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Effects of Dietary Supplementation of Two types of propolis on growth performance, feed utilization, innate immunity and disease resistance of olive flounder *Paralichthys olivaceus*

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Abstract

We investigated the effects of dietary supplementation of two types (powder or liquid) of propolis on innate immunity and disease resistance of olive flounder *Paralichthys olivaceus* against *Edwerdsiella tarda*. A total of 600 fish averaging 30 g were randomly distributed into 24 tanks in groups of 25. Three tanks were assigned to each of eight experimental diets: 0 (Control), 0.25, 0.5, 0.75 and 1 % propolis in powder form and 0.25, 0.5 and 1 % propolis in liquid form (PP0.25, PP0.5, PP0.75, PP1, LP0.25, LP0.5 and LP1, respectively). Fish were fed each experimental diet twice daily for four weeks to apparent satiation. At the end of the feeding trial, lysozyme and myeloperoxidase activities and total immunoglobulin level were significantly higher in fish fed the PP1 and LP0.5 diets compared to those fed the control diet. The PP1 diet was also associated with a significant increase in anti-protease activity compared to the control diet. After challenge with *E. tarda*, fish fed the LP0.5 diet showed numerically higher survival compared to the other groups. This study indicates that non-specific immune responses of olive flounder can be enhanced by dietary supplementation with powder and liquid forms of propolis, and that the optimal level would be 1% in powder form or 0.5% in liquid form. It seemed that growth performance and feed utilization are not affected by the propolis supplementation in diets for olive flounder.

Key words: *Paralichthys olivaceus*, Propolis, Innate immunity, Olive flounder, *Edwerdsiella tarda*

Introduction

Propolis is a resinous material produced by bees using exudates of plants which are mixed with products of their salivary gland and wax. Khalil et al. (2006) reported that more than 300 constituents exist in different types of propolis. Flavonoids, phenolids and various aromatic compounds can be found as main chemical classes among the chemical composition of propolis (Kolankaya et al., 2002). Propolis composition varies depending on the source, but it contains a number of B complex vitamins as well as major and trace minerals (Burdock 1998).

Several studies have previously reported immunostimulatory and anti-inflammatory effects of propolis in mammals (Zhang et al., 2009; Talas and Gulhan et al., 2009). In fishes, propolis has been reported to have an important effect on aquatic environment (Christybapita et al., 2007) and to enhance non-specific immune responses and disease resistance of Nile tilapia through dietary supplementation (Abd-el-Rhaman et al., 2009). Physiological functions of fishes may be boosted by propolis and there may also be health benefits for fish consumers (Talas and Gulhan et al., 2009). Propolis

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is sold mainly in solid or liquid form, produced as water or ethanol extracts (Burdock, 1998).

South Korea is the top global producer of olive flounder; its production exceeds 60% of the annual production of cultured fish (Bai and Kim, 2001; FAO, 2014). However, diseases present a major challenge for olive flounder aquaculture and there is a great deal of research interest in methods for improving the innate immunity of fish via feed manipulation. Immunostimulants have the potential to suppress disease via non-specific defence mechanisms of fishes and to increase aquaculture productivity by helping protect fish from pathogens (Siwicki et al., 1994).

In this study, we investigated the effects of dietary supplementation levels of either powder or liquid forms of propolis on growth, non-specific immune responses and disease resistance against *Edwardsiella tarda* in olive flounder.

Material and Methods

Experimental diets

Eight experimental diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (17.1 kJ/g). A fish meal based diet was formulated and regarded as a control and seven other experimental diets were prepared by dietary propolis supplementaion at levels of 0.25, 0.5, 0.75 and 1 % of powder (PP) form, and 0.25, 0.5 and 1 % of liquid (LP) form (designated as control, PP0.25, PP0.5, PP0.75, PP1, LP0.25, LP0.5 and LP1). All dry ingredients were thoroughly mixed with fish oil and 20-30 % double distilled water. The mixed dough was then extruded through a pellet machine. The pellets were subsequently dried in 25ºC and stored at –20ºC until use. The experimental diets contained approximately 46.2 ± 0.43% crude protein, 14.6 ± 0.33% crude lipid and 9.2 ± 0.11% ash. Propolis was added to the basal diet at the expense of cellulose.

