

# Ethanol Extracts of Yacon and Ginger in Diet of Juvenile Olive Flounder (*Paralichthys olivaceus*): Effect on Growth, Feed Utilization, Body Composition, Plasma Chemistry and Challenge Test against *Vibrio anguillarum*

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## Abstract

Development of natural immunostimulants as dietary additive keeps receiving scientist's interest and administration of ethanol extract of additive is one of the effective methods to concentrate their bioactive compounds to improve their effects. Dietary inclusion effect of ethanol extracts of yacon and ginger on growth, feed utilization, body composition, plasma chemistry of fish and challenge test against *Vibrio anguillarum* compared to a commercial probiotic (Super lacto<sup>®</sup>) was determined. Three hundred and sixty juvenile fish were distributed into 12, 50 L flow-through tanks. Four experimental diets were prepared. Additive-free basal diet was served as the control (CON) diet. The 1% ethanol extracts of yacon and ginger, and 0.5% Super lacto<sup>®</sup> were included into the CON diet, referred to as the YCE, GGE and SUP diets, respectively. Each diet was hand-fed to triplicate groups of fish for 8 weeks. After the 8-week feeding trial, fish were injected with *V. anguillarum* and survival was monitored for the next 7 days. Dietary additives did not affect weight gain, feed utilization, whole body composition and plasma parameters of fish. Survival of fish fed the YCE and GGE diets was significantly higher than that of the fish fed the CON and SUP diets at the end of the 7-day post observation. Ethanol extracts of yacon and ginger can be effectively used as natural immunostimulants for olive flounder at occurrence of *V. anguillarum*.

## Introduction

Olive flounder (*Paralichthys olivaceus*) is one of the most commercially important marine fish species for aquaculture in Eastern Asia, having excellent growth and abundantly raised in Korea, Japan and China (Cho *et al.*, 2013; Kim *et al.*, 2017). The annual aquaculture production of olive flounder reached 43,320 metric tons in 2019 (KOSIS, 2020) occupying 98% of the domestic aquaculture market in Korea (Oh *et al.*, 2019). Recently aquaculture farms are at high risk of mass mortality and poor growth performance of olive flounder due to

frequent outbreak of infectious bacterial and viral diseases (Harikrishnan *et al.*, 2011; Oh *et al.*, 2019; Yang *et al.*, 2019). Those pathogenic infections are posing severe problems in the aquaculture industries and causing huge economic losses to the fish farmers (Bulfon *et al.*, 2015). Among the infections, vibriosis is a fatal haemorrhagic septicaemic disease in marine fish caused by several bacterial species including *Vibrio anguillarum* in Korea (Kim *et al.*, 2014a).

Fish farmers generally use conventional disease controlling methods, such as administration of the synthetic antibiotics or chemotherapy drugs (Park,

2009). However, the synthetic antibiotics are costly and have detrimental effect including environmental pollution, accumulation of the antibiotic residue in the fish tissues and food safety threats for public health (Jones *et al.*, 2004; Cabello, 2006; Park, 2009). Hence, application of the synthetic antibiotics is not permitted by the Korean government for human consumption (Choi *et al.*, 2010) as well as worldwide strict rules have been established to ban or minimize their use in aquaculture (Gabor *et al.*, 2010; Hoseinifar *et al.*, 2018). Since immunity and nutrition are closely related (Martin & Król, 2017), dietary inclusion of environment-friendly and cost effective nonspecific immune modulatory nutrition-balanced feed is highly required as a substitute for the synthetic antibiotics in aquaculture.

Nowadays natural immunostimulants have been used as an alternative to the synthetic antibiotics for controlling fish diseases as they contain several bioactive compounds, such as alkaloids, quinone, terpenoids, lectins, polypeptides, phenolic and polyphenolic, and are also capable of improving fish growth and immunity (Bulfon *et al.*, 2015; Vallejos-Vidal *et al.*, 2016). Since plant-based treatments are easy to prepare and cheaper than the synthetic antibiotics (Harikrishnan *et al.*, 2011; Hossain *et al.*, 2019), their potential effects on growth, survival and/or antimicrobial properties of fish have been increased (Ji *et al.*, 2007a; Ji *et al.*, 2007b; Nya & Austin, 2009; Talpur *et al.*, 2013; Zemheri-Navruz *et al.*, 2019).

