korea with annual production amount of ~37,000 tons (Ministry of Maritime Affairs and Fisheries 2013). South Korea is the top global producer of olive flounder where the production exceeds 50% of annual production of total fish species while the production has been continuously increased through improved culture techniques (Bai and Kim, 2001). However, mortalities caused by infectious bacterial disease have been a main constraint to its culture. Thus, the aim of current study was to investigate the effect of dietary EOP on growth performance, biochemical responses, digestibility, disease resistance and innate immunity in olive flounder (*Paralichthys olivaceus*).

Ⅱ. MATERIALS AND METHODS

2.1 Experimental diets

A basal experimental diet (45% crude protein, 18 MJ/kg diet gross energy) was prepared and regarded as a control and three other diets were prepared by the EOP each in different concentrations. The tested EOP was provided from the AQUA TECHNA companay in France. All dry ingredients were thoroughly mixed and extruded through a pellet machine (SP-50, Geumgang ENG) after adding fish oil and 30% distilled water in each 5 mm and 3mm diameter. The pellets were subsequently crushed into desirable sizes and stored at -20^{o}C until use. The formulation and proximate composition of the basal diet are provided in Table 1. Analyses of crude protein, moisture, and ash in the diets were performed using standard methods (AOAC, 1995) and lipid content was determined by the method of Folch et al. (1957).

Figure 1. Preparation of experimental diets.

Table 1. Formulation of the four experimental diets for olive flounder (%, dry matter basis).

Ingredients	**Diets**			
	CON	EOP-0.1	EOP-0.2	EOP-0.4
Brown FM	46.0	46.0	46.0	46.0
EOP	**0.0**	**0.1**	**0.2**	**0.4**
Soy protein concentrate	5.0	5.0	5.0	5.0
Corn gluten meal	6.0	6.0	6.0	6.0
Wheat flour	31.5	31.4	31.3	31.1
Squid liver oil	4.0	4.0	4.0	4.0
Soybean oil	4.0	4.0	4.0	4.0
Mineral Mix[1]	1.0	1.0	1.0	1.0
Vitamin Mix[2]	1.0	1.0	1.0	1.0
CMC	1.0	1.0	1.0	1.0
Choline chloride	0.5	0.5	0.5	0.5
Dietary nutrient composition(%)				
Moisture	7.6	7.4	7.1	7.2
Protein	48.6	48.6	48.6	48.0
Lipid	13.5	14.0	13.7	13.7
Ash	10.5	11.8	10.8	10.7
Energy, Kcal/kg diet	17.4	17.0	17.1	17.4

[1] Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[2] Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

2.2 Fish and feeding trial

The two feeding trials were conducted at the Marine Science Institute, Jeju National University, Jeju, South Korea. The fish in genetically homogenous stock were obtained from a private hatchery, Jeju Island, and fed a commercial diet for two weeks to be acclimated to the experimental conditions and facilities. The health status of the fish was checked upon arrival and the fish were immediately treated with 100 mg L^{-1} formalin for 20 min. In the first experiment (Exp I), four replicate groups of randomly selected fish (initial mean body weight, 27.6±0.1g) were distributed into a similar rearing system at a density of 30 fish per tank. In the second experiment (Exp II), three replicate groups of randomly selected olive flounder (initial mean body weight, 8.92 ± 0.01 g) were distributed into a similar rearing system at a density of 25 fish per tank. The tanks were supplied with filtered seawater at a flow-rate of 3 L/min and aeration to maintain enough dissolved oxygen. The fish were fed with the experimental diets to apparent satiation (three times a day, 08:30, 13:30, 18:00 h) for 10 and 7weeks for the Exp I and II, respectively. Growth measurement was carried out at every 2 week intervals. Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

Figure 2. Experimental facility.

2.3 Sample collection and analyses

At the end of the feeding trial, all fish in each tank were bulk-weighed and counted to calculate the feed conversion ratio, protein efficiency ratio, and survival. Three fish from each tank were sampled for analysis of whole-body composition. Three fish per tank nine fish per dietary treatment) were randomly captured, anaesthetized in 200 ppm 2-phenoxyethanol solution, and blood samples were taken from the caudal vein with heparinized syringes to determine hematocritand hemoglobin. Additionally, another set of blood samples were taken from the caudal vein of three fish from each tank using non-heparinized syringes, allowed to clot at room temperature for 30 min, and the serum was separated by centrifugation for 10 min at 5000 × *g* and stored at -70℃ for analysis of innate immune response parameters including lysozyme, myeloperoxidase (MPO) and antiprotease activities.

A turbidometric assay was used to determine serum lysozyme levels using the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (pH 6.4, 0.1 M), 200 µl of suspension was placed in each well of a 96-well plate, and 20 µl of serum was added. The reduction in absorbance of samples was determined at 570 nm in a microplate reader after a room temperature incubation for 0 and 30 min (UVM 340, Biochrom**,** Cambridge**,** UK). Hen egg white lysozyme (Sigma) was used for the standard curve. Values are expressed as µg ml^{-1}.

Serum MPO activity was measured according to Quade and Roth (1997). Briefly, 20 µl of serum was diluted with Hanks Balanced Salt Solution (HBSS, Sigma) without Ca^{2+} or Mg^{2+} in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 ml of 4 M sulfuric acid. Finally, optical density was read at 450 nm in a microplate reader.

Serum antiprotease activity was measured according to the method described by Ellis (1990) with slight modifications (Magnadóttir et al., 1999). Briefly, 20 µl of serum was incubated with 20 µl of standard trypsin solution (Type II-S, from porcine pancreas, 5 mg ml^{-1}, Sigma) for 10 min at 22℃. Then, 200 µl of phosphate buffer (0.1 M, pH 7.0) and 250 µl azocasein (2%) (Sigma) were added and incubated for 1 h at 22℃. Five hundred microliters of 10% trichloro acetic acid was added and further incubated for 30 min at 22℃. The mixture was centrifuged at 6000 × *g* for 5 min, and 100 µl of the supernatant was transferred to the wells of a 96 well flat bottomed microplate containing 100 µl of 1 N NaOH. Optical density was read at 430 nm. Buffer replaced the serum for the 100% positive control, and buffer replaced both serum and trypsin for the negative control. The trypsin inhibition percentage was calculated using the following equation:

Trypsin inhibition (%) = $(A_1 - A_2/A_1) \times 100$

where A_1 = control trypsin activity (without serum); A_2 = trypsin activity remaining after adding serum.

Plasma total immunoglobulin(Ig) levels were determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein content was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the immunoglobulin molecules, using a 12% solution of polyethylene glycol (Sigma) where the difference in protein content represents the Ig content.

Figure 3. Checking and blood sampling.

2.4 Digestibility test

For estimation of apparent digestibility coefficient (ADC) of the experimental diets in the first feeding trial, chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 1.0%. All dry ingredients were thoroughly mixed and extruded through a pellet machine (SP-50, Geumgang ENG) in 5 mm diameter after addition of squid liver oil, soybean oil and 20–30% double distilled water. The prepared pellets were freeze-dried at − 40 ℃ for 24 h and maintained in a freezer at − 20 ℃ until used.

The digestibility trial was conducted in four fiberglass fecal collection tanks of 400 L capacity, designed according to Cho et al. (1982). New sets of olive flounder with mean body weight of 135g were stocked into each tank at a density of 40 fish per tank and each group of fish was fed one of the test diets. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. The digestibility trial consisted of three periods of 10 days. In each 10-day period, the fish were allowed to become acclimatized to the feed for the first three days and feces were collected over the next 7 days. Then, diets were randomly changed between tanks and the procedure repeated for two more times, giving a total of three fecal samples for each diet. All feces collected from each tank in each period were pooled and frozen at − 20 ℃ for analysis. After feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed waste and fecal residues. Chromium oxide content of diet and feces samples was analyzed by the method described by Divakaran et al. (2002). The apparent digestibility coefficient of the experimental diets was calculated through the following formula:

ADC of dry matter (%) = 100 - 100 × (% Cr_2O_3 in diet / % Cr_2O_3 in feces)

ADC of protein (%) = 100 - 100 × (% Cr_2O_3 in diet / % Cr_2O_3 in feces) × (% protein in feces / % protein in diet)

Figure 4. Feces collection tanks (Guelph system).