Preparation of powder and liquid propolis

A pure propolis of 20 g was dissolved into 1000 mL of 95% ethanol and incubated in 60ºC for 3h. The solution was filtered through a 0.5 μm filter paper and then 95% purified propolis was extracted by drying, ground and mixed with 5% maltodextrin for the powder propolis. For the liquid propolis, after drying the alcohol in the extract process, water was added into the dried filtered-extract to be used as dietary supplement. The total flavonoids concentration of the final propolis in powder or liquid forms was analyzed to have 2.0% or higher.

Fish and feeding trial

After a 2-week acclimatization period, we randomly distributed 600 fish (initial mean body weight, 30 g) into 24 polyvinyl circular tanks at a density of 25 fish per tank. Each tank was supplied with filtered sea water and aerated to maintain sufficient levels of dissolved oxygen. Triplicate groups of fish were fed one of the experimental diets twice daily (09:00 h and 18:00 h) to apparent satiation for 4 weeks.

Sample collection and analyses

At the end of the feeding trial, all the fish from each tank were bulk weighted to obtain total biomass. Three fish were randomly selected from each tank and anesthetized with 2-Phenoxyethanol solution (200 ppm). We then collected blood samples from caudal veins with heparinized syringes to determine hematocrit and hemoglobin levels. Plasma was separated from whole blood samples by centrifugation at 5,000 *g* for 10 min and stored at –70ºC for determination of total immunoglobulin level (Ig). Another set of blood samples was taken from the caudal veins of three fish from each tank using non-heparinized syringes. The collected blood was then allowed to clot at room temperature for 30 min and the serum was separated by centrifugation at 5,000 *g* for 10 min and stored at –70ºC for subsequent non-specific immune response analyses including lysozyme, superoxide dismutase (SOD), anti-protease and myeloperoxidase (MPO).

Hematocrit was determined by microhematocrit technique described by Brown (1980) and hemoglobin was measured using an automated blood analyzer (SLIM, SEAC Inc., Florence, Italy). Serum lysozyme level was measured using a turbidometric assay (Hultmark, 1980) with slight modifications. Serum MPO activity was measured according to Quade and Roth (1997). SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Serum anti-protease activity was measured according to the method described by Ellis (1990) with slight modifications (Magnadóttir et al., 1999), and total Ig levels in plasma were determined according to the method described by Siwicki and Anderson (1994).

Challenge test

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly captured and injected intraperitoneally with *E. tarda* suspension containing 1×10^5 CFU mL^{-1} (Khosravi et al., 2015). *E. tarda* (ATCC 15947, Korea Collection for Type Cultures) served as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The bacterium, isolated from diseased olive flounder, was cultured in 10 mL BHI broth (Difco, Detroit, MI, USA) containing 1.5% NaCl and incubated with shaking for 24 h at 37°C. Bacterial growth was measured at an optical density of 700 nm followed by plate counting in BHI-NaCl. The isolated bacteria were identified using the API 20E