Yacon (*Smallanthus sonchifolin*) is a native fruit of the Andes (Qian *et al.*, 2006) and contains phenolic compounds (chlorogenic, caffeic and ferulic acids), which have health promoting effect (Simonovska & Vovk, 2003). In addition, yacon exhibits excellent antioxidant property (Yan *et al.*, 1999) and antimicrobial activity against *Bacillus subtilis* (Lin *et al.*, 2003). Improved growth performance and antibacterial activity against *Streptococcus parauberis*, *Edwardsiella tarda* and *S. iniae* in rockfish (*Sebastes schlegeli*) fed the diets containing yacon have been reported (Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018). However, dietary inclusion of yacon did not affect growth, but improved disease resistance of olive flounder against *Streptococcus iniae* (Kim *et al.*, 2019).

Ginger (*Zingiber officinale*) belonging to the family Zingiberaceae is a perennial herb (Karuppiyah & Rajaram, 2012), contains gingerol, gingerdiol and gingerdione, and also possesses potential antioxidant activity (Akbarian *et al.*, 2011). The diet supplemented with ginger achieved higher weight gain, feed utilization and lower cumulative mortality of juvenile rockfish than the control diet containing no additive against *S. parauberis*, *E. tarda* and *S. iniae* (Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018). Ameen *et al.* (2018) found that the aqueous extracts of ginger was the most effective in inhibiting the growth of *Ichthyophonus* sp. *in vitro* compared to linseed (*Linum usitatissimum*), henna (*Lawsonia inermis*) and white turmeric (*Curcuma zedoaria*) in blue tilapia (*Oreochromis aureus*)'s feed.

Improved growth and immunity were also observed in Sobaita sea bream (*Sparidentex hasta*), rainbow trout (*Oncorhynchus mykiss*) and Asian sea bass (*Lates calcarifer*) fed the diets containing ginger (Nya & Austin, 2009; Talpur *et al.*, 2013; Jahanjoo *et al.*, 2018). However, Kim *et al.* (2019) reported that dietary inclusion of 1% yacon, ginger and blueberry powder did not improve either growth performance or feed utilization of olive flounder, but lower cumulative mortality against *S. iniae* infection after the 4<sup>th</sup> and 8<sup>th</sup> week feeding trial.

*Lactobacillus fermentum* is a live bacterium, helps to improve the growth performance and feed utilization of fish, and is actively involved in stimulating specific and non-specific immunity in fish (Allameh *et al.*, 2017). Probiotic can prevent the outbreak of bacterial, viral and parasitic diseases in fish and shellfish (Hoseinifar *et al.*, 2018). *Lactobacillus* gets more attention due to the high antagonistic activity, availability and production of extracellular enzyme (Banerjee & Ray, 2017). Improved growth performance and disease resistance against *S. iniae* in olive flounder were achieved by oral administration of probiotic *Lactococcus lactis* subsp. *lactis* I2 compared to the control diet without supplementation of any additive (Heo *et al.*, 2013). Therefore, *Lactobacillus* is a potential alternative to the synthetic antibiotics and probiotic bacterium, *L. fermentum* (Super lacto<sup>®</sup>) is commercially available in Korea.

In this study, therefore, we investigated the dietary inclusion of the ethanol extracts of yacon and ginger on the growth, feed utilization, chemical composition of the whole body and plasma chemistry of juvenile olive flounder and the challenge test against *V. anguillarum* compared to the commercial probiotic.

## Materials and Methods

### Collection of Fish and Rearing Conditions

Juvenile olive flounder was acclimated to the experimental conditions for 2 weeks before the initiation of the feeding trial after purchasing from a private hatchery (Uljin-gun, Gyeongsang Province, Korea). During the acclimation period, fish were hand fed with a commercial extruded pellet (52% crude protein and 10% crude lipid; WooSung Feed Co. Ltd., Daejeon City, Korea) at the ratio of 2.0–2.5% of total biomass twice a day.