Figure 5. Feces sample collection.

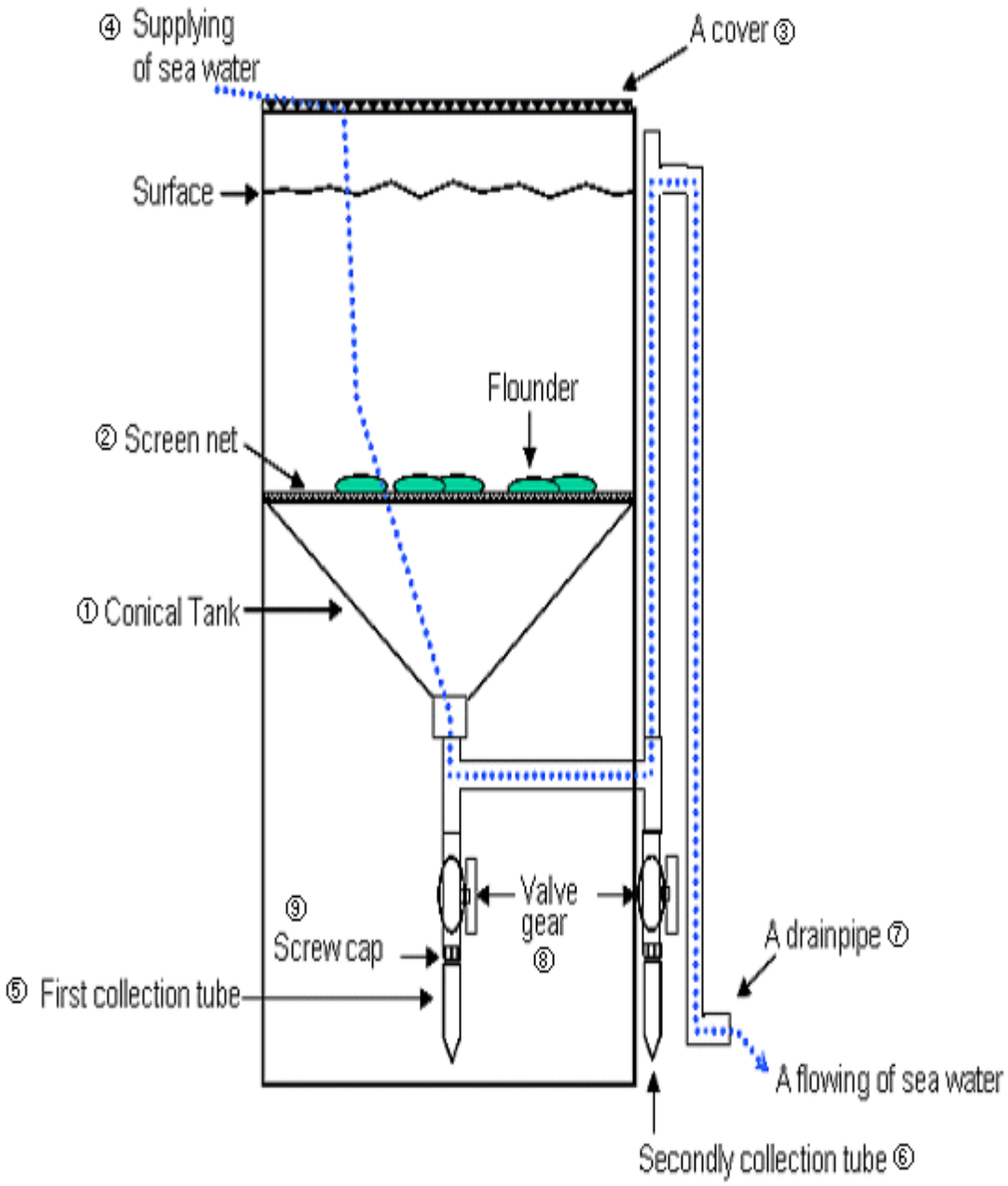

Figure 6. The diagram of Guelph system.

2.5 Challenge test

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly captured and subjected to a bacterial challenge. *E. tarda* (ATCC 15947, Korea Collection for Type Cultures) was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The bacterium was cultured in 10 mL BHI broth (Difco, Detroit, MI, USA) with 1.5% NaCl and incubated with shaking for 24h at 37℃. Bacterial growth was measured at an optical density of 700nm followed by plate counting in BHI-NaCl. The isolated bacteria were identified using API 20E commercial identification kit (97.4% accuracy) (bioMérieux, Marcy l'Etoile, France). The bacterium concentration was determined by plate counting on BHI agar. Fish were injected intraperitoneally with *Edwardsiella tarda* suspension containing 1×10^{6} CFU mL^{-1} for Exp II, respectively. The pathogenic dose of bacterium had previously been determined in a preliminary test using fish of a similar size. After injection, the respective fish groups (15 fish) were distributed into plastic tanks of 65 L capacity in triplicates and their mortality was monitored and recorded for 10 days. Relative percent survival was calculated using the following formula:

Relative % survival = [(mortality (%) control - Mortality (%) Treatment) / Mortality (%) Control] × 100

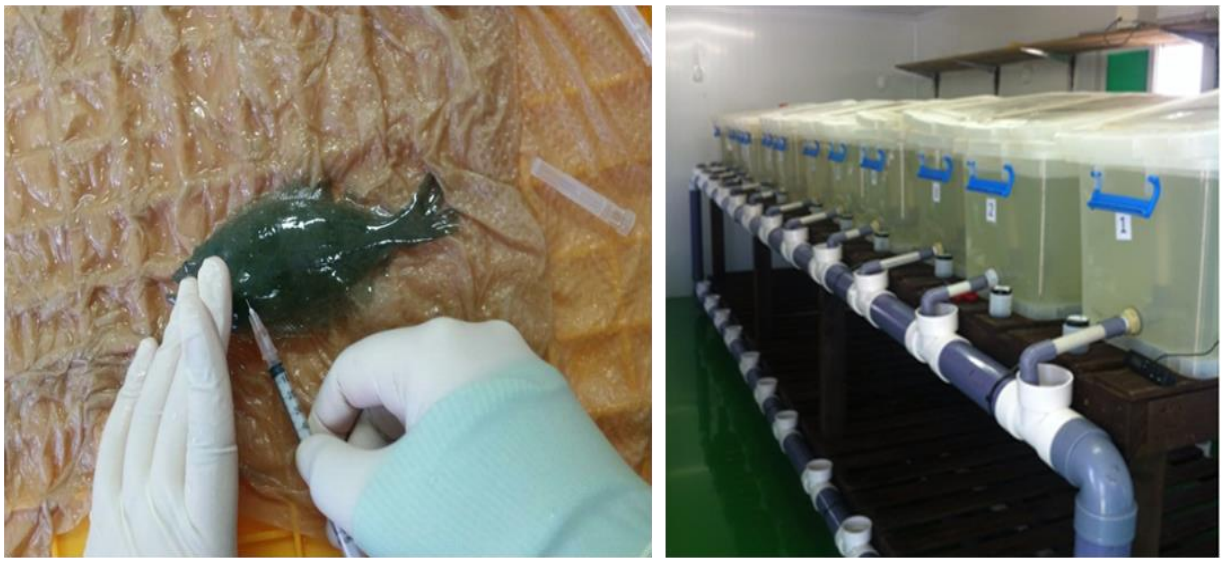

Figure 7. Intraperitioneal injection of olive flounder with *E. tarda*.

2.6 Statistical analysis

All diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using DUNCAN test at the 5% level of significance ($P < 0.05$). Data are presented as mean ± SD. Percentage data were arcsine transformed before analysis.

Ⅲ. RESULT

3.1 Experiment Ⅰ

Growth performance and feed utilization of olive flounder fed the four experimental diets were shown in Table 2. FCR of 0.2% EOP fed fish was significantly lower than that of the fish fed the control and 0.4% EOP diets. PER of 0.2% EOP fed group was significantly higher than that of fish fed the control, EOP-0.1 and EOP-0.4 diets. However, final body weight, weight gain, feed intake and specific growth rate were not significantly affected by the dietary EOP supplementation, even though there was an increasing tendency.