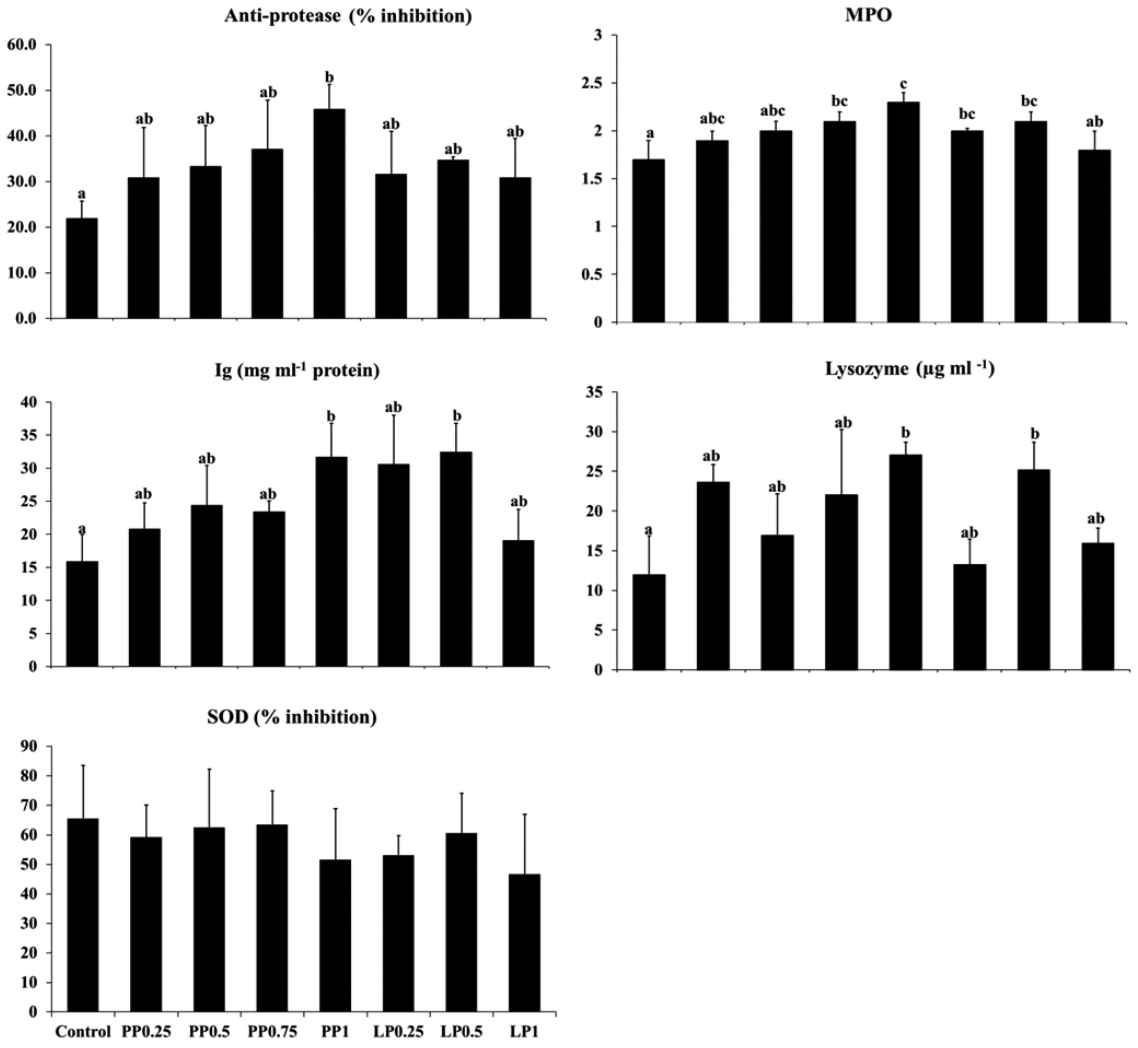

Fig. 1. Non-specific immune responses of olive flounder fed the eight experimental diets for 4 weeks. Different letters on the bars indicates significant difference ($P < 0.05$) between groups: Control, 0% propolis; PP0.25, 0.25 % propolis powder; PP0.5, 0.5% propolis powder; PP0.75, 0.75% propolis powder; PP1, 1% propolis powder; LP0.25, 0.25% propolis liquid; LP0.5, 0.5% propolis liquid; and LP1, 1% propolis liquid.

commercial identification kit (bioMérieux, Marcy l'Etoile, France). After injection, the fish were distributed into plastic tanks of 65 L capacity and their mortality was monitored and recorded for 6 days.

Statistical analysis

All treatments were assigned using a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in means were identified with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$ and data are presented as the mean ± standard deviation (SD).

Results

The growth performance and feed utilization of fish were not significantly affected by dietary treatments (Table 1). No significant differences were found in the hematological parameters of fish fed the experimental diets. However, numerically higher hematocrit and hemoglobin values were detected in fish fed the LP0.5 diet.

The non-specific immune responses of fish are provided in Fig. 1. Dietary supplementation of the propolis at the level of 0.75 and 1% in powder form and 0.25 and 0.5% in liquid form resulted in significantly higher MPO activity compared to the control group. Significantly higher lysozyme activity and Ig levels were found in fish fed the PP1 and LP0.5 diets than in fish fed the control diet. Anti-protease activity was sig-

nificantly higher in fish fed the PP1 diet compared to fish fed the control diet. However, SOD activity did not significantly differ among any of the treatments.