Three hundred and sixty juvenile fish (an initial body weight; 7.2±0.02 g) were randomly distributed into 12, 50-L flow-through tanks (30 fish per tank). Sand-filtered seawater was supplied throughout the feeding trial at temperature ranging from 10.9–23.8°C (mean±SD: 20.9±1.90°C) and flow rate was 1.43 L min<sup>-1</sup> tank<sup>-1</sup>. Proper aeration was supplied to each tank, and the photoperiod followed natural condition. Each diet was randomly assigned to triplicate tanks of fish and hand-fed twice daily at 09:00 a.m. and 5:00 p.m. at a

visual satiation level for 8 weeks. Satiation feeding was determined by the point of cessation of voluntary feeding activity by fish. Pellet size (diameter: 2 mm) was adjusted, and appropriate size pellet (4 mm) was fed as the fish grew. Dead fish were removed daily and the bottom of the tanks was daily cleaned.

### Experimental Diets

Four experimental diets were prepared in triplicates (Table 1). Sixty percent fish meal and 7.5% fermented soybean meal were used as the protein source in the control (CON) diet. Twenty-four percent wheat flour and 4% squid liver and 2% soybean oils were used as the carbohydrate and lipid sources, respectively in the CON diet. No additive was included in the CON diet.

Yacon and ginger were purchased from Tongjong Village (Seoul, Korea) and supplied to Clinical PK & PD LAB, College of Veterinary Medicine, Kyungpook National University (Daegu, Korea). Yacon and ginger were filtered through a filter paper (Whatsman filter paper) prior to extraction with absolute ethanol (1:10 v/v), and then concentrated under reduced pressure using a rotary evaporator. The ethanol extracts of 1% yacon and ginger and 0.5% commercial probiotic (Super lacto<sup>®</sup>) based on recommendation of the manufacturer were included into the experimental diets instead of an equal amount of water, referred to as the YCE, GGE and

SUP diets, respectively. All experimental diets satisfied the dietary nutrient requirements for olive flounder (Lee *et al.*, 2000; Kim *et al.*, 2002; Lee *et al.*, 2002).

All ingredients of each diet were well mixed and pelletized by laboratory pellet extruder (Dongsung mechanics, Busan, Korea). The diets were dried at room temperature overnight and stored at -20°C until use.

### Biological Measurements

At the end of the 8-week feeding trial, all surviving fish in each tank were starved for 24 h, anaesthetized by ethyl aminobenzoate at a concentration of 50 ppm, and then harvested and collectively weighed. Growth performance of fish was evaluated with the following response variables: weight gain (%) = (final weight - initial weight) × 100/initial weight; specific growth rate (SGR) (% body weight gain/day) = [(ln (Wf) - ln (Wi))/days of feeding trial] × 100, where ln (Wf) = natural log of the final mean weight of fish and ln (Wi) = natural log of the initial mean weight of fish. Feed efficiency ratio (FER), protein efficiency ratio (PER), protein retention (PR), condition factor (CF) and hepatosomatic index (HSI) were calculated as follows: FER = Weight gain of fish/feed consumed, PER = Weight gain of fish/protein consumed, PR = Protein gain × 100/protein consumed, CF = Fish weight × 100/total length<sup>3</sup> and HSI = Liver weight × 100/fish weight.

**Table 1.** Formulation of the experimental diets (% DM basis)

	Experimental diets			
	CON	YCE	GGE	SUP
Ingredients (% DM)				
Fish meal*	60	60	60	60
Fermented soybean**	7.5	7.5	7.5	7.5
Wheat flour	24	24	24	24
Super lacto****	0	0	0	0.5
Ethanol extracts of yacon****		1		
Ethanol extracts of ginger****			1	
Squid liver oil	4	4	4	4
Soybean oil	2	2	2	2
Choline	0.5	0.5	0.5	0.5
Vitamin premix*****	1	1	1	1
Mineral premix*****	1	1	1	1
Nutrients (% DM)				
Dry matter	98.5	98.2	97.8	98.1
Crude protein	48.5	49.3	48.3	49.6
Crude lipid	12.8	12.9	14.7	13.2
Ash	13.3	13.9	13.6	13.5

\*Fish meal was purchased from Abank Co Ltd. (Seoul, Korea).

\*\*Fermented soybean meal was supplied by CJ CheilJedang Corp. (Seoul, Korea).

\*\*\*\*Super lacto<sup>®</sup> (a commercial probiotic, *Lactobacillus fermentum*) was purchased from Chang-Jo Biotec Co Ltd. (Jeju, Korea), which was an aqueous type and included into the experiment diets instead of the same amount of water.