Effects of dietary EOP levels on proximate composition of the whole-body (% wet basis) after the feeding trial are presented in Table 3. The results of moisture, protein, ash and lipid contents in the whole-body and muscle did not show any significant differences among the dietary treatments.

Hemoglobin, hematocrit, total protein, glucose, total cholesterol and trigryceride amount of fish fed experiment diets were not showed any significant differences compared to the control diet (Table 4). However, EOP-0.1 diet group showed significantly higher triglyceride amount compared to the EOP-0.4 diet and it is numerically higher than the other experimental diets.

Non-specific immune response parameters of olive flounder fed the four experimental diets were shown in Figure 8. Lysozyme activities of olive flounder was not significantly affected by the dietary treatments. However, plasma Ig level of EOP-0.1 and EOP-0.2 diets significantly higher than that of the fish fed control and EOP-0.4 diets. All the EOP supplemented groups showed numerically higher Ig values than the control group. Also,

serum anti-protease activity was significantly higher in EOP-0.1 diet compared to that of fish fed the control diet and all the EOP fed groups showed significantly higher serum MPO activity than the control group while EOP-0.1 diet fed group resulted in the highest activity than the other fish groups.

ADC of dry matter and protein were significantly increased in all the EOP diets than those of the control diet (Figure 9).

Table 2. Growth performance and feed utilization of olive flounder fed the four experimental diets containing four different EOP levels for 10 weeks.

	Diets			
	CON	**EOP-0.1**	**EOP-0.2**	**EOP-0.4**
IBW[1]	27.4±0.27	27.6±0.04	27.7±0.16	27.4±0.06
FBW[2]	132±8.86	140±3.00	144±6.74	134±8.38
WG(%)[3]	381±34.6	406±10.2	418±24.3	389±30.8
FI[4]	164±4.45	163±9.56	166±17.4	164±5.13
FCR[5]	1.28±0.05^{b}	1.19±0.02^{ab}	1.13±0.07^{a}	1.27±0.03^{b}
SGR(%)[6]	2.18±0.10	2.25±0.03	2.28±0.06	2.20±0.09
PER[7]	1.66±0.07^{a}	1.78±0.03^{ab}	1.88±0.12^{b}	1.67±0.04^{a}
Survival	90.0±9.48	97.1±2.33	86.4±8.21	88.6±7.00

Mean values of triplicate groups are presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P< 0.05$).

[1]IBW (g) = Initial body weight

[2]FBW (g) = Final body weight

[3]Weight gain (%) = (final body weight – initial body weight) / initial body weight x 100

[4] Feed intake (g/fish) = dry feed consumed (g)/fish

[5]Feed conversion ratio = dry feed fed / wet weight gain

[6] Specific growth rate (%) = 100 x [ln(final body weight) – ln(initial body weight)] / days

[7] Protein efficiency ratio = wet weight gain / total protein given

Table 3. Whole-body proximate composition of olive flounder fed the four experimental diets for 10 weeks.

	Diets			
	CON	**EOP-0.1**	**EOP-0.2**	**EOP-0.4**
Moisture (%)	71.8±0.2	72.0±0.1	72.8±0.2	72.5±1.1
Protein (%)	66.5±1.2	65.2±1.1	68.5±2.4	66.5±1.2
Ash (%)	12.6±0.8	11.3±0.5	12.0±1.3	11.9±0.8
Lipid (%)	19.1±2.7	17.1±2.8	16.8±1.4	18.0±1.6

Table 4. Hematological and biochemical characteristics of olive flounder fed the four experimental diets containing four different EOP levels for 10 weeks.

	Diets			
	CON	**EOP-0.1**	**EOP-0.2**	**EOP-0.4**
Hemoglobin	8.81±0.44	9.04±0.42	9.19±0.49	9.35±0.31
Hematocrit	32.5±1.1	33.4±2.4	32.6±3.1	31.6±0.8
Total protein	2.39±0.39	2.41±0.61	2.37±0.51	2.76±0.73
Total cholesterol	265±34	252±44	284±81	232±42
Triglyceride	353±445^{ab}	413±53^{b}	392±19^{ab}	308±57^{a}
Glucose	72.8±2.72	73.5±5.18	70.8±8.53	70.3±2.49
ALT	54.4±14.9	54.0±8.58	57.5±19.65	56.6±16.33
AST	70.1±18.8	58.3±16.6	63.4±12.9	48.9±20.9

Mean values of triplicate groups are presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P< 0.05$).

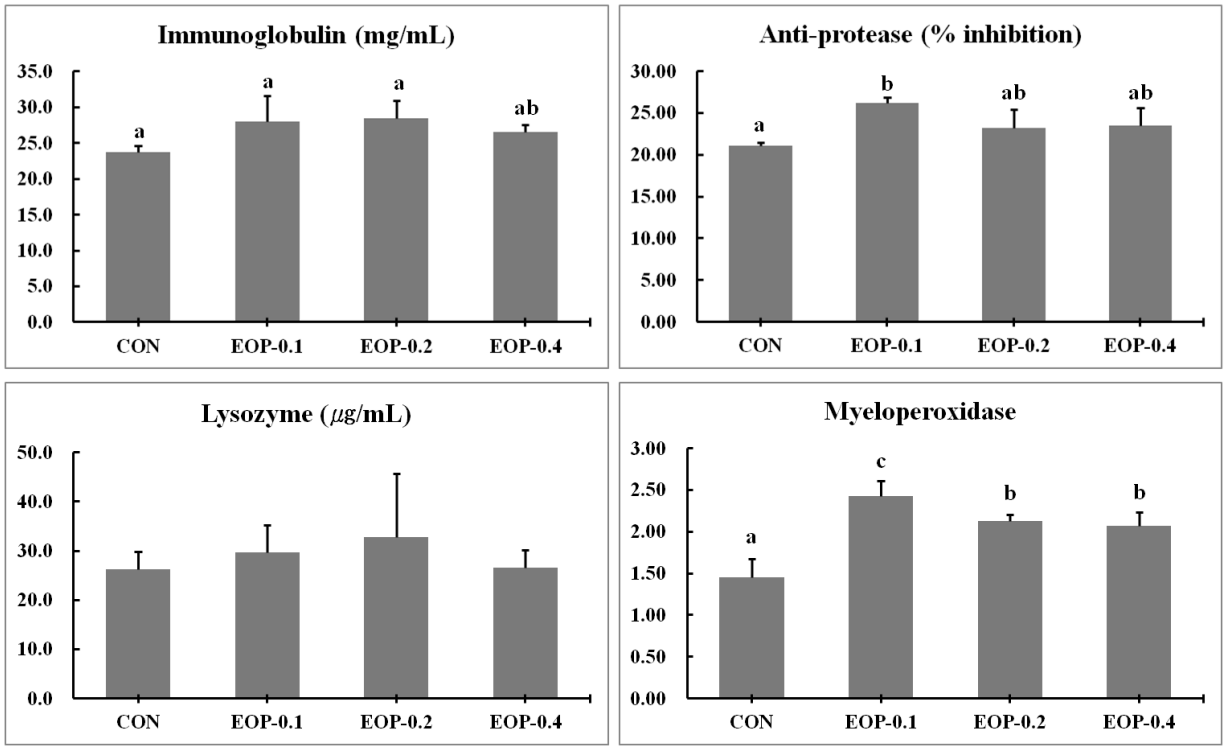

Figure 8. Non-specific immune response parameters of olive flounder fed the four experimental diets containing four different EOP levels for 10 weeks.