During the challenge test, the first dramatic mortality was observed on the third day after injection. While fish fed the LP0.5 diet showed slightly higher disease resistance than the control group, the difference was not significant.

Discussion

Dietary propolis supplementation has been shown to significantly enhance the growth of juvenile rainbow trout (Deng et al., 2011) and Nile tilapia (Abd-El-Rhman et al., 2009; Abbass et al., 2012). Furthermore, Bae et al. (2012) suggested that the required level of dietary crude propolis might be lowered to encourage optimal growth of juvenile eels (*Anguilla japonica*) than to induce the optimal immune response; they concluded that 0.25-0.5% dietary propolis was sufficient to optimize growth. Although the present study used propolis doses similar to those of Bae et al. (2012) and Abd-El-Rhman et al. (2009), we found no significant effect of propolis supplementation on growth performance or feed utilization efficiency among juvenile olive flounder. Similarly, Cuesta et al. (2005) found no significant effect of propolis at dosages of 0.1 g or 10 g propolis/kg^{-1} diet on the growth rate of gilthead sea bream. Despite the limited evidence available, it seems that the efficacy of propolis in enhancing fish growth rates and feed utilization efficiency may vary depending upon the type of propolis product and/or fish species. Therefore, further investigation is required to elucidate the precise effects of propolis products in different forms, as their content and composition may differ according to their source.

In the present study, hemoglobin and hematocrit values were not significantly affected by propolis supplementation. Conversely, dietary supplementation of propolis at 2% and 4% has been found to significantly decrease the hematocrit level of juvenile eels (Bae et al., 2012). Similarly, Yonar et al. (2012) found that oral intake of propolis significantly increased the leukocyte counts of juvenile common carp compared to those of fish without supplementation. Yonar et al. (2012) aimed to evaluate the effects of propolis on chlorpyrifos-induced changes in the hematological parameters of common carp; they suggested that propolis may help fish combat stress resulting from exposure to toxic materials such as chlorpyrifos.

MPO is an enzyme that produces hypochlorous acid from oxidative radicals to destroy bacteria and pathogens. It is released mainly from the azurophilic granules of neutrophils during oxidative respiratory bursts (Das et al., 2013). Unfortunately, there are no previous reports regarding the effects of propolis on MPO activity in olive flounder. However, in the present study, the MPO activity of olive flounder fed propolis (in either powder or liquid form) was significantly higher than that of the control group, and the largest effect was observed in the 1% propolis powder-fed group.

Serum lysozyme activity can be used to measure the innate immune response in fish, as it is involved in defense against microorganisms (Galindo et al., 2003). Lysozyme is a hydrolytic enzyme that enhances the immune response of animals, including fish, and restricts bacterial growth by attacking peptidoglycan in bacterial cell walls. Ig is a protein produced by plasma B-cells that has the ability to recognize and neutralize foreign objects (Solem and Stenvik, 2006). In the present study, significantly higher serum lysozyme activity and plasma Ig were observed in fish fed 1% propolis in powder form and 0.5% propolis in liquid form. In concordance with this result, Bae et al. (2012) found that 1% propolis supplementation improved the serum and mucus lysozyme level of *Anguila japonica*, but more than 1% supplementation decreased the lysozyme activity of eels. Furthermore, ethanolic extract and

Table 1. Growth performance of olive flounder *Paralichthys olivaceus* (Initial body weight, 30 g) fed the eight experimental diets for 4 weeks[1]