\*\*\*\*Ethanol extracts of yacon and ginger were supplied from Clinical PK & PD LAB, College of Veterinary Medicine, Kyungpook National University (Daegu, Korea) and included into the experimental diets instead of the same amount of water.

\*\*\*\*\*Vitamin premix contained the following amount which were diluted in cellulose (g/kg mix): L-ascorbic acid, 121.2; DL- $\alpha$ -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-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

\*\*\*\*\*Mineral premix contained the following ingredients (g/kg mix): MgSO<sub>4</sub>·7H<sub>2</sub>O, 80.0; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 370.0; KCl, 130.0; ferric citrate, 40.0; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 20.0; Ca-lactate, 356.5; CuCl, 0.2; AlCl<sub>3</sub>·6H<sub>2</sub>O, 0.15; KI, 0.15; Na<sub>2</sub>Se<sub>2</sub>O<sub>3</sub>, 0.01; MnSO<sub>4</sub>·H<sub>2</sub>O, 2.0; CoCl<sub>2</sub>·6H<sub>2</sub>O, 1.0.

### Analytical Procedures of the Experimental Diets and Fish

After the 8-week feeding trial, 10 fish from each tank were randomly sampled and frozen at  $-20^{\circ}\text{C}$  for chemical analysis. Chemical analysis of the experimental diets and whole body of olive flounder was done according to AOAC (1995) method. Crude protein was measured by the Kjeldahl method (Kjeltec 2100 Distillation Unit; Foss Tecator, Hoganas, Sweden), crude lipid was determined using an ether-extraction method (Soxtec TM 2043 Fat Extraction System; Foss Tecator), moisture was determined by oven drying at  $105^{\circ}\text{C}$  for 24 h, and ash was determined using a muffle furnace at  $550^{\circ}\text{C}$  for 4 h.

### Plasma Chemistry Analysis

Fish were starved for 24 h at the end of 8-week feeding trial prior to analyzing the plasma chemistry. Five fish were randomly chosen from each tank, anesthetized with ethyl aminobenzoate at a concentration of 50 ppm and then blood samples were taken by heparinized syringe from the caudal vein of fish. Plasma was collected after centrifugation at 7,000 g for 10 min and stored at  $-70^{\circ}\text{C}$  as separate aliquots. Finally, total protein, total cholesterol, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and triglycerides were analyzed by

an automatic chemistry system (HITACHI 7180/7600-210, Hitachi, Japan).

### Challenge Test

At the end of 8-week feeding trial, 15 fish of externally normal appearance shown to be free from bacterial infection were selected from each tank and stocked into 12 static 50 L tanks. The fish were used for the *V. anguillarum* challenge and water was not exchanged. The pathogenic gram-positive *V. anguillarum* (FP5208) reference strain isolated from olive flounder was used for the challenge.

The culture suspension of *V. anguillarum* was grown on agar for 24 h, collected, washed and suspended in sterile 0.85% saline solution, and then counted. Fish were then artificially infected by intraperitoneal injection with 0.1 mL of pathogenic *V. anguillarum* culture suspension containing  $4.2 \times 10^6$  cells/mL. The fish were monitored for 7 days post-infection; dead fish were removed every 6 h for the first four days and every 12 h for the remainder of the study. Fish were starved throughout the 7-day challenge test.

### Statistical Analysis

Significant differences among the means of treatment were determined by one-way ANOVA and Duncan's multi-range test (Duncan, 1955) using SPSS

**Table 2.** Survival (%), weight gain (%) and specific growth rate (SGR, %/day) of the olive flounder fed the experimental diets for 8 weeks

Experimental diets	Initial weight (g/fish)	Final weight (g/fish)	Survival (%)	Weight gain* (%)	SGR** (%/day)
CON	7.2±0.03	29.6±0.38	100±0.00	310.2±4.20	2.52±0.018
YCE	7.2±0.02	30.1±0.20	100±0.00	317.2±3.13	2.55±0.013
GGE	7.2±0.02	30.4±0.08	100±0.00	320.0±0.40	2.56±0.002
SUP	7.2±0.01	30.1±0.15	100±0.00	316.0±2.58	2.55±0.011

Values (means of triplicate ± SE) with different superscripts are significantly different ( $P < 0.05$ ).