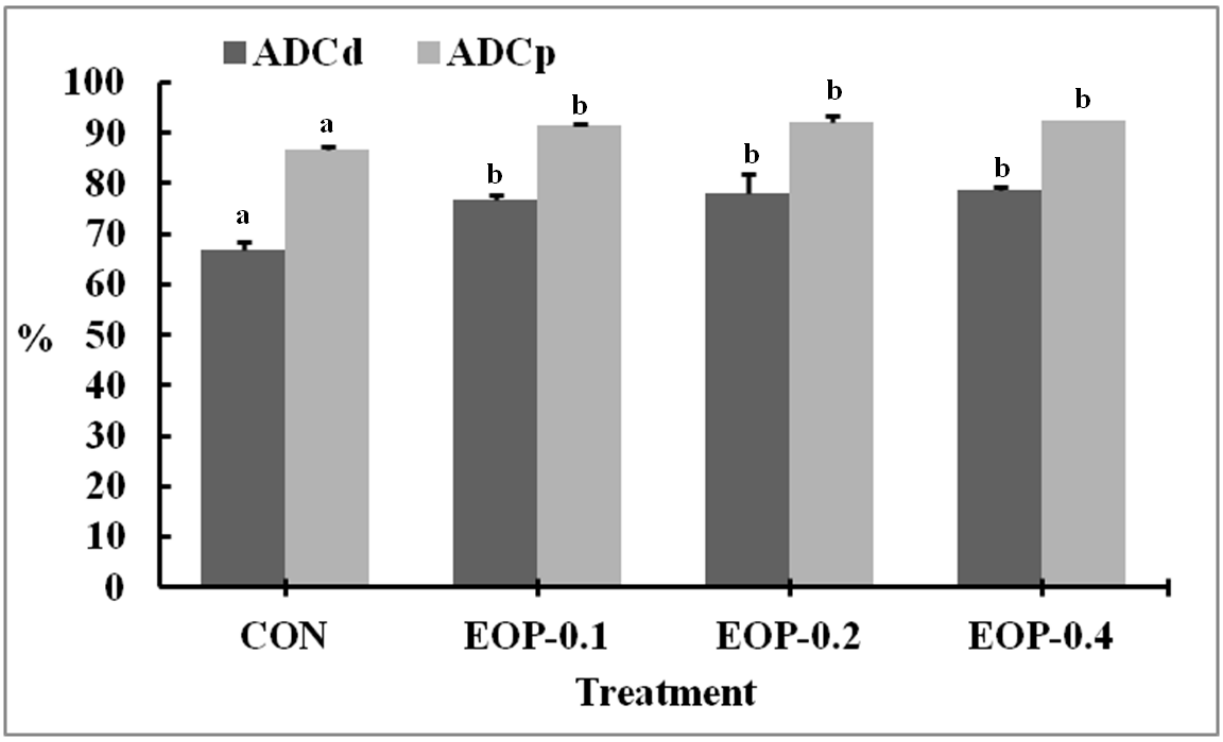

Figure 9. Apparent digestibility coefficients of dry matter (ADCd) and apparent digestibility coefficients of protein (ADCp) of experimental diets containing four different EOP levels.

3.2 Experiment Ⅱ

Growth performance and feed utilization of olive flounder fed the four experimental diets were shown in Table 5. FCR of fish fed the control diet was significantly lower than that of fish fed the EOP-0.2 and no significant differences were resulted by EOP-0.1 and EOP-0.4 diet fed fish compared to the control group. PER of fish fed EOP-0.2 diet was significantly lower than that of fish fed the control diet and no significant differences were observed in EOP-0.1 and EOP-0.4 diet groups compared to the control diet. However, final body weight, weight gain, feed intake and specific growth rate were not significantly affected by dietary EOP supplementation.

Hemoglobin of all the EOP fed fish was significantly higher than that of fish fed the control diet. Hematocrit of fish fed EOP-0.1 diet was significantly higher than that of fish fed the control (Table 6). Also, EOP-0.1 diet group showed significantly higher total cholesterol amount compared to EOP-0.2 diet group. However, total protein, glucose, ALT and AST activies of fish fed experimental diets were not significantly different compared to those of fish fed the control diet.

Non-specific immune response parameters of olive flounder fed the four experimental diets were shown in Figure 10. Anti-protease activity of EOP-0.2 and EOP-0.4 diet groups and MPO activity of fish fed EOP-0.4 diet were significantly higher than those of fish fed the control diet. However, Ig level and lysozyme activity of olive flounder were not significantly affected by the dietary EOP supplementation. Survival rate of olive flounder fed the four experimental diets after challenge with *Edwardsiella tarda* was shown in Figure 11. EOP-0.4 diet fed fish resulted in the highest survival rate after 11 days of challenge and the survival rates of EOP-0.1 and EOP-0.2 diet fed fish were lower than that of fish fed the control diet while EOP-0.1 diet fed group resulted in the lowest survival than the other fish groups.

Table 5. Growth performance and feed utilization of olive flounder fed the four experimental diets for 7 weeks.

	Diets			
	CON	**EOP-0.1**	**EOP-0.2**	**EOP-0.4**
IBW[1]	8.91±0.00	8.92±0.01	8.93±0.02	8.92±0.00
FBW[2]	33.4±1.1	32.9±3.8	32.3±2.5	35.1±0.6
WG(%)[3]	274±12	269±43	261±29	293±7
FI[4]	30.6±0.5	31.9±1.6	31.5±0.4	32.1±0.1
FCR[5]	1.26±0.06	1.37±0.20	1.37±0.09	1.23±0.06
SGR(%)[6]	2.69±0.06	2.66±0.24	2.62±0.16	2.79±0.04
PER[7]	1.59±0.08^{b}	1.42±0.20^{ab}	1.33±0.08^{a}	1.48±0.07^{ab}
Survival	98.7±2.31	94.7±2.31	97.3±2.31	98.7±2.31

Mean values of triplicate groups are presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P< 0.05$).

[1]IBW (g) = Initial body weight

[2]FBW (g) = Final body weight

[3]Weight gain (%) = (final body weight – initial body weight) / initial body weight x 100

[4] Feed intake (g/fish) = dry feed consumed (g)/fish

[5]Feed conversion ratio = dry feed fed / wet weight gain

[6] Specific growth rate (%) = 100 x [ln(final body weight) – ln(initial body weight)] / days

[7] Protein efficiency ratio = wet weight gain / total protein gain

Table 6. Hematological and biochemical characteristics of olive flounder fed the four experimental diets for 7 weeks.

	Diets			
	CON	**EOP-0.1**	**EOP-0.2**	**EOP-0.4**
Hemoglobin	5.44±0.39^{a}	6.46±0.36^{b}	6.68±0.66^{b}	6.52±0.29^{b}
Hematocrit	21.0±1.3^{a}	25.2±1.8^{b}	23.3±1.7^{ab}	23.7±0.8^{ab}
Total protein	3.49±0.05	3.74±0.77	3.43±0.14	3.89±0.73
Total cholesterol	264±50^{ab}	304±15^{b}	233±40^{a}	288±18^{ab}
Glucose	9.25±4.87	13.6±2.2	17.2±6.1	12.1±1.8
ALT	13.7±1.3	14.7±2.3	20.5±10.5	15.4±0.4
AST	16.0±3.5	14.7±2.3	17.2±8.5	12.0±2.7

Mean values of triplicate groups are presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P< 0.05$).