	FBW[2] (g)	WG[3] (%)	FI[4] (g/fish)	FCR[5]	SGR (%)[6]	Survival (%)
Control	52 ± 1.3	72 ± 4.9	21.3 ± 0.3	0.98 ± 0.06	2.25 ± 0.12	94.7 ± 4.6
PP0.25	52 ± 0.8	73 ± 3.6	22.6 ± 0.8	1.03 ± 0.07	2.27 ± 0.09	86.7 ± 4.6
PP0.5	53 ± 3.2	75 ± 8.5	26.8 ± 5.1	1.19 ± 0.15	2.33 ± 0.20	72.0 ± 21.2
PP0.75	53 ± 1.4	78 ± 4.6	26.7 ± 6.0	1.14 ± 0.19	2.39 ± 0.11	73.3 ± 22.7
PP1	51 ± 4.3	70 ± 14.6	25.5 ± 4.4	1.29 ± 0.53	2.19 ± 0.37	74.7 ± 16.7
LP0.25	50 ± 0.9	65 ± 1.2	26.0 ± 3.7	1.32 ± 0.20	2.10 ± 0.03	72.0 ± 17.4
LP0.5	53 ± 3.6	77 ± 12.9	21.6 ± 1.0	0.95 ± 0.18	2.38 ± 0.30	92.0 ± 4.0
LP1	51 ± 3.7	71 ± 11	21.0 ± 1.0	1.00 ± 0.15	2.22 ± 0.27	96.0 ± 6.9

[1]Values are presented as mean ± SD. Values having different superscript letters in the same colomn are significantly different ($P < 0.05$) between groups: Control, 0% propolis; PP0.25, 0.25 % propolis powder; PP0.5, 0.5% propolis powder; PP0.75, 0.75% propolis powder; PP1, 1% propolis powder; LP0.25, 0.25% propolis liquid; LP0.5, 0.5% propolis liquid; and LP1, 1% propolis liquid.
[2]FBW: final body weight
[3]Weight gain (%) = 100× (final mean body weight – initial mean body weight) / initial mean body weight
[4]Feed intake = dry feed consumed (g) / fish
[5]Feed conversion ratio = feed intake (g) / weight gain (g)
[6]SGR (%/day) = 100 × (ln final body weight (g) – ln initial body weight (g)) / experimental period (day)

crude propolis significantly increase the serum lysozyme activity of Nile tilapia (Abd-El-Rhman et al., 2009), and the lysozyme activity of Chinese sucker has also been increased by supplementation with a mixture of propolis and *Herba epimedii* extract (Zhang et al., 2009). Yonar et al. (2011) found that the plasma Ig level of rainbow trout significantly increased after oral administration of propolis. In other studies, propolis was found to stimulate Ig production in rats regardless of season and origin (Sforcin, 2005, 2007), and an ethanolic extract of propolis was shown to increase Ig production in mice (Scheller et al., 1988).

Anti-protease is an enzyme inhibitor found in serum that may play an important role in the defense against bacteria (Ellis, 2001). Propolis is able to inhibit the action of bacterial protease enzymes (Bulman et al., 2011). In the present study, we found significantly higher anti-protease activity in fish fed 1% propolis powder than the control group.

Yonar et al. (2011) found that SOD activity in plasma, liver and kidneys was not significantly increased by supplementation with ethanolic extract of propolis in rainbow trout. Similarly, in the present study, we observed no significant difference in serum SOD activity among treatment groups.

Bae et al. (2012) indicated that higher levels of propolis supplementation can decrease innate immunity in Japanese eels. Reductions in certain immune parameters have also been observed in Nile tilapia (Abd-El-Rhman, 2009; Dotta et al., 2014). Burdock (1998) reported a toxic effect of high levels of propolis supplementation in rabbits and mice. However, in the present study, no toxic effect was found in fish fed up to 1.0% propolis. Further research is needed to verify any toxic effect of high levels of dietary propolis in fish.

The results of the challenge test in the present study were not statistically significant; although the survival rate of fish fed 0.5% propolis (liquid form) was higher than that of fish fed other diets. The challenged fish died very quickly, which made it difficult to detect significant differences among groups, even though we used a dose of pathogen validated in an earlier study (Khosravi et al., 2015). Variables such as temperature, salinity, pH, and host nutrition and hormonal changes can affect the virulency of *E. tarda* (Sakai et al., 2009). It is possible that one or more of these variables increased the virulency of *E. tarda* in our experiment, resulting in the failure of the challenge test. Therefore, further research is required to determine the effects of dietary supplementation of propolis on the resistance of olive flounder to *E. tarda* and to elucidate the effects of confounding variables.

In conclusion, the non-specific immune response of olive flounder can be enhanced by dietary supplementation with propolis. The optimal dosage is likely to be approximately 1% (powder form) or 0.5% (liquid form) in the olive flounder diet. However, we found no evidence that the growth or feed utilization efficiency of olive flounder was affected by propolis.

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