\*Weight gain (%) = (final weight – initial weight) × 100/initial weight

\*\*SGR (%/day) =  $[(\ln(W_f) - \ln(W_i)) / \text{days of feeding trial}] \times 100$ , where  $\ln(W_f)$  = natural log of the final mean weight of fish and  $\ln(W_i)$  = natural log of the initial mean weight of fish.

Refer to Table 1 for diet information.

**Table 3.** Feeding consumption (g/fish), feed efficiency ratio (FER), protein efficiency ratio (PER), protein retention (PR), condition factor (CF) and hepatosomatic index (HSI) of olive flounder fed the experimental diets for 8 weeks

Experimental diets	Feed consumption (g/fish)	FE*	PER**	PR***	CF****	HSI*****
CON	25.3±0.34	0.88 ±0.003	1.82±0.005 <sup>a</sup>	38.3±0.16	0.81±0.003	1.62±0.006 <sup>b</sup>
YCE	26.0±0.33	0.88±0.003	1.78±0.007 <sup>b</sup>	37.6±0.12	0.80±0.003	1.65±0.003 <sup>a</sup>
GGE	26.4±0.06	0.88±0.001	1.82±0.003 <sup>a</sup>	38.1±0.31	0.80±0.000	1.65±0.003 <sup>a</sup>
SUP	25.8±0.20	0.89±0.002	1.79±0.004 <sup>b</sup>	37.5±0.24	0.81±0.003	1.63±0.003 <sup>a</sup>

Values (means of triplicate ± SE) with different superscripts are significantly different ( $P < 0.05$ ).

\*Feed efficiency ratio (FER) = Weight gain of fish/feed consumed.

\*\*Protein efficiency ratio (PER) = Weight gain of fish/protein consumed.

\*\*\*Protein retention (PR) = Protein gain × 100/protein consumed.

\*\*\*\*Condition factor (CF) = Fish weight × 100/total length<sup>3</sup>.

\*\*\*\*\*Hepatosomatic index (HSI) = Liver weight × 100/fish weight.

Refer to Table 1 for diet information.

program version 19.0 (SPSS Michigan Avenue, Chicago, IL, USA). All percentage data were arcsine-transformed prior to statistical analysis. Survival of fish during the 7-day post observation period after *S. iniae* infection was analyzed by using Kaplan-Meier survival curve, Log-rank and Wilcoxon tests.

## Results

All fish remained alive at the end of the 8-week feeding trial (Table 2). Weight gain ranging from 310.2 to 320.0% and SGR ranging from 2.52 to 2.56%/day were not significantly ( $P>0.05$ ) different among the experimental diets.

Feed consumption (g/fish) ranging from 25.3 to 26.4 g, FER ranging from 0.88 to 0.89, PR ranging from 37.5 to 38.3 and CF ranging from 0.80 to 0.81 were not significantly ( $P>0.05$ ) affected by the experimental diets (Table 3).

Significantly ( $P<0.05$ ) higher protein efficiency ratio (PER) was achieved in fish fed the CON and GGE diets than that of fish fed the SUP and YCE diets. Significantly ( $P<0.05$ ) lower HSI was obtained in fish fed the CON diet than that of fish fed all other diets.

Dry matter ranging from 29.7 to 29.9%, crude protein ranging from 65.6 to 65.8%, crude lipid ranging from 8.8 to 9.0%, and ash content ranging from 14.0 to 14.7% of the whole body of fish were not significantly ( $P>0.05$ ) affected by the experimental diets (Table 4).

The plasma total protein ranging from 3.24 to 4.49 g/dL, total cholesterol ranging from 173.3 to 208.0 mg/dL, GOT ranging from 42.7 to 68.0 IU/L, GPT ranging from 2.0 to 2.7 IU/L and triglyceride ranging from 174.3 to 338.7 mg/dL were not significantly ( $P>0.05$ ) affected by the experimental diets (Table 5).

Mortality of olive flounder started to show at 42 h after *V. anguillarum* infection after the 8-week feeding trial (Figure 1). Fish fed the YCE and GGE diets showed significantly higher survival than that of fish fed the CON

and SUP diets at the end of the 7-day post observation after infection of *V. anguillarum* ( $P<0.005$  for Log-rank test).