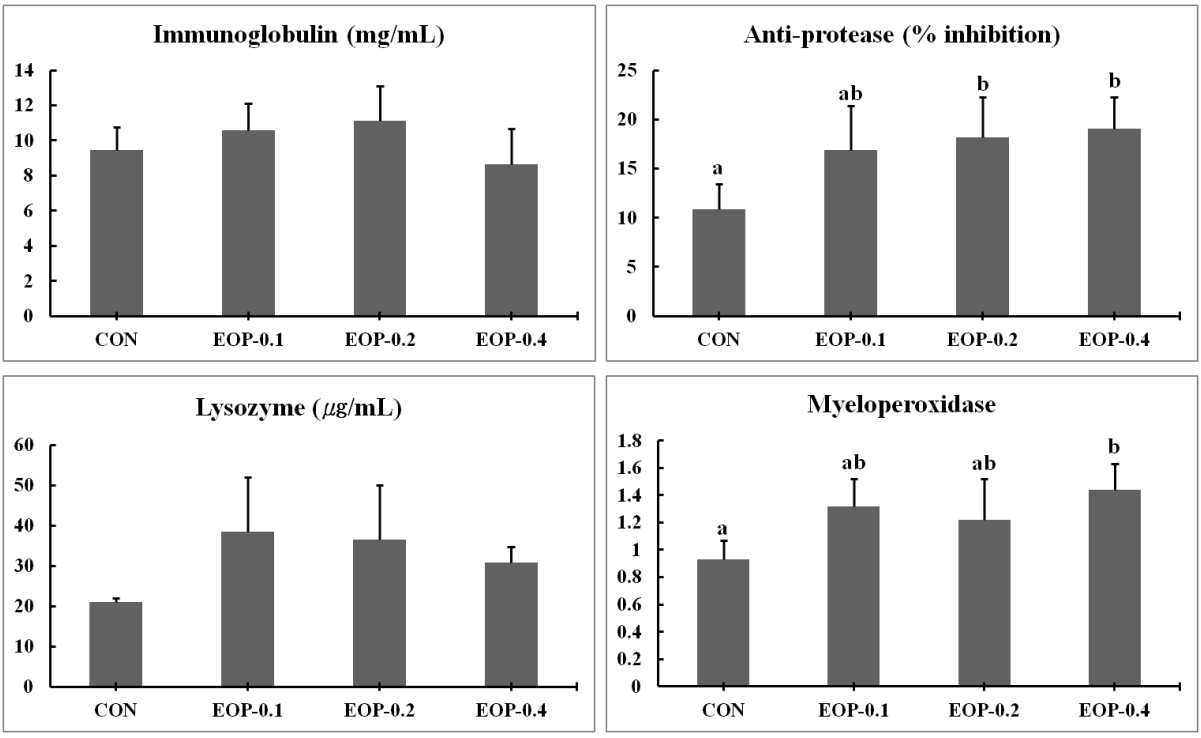

Figure 10. Non-specific immune response parameters of olive flounder fed the four experimental diets containing four different EOP levels for 7 weeks.

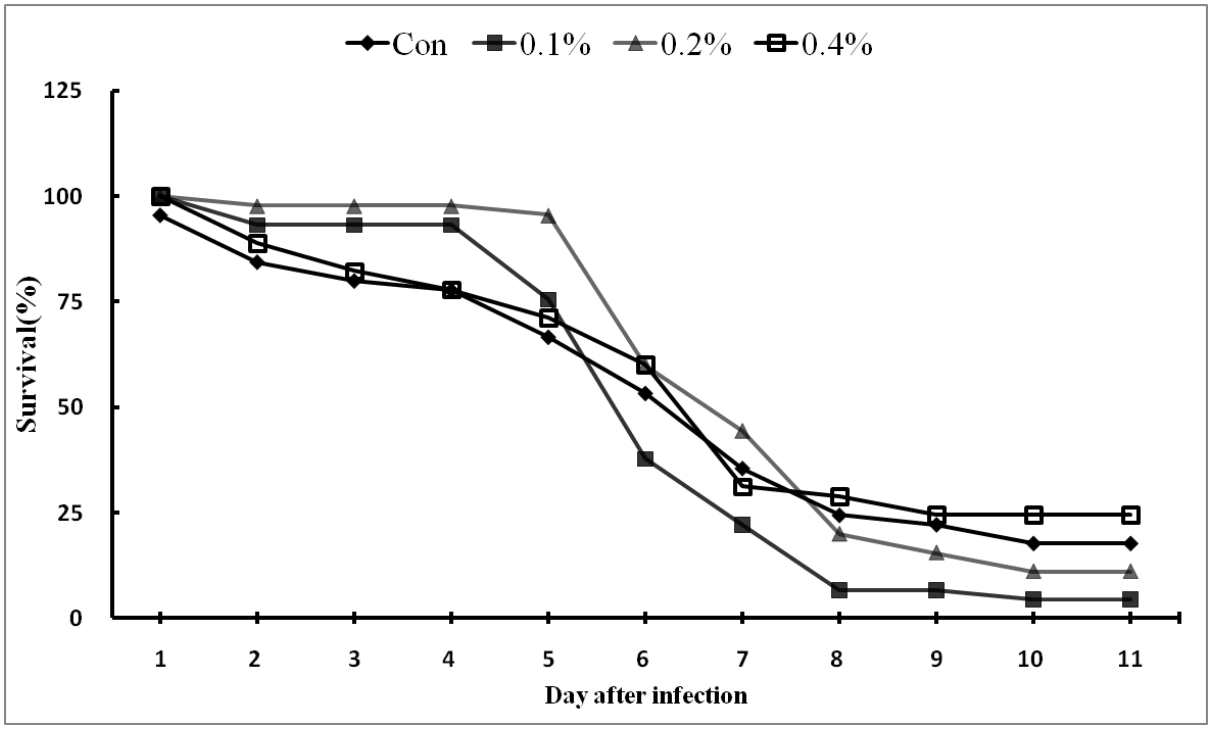

Figure 11. Survival rate of olive flounder fed the four experimental diets after challenge with *Edwardsiella tarda*.

Ⅳ. DISCUSSION

This study was conducted to evaluate the effects of dietary supplementation of mixture of essential oils and prebiotics (EOP) on growth performance, feed utilization, innate immunity, digestibility and disease resistance of juvenile olive flounder. Result of the present study showed that dietary supplementation of EOP exerts a positive effect on innate immunity and digestibility in juvenile olive flounder. In the present study, the first feeding trial showed that feed utilizations of olive flounder was affected by dietary EOP levels. However, growth performance was not significantly affected by the dietary EOP supplementation, even though there was an increasing tendency. Similar results were observed when prebiotic were supplemented to hybrid striped bass (*Morone chrysops* × *M. saxatilis*) diets (Li and Gatline).

The EOP consists of essential oils and prebioitcs. Prebiotics have been reported to enhance fish performance by favoring the predominance of beneficial gut microflora to improve nutrient assimilation, feed conversion, growth and immunity (Vázquez et al., 2006; Grisdale-Helland et al., 2008; Ringø et al., 2010). The beneficial bacteria in the gastrointestinal tract can produce excess amount of exoenzymes that can increase nutrient digestibility and promote better health conditions (Vine et al., 2006; Yanbo and Zirong, 2006).

Result of the present study revealed that EOP can increase the digestibility of protein in a fish meal based diet by olive flounder. The ADC for protein and dry matter in the EOP-supplemented diets were significantly higher compared to those of fish fed the control diet. The protein ADC values for the control diet in the present study were also similar to those reported for other various species (Glencross et al., 2005; Burr et al., 2008; Ø verland et al., 2009; Lee et al., 2010; Bui et al., 2014). Whole-body composition of fish was not significantly influenced by the supplementation of EOP. Similarly, Zeppenfeld et al., (2014)

found that the muscle protein content of the silver catfish was not affected by addition of essential oil to the water for 6h.

In Exp I, hemoglobin count was significantly increased by EOP supplementation and significantly higher hematocrit was shown by EOP-0.1 fed group. Hematological and biochemical variables are the most significant physiological indicators the of health status of fish health (Blaxhall and Daisley, 1973; Campbell. 2004; Basusta, 2005). Similar results were obtained by supplementing essential oils to other fish species by Acar et al in 2015.

In Exp I, plasma immunoglobulin level was increased significantly by EOP supplementation. The Ig can elevate innate immunity in fish as explained by Ross et al. (1998) that soluble forms of immunoglobulin play a role as an immune effectors.

Present results indicated that dietary EOP can enhance anti-protease activity in olive flounder. It has been demonstrated that anti-protease activity can be increased by dietary supplementation of essential oils. Rainbow trout (*Oncorhycus mykiss*) showed significant improvement in anti-protease activity following supplementation with black cumin oil (*Nigella sativa*) (Awad et al., 2013). Since EOP contains essential oils, it might have a possible effect on fish immunity elevation.

MPO is contained in the poly-morphonuclear neutrophils, monocytes, and macrophages (Klebanoff, 1992) and involved in microbicidal activity which influences fish neutrophil ability to kill microorganisms (Siwicki and Anderson, 1993). Therefore, myeloperoxidase inhibition might result in an anti-inflammatory effect (De Mello et al., 2012). In the present study myeloperoxidase activity was increased significantly by EOP. In similar studies conducted with essential oil and plant extract supplementations, elevated MPO activities has been found in rainbow trout fed with black cumin oil supplemented diets for 14 days (Awad et al., 2013). Also, higher MPO activity was shown in *O. mossambicus* fed diets supplemented with herbal extracts (Alexander et al., 2010; Wu et al., 2013).

In the current study juvenile olive flounder fed the EOP-0.1 and EOP-0.2 diet showed dramatically higher lysozyme activity compared to that of the control group. Lysozyme has bactericidal activity and can act as an opsonin to activate phagocytosis as explained by Jollès and Jollès (1984). Lysozyme activities of channel catfish has been enhanced when the fish fed with Orego-Stim® (commercial product containing natural essential oil from *Origanum heracleoticum* L.) (Zheng et al., 2009). Similarly, black cumin oil supplementation to rainbow trout had showed an increase in lysozyme activity (Awad et al., 2013). Elevated lysozyme activity has been reported in *O. mossambicus* after supplementing the diets with *Tinospora cordifolia* and *Solanum trilobatum* extracts (Sudhakaran et al., 2006; Divyagnaneswari et al., 2007).

Regarding the bacterial challenge with *Edwardsiella tarda,* 0.4% treatment showed higher survival rate compared to the control group. *E. tarda* causes a severe pre-harvest economic loss in olive flounder industry in Korea. And also, bacterial disease was one of the biggest concerns to be overcome in the world aquaculture. Therefore, many studies have been conducted around the world to enhance immunity and disease resistance of cultured fish with aromatic plant extracts, essential oils and prebiotics. In previous studies, some kinds of essential oils have been confirmed for having antibacterial potential (McKay and Blumberg, 2006; Bakkali et al., 2008; Oliveira et al., 2009). Also, prebiotics, such as lactose has been reported to promote lactic acid-producing bacteria and prevent the expansion of potential pathogens in certain human diseases (Szilagyi, 2002). Similarly, improvement of bacterial disease resistance with the aid of prebiotics has been reported in tilapia (Acar et al., 2015). In conclusion, results of this study reveals that feed utilization, protein digestibility, hematological parameters, non-specific immune responses and disease resistance of olive flounder can be enhanced by dietary supplementation of EOP.

Ⅴ. SUMMARY

This study was conducted to evaluate the effects of dietary supplementation of mixture of essential oils and prebiotics (EOP) on growth performance, feed utilization, innate immunity, digestibility and disease resistance of juvenile olive flounder.

In Exp-I, quadruplicate groups of juvenile olive flounders were fed four experimental diets containing EOP at levels of 0, 0.1, 0.2 and 0.4% for 10 weeks. Weight gain was numerically increased with the EOP supplementation while significantly lower FCR and higher PER were detected in fish fed 0.2% EOP. Plasma triglycerides, immunoglobulin, anti-protease activity and myleoperoxidase activity were significantly elevated in 0.1% EOP fed group.

In EXP-II, another set of juvenile olive flounders were fed the same experimental diets for 7 weeks. Growth was not affected. But, hemoglobin, hematocrit and plasma cholesterol levels were significantly increased by 0.1% supplementation and also anti-protease activity and myleoperoxidase activity were significantly elevated with the EOP supplementations. Both dry matter and protein digestibility were increased significantly by 0.1% EOP. EOP fed fish showed high disease resistance compared to the control group at the end of the challenge test.

Therefore, this study indicates that dietary supplementation of EOP can enhance protein efficiency, innate immunity and disease resistance of juvenile olive flounder and the optimum supplementation level would be approximately 0.1-0.2%.

Ⅵ. REFERENCE

Acar, Ü., Kesbiç, O. S., Yılmaz, S., Gültepe, N., & Türker, A. (2015). Evaluation of the effects of essential oil extracted from sweet orange peel (*Citrus sinensis*) on growth rate of tilapia (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus iniae*. Aquaculture, 437, 282-286.

Alcaide, E., Blasco, M. D., & Esteve, C. (2005). Occurrence of drug-resistant bacteria in two European eel farms. Applied and environmental microbiology, 71(6), 3348-3350.

Alexander, C. P., Kirubakaran, C. J. W., & Michael, R. D. (2010). Water soluble fraction of *Tinospora cordifolia* leaves enhanced the non-specific immune mechanisms and disease resistance in *Oreochromis mossambicus*. Fish & shellfish immunology, 29(5), 765-772.

AOAC (Association of Official Analytical Chemists), 1995. Official Methods of Analysis. 16thedn. Association of Official Analytical Chemists, Arlington, Virginia.

Awad, E., Austin, D., & Lyndon, A. R. (2013). Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture, 388, 193-197.

Bai, S.C., Kim, K.W., 2001. Present status and future prospects of aquaculture in Korea. World Aquaculture. 32, 28–32.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils–a review. Food and chemical toxicology, 46(2), 446-475.

Başusta, G. A. (2005). Fish hematology and hematological techniques. Research Techniques in Fish Biology (in Turkish), Nobel Publications, Ankara, 275-300.

Birkbecka, T. H., & Ringø, E. (2005). 10 Pathogenesis and the gastrointestinal tract of growing fish. *Microbial ecology in growing animals*, *2*, 208.

Blaxhall, P. C., & Daisley, K. W. (1973). Routine haematological methods for use with fish blood. Journal of Fish Biology, 5(6), 771-781.

Bui, H. T. D., Khosravi, S., Fournier, V., Herault, M., & Lee, K. J. (2014). Growth performance, feed utilization, innate immunity, digestibility and disease resistance of juvenile red seabream (*Pagrus major*) fed diets supplemented with protein hydrolysates. Aquaculture, 418, 11-16.

Burr, G., Hume, M., Neill, W. H., & Gatlin III, D. M. (2008). Effects of prebiotics on nutrient digestibility of a soybean-meal-based diet by red drum *Sciaenops ocellatus* (Linnaeus). Aquaculture Research, 39(15), 1680-1686.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology, 94(3), 223-253.

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing

problem for human and animal health and for the environment. Environmental microbiology, 8(7), 1137-1144.

Campbell, A.C., Buswell, J.A., (1983). The intestinal microflora of farmed Dover sole (*Solea solea*) at different stages of fish development. J. Appl. Bacteriol. 55, 215-223.

Campbell, T. W. (2004, November). Hematology of lower vertebrates. In Proceedings of the 55th Annual Meeting of the American College of Veterinary Pathologists & 39th Annual Meeting of the American Society of Clinical Pathology (p. 1104).

Casula, G., & Cutting, S. M. (2002). Bacillus probiotics: spore germination in the gastrointestinal tract. Applied and environmental microbiology, 68(5), 2344-2352.

Cha, J. H., Rahimnejad, S., Yang, S. Y., Kim, K. W., & Lee, K. J. (2013). Evaluations of Bacillus spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against Streptococcus iniae and as water additives. Aquaculture, 402, 50-57.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., ... & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. Journal of Environmental Quality, 38(3), 1086-1108.

Cho, C. Y., Slinger, S. J., & Bayley, H. S. (1982). Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology Part B:

Comparative Biochemistry, 73(1), 25-41.

Dambolena, J. S., López, A. G., Cánepa, M. C., Theumer, M. G., Zygadlo, J. A., & Rubinstein, H. R. (2008). Inhibitory effect of cyclic terpenes (limonene, menthol, menthone and thymol) on *Fusarium verticillioides* MRC 826 growth and fumonisin B1 biosynthesis. Toxicon, 51(1), 37-44.

De Kivit, S., Tobin, M. C., Forsyth, C. B., Keshavarzian, A., & Landay, A. L. (2014). Regulation of intestinal immune responses through TLR activation: implications for pro- and prebiotics. Frontiers in immunology, 5.

De Melo, G. A. N., Fonseca, J. P., Farinha, T. O., do Pinho, R. J., Damião, M. J., Grespan, R., ... & Cuman, R. K. N. (2012). Anti-inflammatory activity of *Salvia officinalis* L. Journal of Medicinal Plants Research, 6(35), 4934-4939.

Dimitroglou, A., Merrifield, D. L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., ... & Davies, S. J. (2011). Microbial manipulations to improve fish health and production–a Mediterranean perspective. Fish & Shellfish Immunology, 30(1), 1-16.

Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R., & Davies, S. J. (2010). Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). Aquaculture, 300(1), 182-188.

Divakaran, S., Obaldo, L. G., & Forster, I. P. (2002). Note on the methods for determination

of chromic oxide in shrimp feeds. Journal of agricultural and food chemistry, 50(3), 464-467.

Divyagnaneswari, M., Christybapita, D., & Michael, R. D. (2007). Enhancement of nonspecific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. Fish & shellfish immunology, 23(2), 249-259.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of applied microbiology, 88(2), 308-316.