## Discussion

As frequent outbreak of bacterial, fungal and viral diseases have become a primary constraint to sustainable aquaculture production because of the associated risk of economic losses and food security in Korea (Shin *et al.*, 2006; Jung *et al.*, 2008; Harikrishnan *et al.*, 2011) as well as in the worldwide (Leung & Bates, 2013; Bulfon *et al.*, 2015; FAO, 2019), plant-based remedy is a promising way to treat infectious fish diseases and has received increasing attention worldwide (Yilmaz, 2019; Zemheri-Navruz *et al.*, 2019). Yacon and ginger are rich in some anti-oxidative and antimicrobial compounds (Yan *et al.*, 1999; Lin *et al.*, 2003; Ghasemzadeh *et al.*, 2010; Talpur *et al.*, 2013; Sugahara *et al.*, 2015; Kim *et al.*, 2016; Kim *et al.*, 2018) and are potential substitutes for the synthetic antibiotics in aquafeed to enhance the non-specific immunity of fish.

Growth performance (weight gain and SGR) of olive flounder were not affected by the experimental diets in this study, being coincidence with other studies showing that none of the dietary additives [5% kelp meal, 10% krill meal, 3% onion, 1% of each garlic, citrus, ginger, mugwort, licorice and wasabi powder, and the mixture of 0.2% of garlic, citrus, ginger, mugwort, licorice and wasabi and 0.5% onion] (Kim *et al.*, 2011) or 6% *Sargassum fusiforme* and *Ecklonia cava* powder (Kim *et al.*, 2014b)] affected the growth performance and feed efficiency of juvenile olive flounder. Similarly, Cho *et al.* (2013) demonstrated that the survival and weight gain of olive flounder were not improved by the dietary inclusion of *Scutellaria baicalensis* extract, but the SGR was higher in fish fed the diet containing 2% *S. baicalensis* extract compared to that of fish fed the diets

**Table 4.** Proximate composition (% dry matter basis) of the whole body of olive flounder fed the experimental diets for 8 weeks

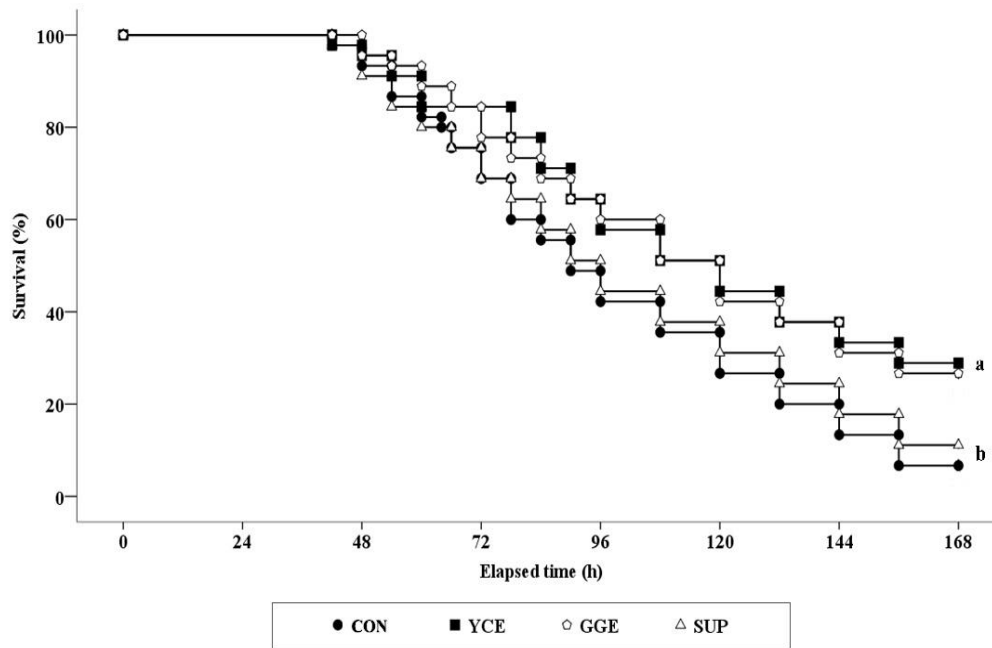
Experimental diets	Dry matter	Crude protein	Crude lipid	Ash
CON	29.8±0.09	65.7±0.14	8.9±0.11	14.3±0.24
YCE	29.9±0.09	65.6±0.22	9.0±0.15	14.7±0.14
GGE	29.7±0.10	65.8±0.43	8.8±0.22	14.2±0.34
SUP	29.8±0.08	65.6±0.10	8.9±0.10	14.0±0.18

Values (means of triplicate ± SE) with different superscripts are significantly different ( $P<0.05$ ). Refer to Table 1 for diet information.