Dorman, H. J. D., Peltoketo, A., Hiltunen, R., & Tikkanen, M. J. (2003). Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected *Lamiaceae* herbs. Food chemistry, 83(2), 255-262.

EL-DAKAR, A. Y., Shalaby, S. M., & Saoud, I. P. (2007). Assessing the use of a dietary probiotic/prebiotic as an enhancer of spinefoot rabbitfish Siganus rivulatus survival and growth. Aquaculture Nutrition, 13(6), 407-412.

Ellis, A. E. (1990). Serum antiproteases in fish. Techniques in fish immunology, 1, 95-99.

Escobar, P., Milena Leal, S., Herrera, L. V., Martinez, J. R., & Stashenko, E. (2010). Chemical composition and antiprotozoal activities of Colombian *Lippia* spp essential oils and their major components. Memórias do Instituto Oswaldo Cruz, 105(2), 184-190.

Fabri, R. L., Nogueira, M. S., Moreira, J. D. R., Bouzada, M. L. M., & Scio, E. (2011).

Identification of antioxidant and antimicrobial compounds of *Lippia* species by bioautography. Journal of medicinal food, 14(7-8), 840-846.

Folch, J., Lee, M., and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Food and Agriculture Organization of the United Nations (FAO). 2001. Health and Nutritional Properties of Probiotics in Food including Power Milk with Live Lactic Acid Bacteria, http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf

Franz, C., Baser, K. H. C., & Windisch, W. (2010). Essential oils and aromatic plants in animal feeding–a European perspective. A review. Flavour and Fragrance journal, 25(5), 327-340.

Gatesoupe, F. J. (2010). Probiotics and other microbial manipulations in fish feeds: prospective health benefits. Bioactive Foods in Promoting Health. Probiotics and Prebiotics, 541-552.

Glencross, B., Evans, D., Dods, K., McCafferty, P., Hawkins, W., Maas, R., & Sipsas, S. (2005). Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss*, using either stripping or settlement faecal collection methods. Aquaculture, 245(1), 211-220.

Grisdale-Helland, B., Helland, S. J., & Gatlin, D. M. (2008). The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or

galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmo salar*). Aquaculture, 283(1), 163-167.

Groff, J. M., & Lapatra, S. E. (2000). Infectious diseases impacting the commercial culture of salmonids. Journal of Applied Aquaculture, 10(4), 17-90.

Haldar, S., Kar, B., Dolai, N., Kumar, R. S., Behera, B., & Haldar, P. K. (2012). In vivo anti–nociceptive and anti–inflammatory activities of *Lippia* alba. Asian Pacific Journal of Tropical Disease, 2, S667-S670.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of applied microbiology, 86(6), 985-990.

Harikrishnan, R., Balasundaram, C., Jawahar, S., & Heo, M. S. (2011). Solanum nigrum enhancement of the immune response and disease resistance of tiger shrimp, *Penaeus monodon* against *Vibrio harveyi*. Aquaculture, 318(1), 67-73.

Hoseinifar, S. H., Sharifian, M., Vesaghi, M. J., Khalili, M., & Esteban, M. Á. (2014). The effects of dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and growth performance of Caspian white fish (*Rutilus frisii* kutum) fry. Fish & shellfish immunology, 39(2), 231-236.

Huang, A. G., Yi, Y. L., Ling, F., Lu, L., Zhang, Q. Z., & Wang, G. X. (2013). Screening of plant extracts for anthelmintic activity against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*). Parasitology research, 112(12), 4065-4072.

Hultmark, D. 1980. Insert immunity: purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur. J. Biochem. 106, 7-16.

Hyronimus, B., Le Marrec, C., & Urdaci, M. C. (1998). Coagulin, a bacteriocin-like-inhibitory substance produced by Bacillus coagulans I. Journal of applied microbiology, 85(1), 42-50.

Isman, M. B., & Machial, C. M. (2006). Pesticides based on plant essential oils: from traditional practice to commercialization. Advances in phytomedicine, 3, 29-44.

Jollès, P., & Jollès, J. (1984). What's new in lysozyme research?. Molecular and cellular biochemistry, 63(2), 165-189.

Kaushik, S. J. and C. B. Cowey., 1991. Dietary factors affecting nitrogen excretion by fish. In: Nutritional Strategies and Aquaculture Waste (Ed. C. B. Cowey and C. Y. Cho). University of Guelph, Canada, pp. 3-19.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., & Gibson, L. (2008). Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture, 274(1), 1-14.

Klebanoff, S. J. (1992). Oxygen metabolites from phagocytes. Inflammation: basic principles and clinical correlates, 541.

Lee, K. J., Powell, M. S., Barrows, F. T., Smiley, S., Bechtel, P., & Hardy, R. W. (2010). Evaluation of supplemental fish bone meal made from Alaska seafood processing byproducts and dicalcium phosphate in plant protein based diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 302(3), 248-255.

Lee, S. J., Umano, K., Shibamoto, T., & Lee, K. G. (2005). Identification of volatile components in basil (*Ocimum basilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. Food Chemistry, 91(1), 131-137.

Li, P., & Gatlin, D. M. (2004). Dietary brewers yeast and the prebiotic Grobiotic™ AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops*× *M. saxatilis*) to Streptococcus iniae infection. Aquaculture, 231(1), 445-456.

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in microbiology, 5.

Lundén, T., Lilius, E. M., & Bylund, G. (2002). Respiratory burst activity of rainbow trout (*Oncorhynchus mykiss*) phagocytes is modulated by antimicrobial drugs. Aquaculture, 207(3), 203-212.

Magnadóttir, B., Jónsdóttir, H., Helgason, S., Björnsson, B., Jørgensen, T. Ø ., & Pilström, L. (1999). Humoral immune parameters in Atlantic cod (*Gadus morhua* L.): I. The effects

of environmental temperature. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 122(2), 173-180.

McKay, D. L., & Blumberg, J. B. (2006). A review of the bioactivity and potential health benefits of peppermint tea (*Mentha piperita* L.). Phytotherapy Research, 20(8), 619-633.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T., Bøgwald, J., ... & Ringø, E. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture, 302(1), 1-18.

Moreira, M. R., Ponce, A. G., Del Valle, C. E., & Roura, S. I. (2005). Inhibitory parameters of essential oils to reduce a foodborne pathogen. LWT-Food Science and Technology, 38(5), 565-570.

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. Fish & Shellfish Immunology, 29(1), 2-14.

Nayak, S. K., Swain, P., & Mukherjee, S. C. (2007). Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). Fish & shellfish immunology, 23(4), 892-896.

Nogueira, M. A., Diaz, G., & Sakumo, L. (2009). Caracterização química e atividade biológica do óleo essencial de *Lippia* alba cultivada no Paraná. Revista de Ciências Farmacêuticas Básica e Aplicada, 28(3), 273-278.

Oliveira, D. R., Leitao, G. G., Santos, S. S., Bizzo, H. R., Lopes, D., Alviano, C. S., ... & Leitao, S. G. (2006). Ethnopharmacological study of two *Lippia* species from Oriximiná, Brazil. Journal of Ethnopharmacology, 108(1), 103-108.

Oliveira, V. C., Moura, D. M., Lopes, J. A., de Andrade, P. P., da Silva, N. H., & Figueiredo, R. C. (2009). Effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham., and *Ocimum gratissimum* L. on growth and ultrastructure of *Leishmania chagasi* promastigotes. Parasitology research, 104(5), 1053-1059.

Oussalah, M., Caillet, S., Saucier, L., & Lacroix, M. (2006). Antimicrobial effects of selected plant essential oils on the growth of a *Pseudomonas putida* strain isolated from meat. Meat science, 73(2), 236-244.

Ø verland, M., Sørensen, M., Storebakken, T., Penn, M., Krogdahl, Å ., & Skrede, A. (2009). Pea protein concentrate substituting fish meal or soybean meal in diets for Atlantic salmon (*Salmo salar*)—effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality. Aquaculture, 288(3), 305-311.

Petersen, A., Andersen, J. S., Kaewmak, T., Somsiri, T., & Dalsgaard, A. (2002). Impact of integrated fish farming on antimicrobial resistance in a pond environment. Applied and Environmental Microbiology, 68(12), 6036-6042.

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology, 58(3), 239-248.

Rawles, S. D., Kocabas, A., Gatlin, D. M., Du, W. X., & Wei, C. I. (1997). Dietary Supplementation of Terramycin and Romet-30 Does Not Enhance Growth of Channel Catfish But Does Influence Tissue Residues. Journal of the World Aquaculture Society, 28(4), 392-401.

Ringø, E., Myklebust, R., Mayhew, T. M., & Olsen, R. E. (2007). Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. Aquaculture, 268(1), 251-264.

Ringø, E., Olsen, R. E., Gifstad, T. Ø., Dalmo, R. A., Amlund, H., HEMRE, G. I., & Bakke, A. M. (2010). Prebiotics in aquaculture: a review. Aquaculture Nutrition, 16(2), 117-136.

Ross, D. A., Wilson, M. R., Miller, N. W., Clem, L. W., Warr, G. W., Miller, N. W., & Warr, G. W. (1998). Evolutionary variation of immunoglobulin μ heavy chain RNA processing pathways: origins, effects, and implications. Immunological reviews, 166(1), 143-151.

Sáenz de Rodrigáñez, M. A., DÍAZ-ROSALES, P., Chabrillón, M., Smidt, H., Arijo, S., LEÓN-RUBIO, J. M., ... & Moyano, F. J. (2009). Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858). Aquaculture Nutrition, 15(2), 177-185.

SHARIF, R. M., Haghighi, M., & Assaeian, H. (2011). THE LETHAL CONCENTRATION (LCSO) OF ZATARIA MULTIFLORA ESSENTIAL OIL IN FRIES OF RAINBOW TROUT (ONCORHYNCHUS MYKISS).

Sharma, N., & Tripathi, A. (2006). Fungitoxicity of the essential oil of Citrus sinensis on post-harvest pathogens. World Journal of Microbiology and Biotechnology, 22(6), 587-593.

Siwicki, A. K., & Anderson, D. P. (1993). An easy spectrophotometric assay for determining total protein and immunoglobulin levels in fish sera: correlation to fish health. Tech Fish Immunol, 3, 23-30.

Soltani, M. A. H. D. I., Esfandiary, M., Khazraeenia, S., & Sajadi, M. M. (2009). Effects of *Zataria multiflora* essential oil on rainbow trout (*Oncorhynchus mykiss*) egg hatchability and survival of larvae compared with hydrogen peroxide and malachite green. Journal of Veterinary Research, 64(2), Pe127-Pe134.

Soltani, M., Zarifmanesh, T., & Zorriehzahra, S. J. (2013). Effect of *Zataria multiflora* essential oils on rainbow trout (*Oncorhynchus mykis*) complement component activity and lysozyme. ISFJ, 21(4), 13-23.

Steverding, D., Morgan, E., Tkaczynski, P., Walder, F., & Tinsley, R. (2005). Effect of Australian tea tree oil on Gyrodactylus spp. infection of the three-spined stickleback *Gasterosteus aculeatus*. Diseases of aquatic organisms, 66(1), 29.

Stickney, R.R., Shumway, S.E., (1974). Occurrence of cellulase activity in the stomachs of fishes. J Fish Biol. 6, 779-790.

Sudhakaran, D. S., Srirekha, P., Devasree, L. D., Premsingh, S., & Michael, R. D. (2006).

Immunostimulatory effect of *Tinospora cordifolia* Miers leaf extract in *Oreochromis mossambicus*. Indian journal of experimental biology, 44(9), 726.

Sun, Y. Z., Yang, H. L., Ma, R. L., & Lin, W. Y. (2010). Probiotic applications of two dominant gut Bacillus strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. Fish & shellfish immunology, 29(5), 803-809.

Szilagyi, A. (2002). Lactose—a potential prebiotic. Alimentary pharmacology & therapeutics, 16(9), 1591-1602.

Teitelbaum, J. E., & Walker, W. A. (2002). Nutritional Impact of Pre-and Probiotics as Protective Gastrointestinal Organisms*. Annual Review of Nutrition, *22*(1), 107-138.

Tseng, D. Y., Ho, P. L., Huang, S. Y., Cheng, S. C., Shiu, Y. L., Chiu, C. S., & Liu, C. H. (2009). Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. Fish & Shellfish Immunology, 26(2), 339-344.

Vázquez, M. J., Alonso, J. L., Domínguez, H., & Parajó, J. C. (2006). Enhancing the potential of oligosaccharides from corncob autohydrolysis as prebiotic food ingredients. Industrial Crops and Products, 24(2), 152-159.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. Microbiology and molecular biology reviews,

64(4), 655-671.

Vine, N. G., Leukes, W. D., & Kaiser, H. (2006). Probiotics in marine larviculture. FEMS microbiology reviews, 30(3), 404-427.

Wang, Y. B., Li, J. R., & Lin, J. (2008). Probiotics in aquaculture: challenges and outlook. Aquaculture, 281(1), 1-4.

Wu, Y. R., Gong, Q. F., Fang, H., Liang, W. W., Chen, M., & He, R. J. (2013). Effect of *Sophora flavescens* on non-specific immune response of tilapia (GIFT *Oreochromis niloticus*) and disease resistance against *Streptococcus agalactiae*. Fish & shellfish immunology, 34(1), 220-227.

Yanbo, W., & Zirong, X. (2006). Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. Animal feed science and technology, 127(3), 283-292.

Zeppenfeld, C. C., Toni, C., Becker, A. G., dos Santos Miron, D., Parodi, T. V., Heinzmann, B. M., ... & da Cunha, M. A. (2014). Physiological and biochemical responses of silver catfish, Rhamdia quelen, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton. Aquaculture, 418, 101-107.

Zheng, Z. L., Tan, J. Y., Liu, H. Y., Zhou, X. H., Xiang, X., & Wang, K. Y. (2009). Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*).

Aquaculture, 292(3), 214-218.

VII. ACKNOWLEDGEMENTS

어느새 2 년이란 시간이 훌쩍 지나갔습니다. 짧으면 짧다고, 길면 길다고 할 수도 있는 시간 동안 저를 이끌어주시고 격려해준 많은 분들이 계십니다. 우선 늘 저의 부족한 점을 고칠 수 있게끔 지도해주시고 격려해주시어 이 자리 까지 올 수 있게 해주신 이경준 지도교수님께 고개 숙여 깊은 감사를 드립니다. 무엇보다 교수님의 열정과 리더쉽, 학자로서의 모습 그리고 제자들을 사랑하는 마음을 항상 기억하도록 하겠습니다. 수업 뿐만 아니라 평소에도 관심을 주시고 많은 가르침을 주신 송춘복 교수님, 최광식 교수님, 이제희 교수님, 허문수 교수님, 여인규 교수님, 전유진 교수님, 김기영 교수님, 정석근 교수님, 박상율 교수님에게도 깊은 감사의 말씀을 드립니다.

석사기간 동안 부족한 저를 도와 무사히 연구를 끝낼 수 있게 도와준 초롱이, 항상 억척스럽게 일을 잘 해결해나가는 유정이, 항상 누구보다 열심히 도와주고 고생했을 문호와 재형이, 힘든 타지 생활을 하면서도 자신보다 남을 더 생각해 준 사나즈 누나, 바쁜 와중에 논문을 다듬어준 부디와 마린트에게 진심으로 감사의 말씀 드립니다. 또한 새우양식을 하러 떠난 창형이와 혜지, 지금은 군복무 중인 재민이, 그리고 다른 실험실원 분들에게도 이 글을 빌어 진심으로 감사의 마음을 전합니다.

정말로 많은 분들의 도움을 통해 제가 이 자리에 올 수 있었던 것 같습니다. 소리 없이 저를 응원해 준 친구들에게도 감사의 말씀을 드리며 특히 제가 힘들 때 한 호흡 쉬어 갈 수 있게끔 해준 재윤이와 옆에서 힘을 실어 준 현지 에게도 감사의 마음을 전합니다.

끝으로 저를 앞에서 끌어주시고 뒤에서 받쳐주시며 제가 가는 길을 지지해주신 존경하고 사랑하는 아버지, 어머니와 누나들과 매형들 그리고 귀여운 제 조카들 모두 감사의 인사를 드립니다.