**Table 5.** Plasma chemistry of olive flounder fed the experimental diets for 8 weeks

Experimental diets	Total protein (g/dL)	Total cholesterol (mg/dL)	GOT (IU/L)	GPT (IU/L)	Triglyceride (mg/dL)
CON	3.24±0.11	208.0±3.21	42.7 ± 5.81	2.3 ± 0.33	174.3±18.84
YCE	3.26±0.10	196.3±3.76	54.3 ± 4.98	2.0±0.58	287.3±90.36
GGE	3.41±0.22	189.3±12.72	68.0±3.51	2.3±0.33	231.7±37.57
SUP	4.49±0.54	173.3±14.50	57.0±8.14	2.7±0.67	338.7±105.53

Values (means of triplicate ± SE) with different superscripts are significantly different ( $P<0.05$ ). Refer to Table 1 for diet information.



**Figure 1.** Kaplan-Meier survival curves of juvenile olive flounder infected with *Vibrio anguillarum* after the 8-week feeding trial (means of triplicate  $\pm$  SE) (Different letters within the same elapsed time indicated the significant differences among the experimental diets at the significance level of  $P=0.05$ ). Refer to Table 1 for diet information ( $P<0.005$  for Log-rank test).

containing other concentration (0, 0.5, 1, 3 and 5% of *S. baicalensis* extract) for 8 weeks. Conversely, Kim *et al.* (2018) showed that rockfish fed the diets containing 1% yacon, ginger and blueberry exhibited higher weight gain compared to that of fish fed the control diet containing 0.01% commercial antioxidant (ethoxyquin) in the 8-week feeding trial. The differences in growth performance of fish could be related with the fish species, dose and type (powder or liquid) of additives and the nutritional or physiological state of fish (Harikrishnan *et al.*, 2011; Kim *et al.*, 2013b; Kim *et al.*, 2019).

No differences were found in feed consumption, FER and PR of olive flounder in this study. Similarly, Kim *et al.* (2019) reported that dietary inclusion of 1% yacon, ginger and blueberry powder did not affect either growth performance or feed utilization of olive flounder. Kim *et al.* (2014b) also reported that the feed consumption, FE and PER of olive flounder were not affected by the dietary inclusion of *S. fusiforme* and *E. cava* powder in the 6-week feeding trial. Feed utilization of fish was also unaffected by dietary inclusion of phyto-additives (ginger, garlic, citrus, onion, mugwort, licorice, wasabi and *S. baicalensis* extracts) (Cho *et al.*, 2007; Kim *et al.*, 2011). Unlike these studies, however, feed utilization (feed consumption, FER, PER and PR) of rainbow trout and olive flounder was largely affected by the dietary inclusion of ginger and yacon (Nya & Austin, 2009; Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018).

Lower HSI of fish fed the CON diet compared to all the other diets (SUP, YCE and GGE) in this study

supported by Kim *et al.* (2013a)'s study showing that the HSI was higher in olive flounder fed the diets containing various chemical additives, such as solid sulfur (0.5 and 1%), fucoidan (0.2 and 0.4%) and glucan (0.1%) compared to that of fish fed the control diet with no additive in the 6-week feeding trial. In contrast, Cho *et al.* (2007) showed that greater HSI was found in olive flounder fed the control diet with no additive compared to the diets containing various sources of green tea. However, further research will facilitate to find out the precise reason behind the higher HSI in olive flounder fed the phyto-additives.

None of the whole body chemical composition, plasma total protein, cholesterol, GOT, GPT and triglyceride level was affected by the experimental diets in this study. Similarly, the chemical composition and plasma chemistry of fish was not affected by dietary inclusion of additives (Kim *et al.*, 2019). In addition, insignificant effect on the proximate composition (Ji *et al.*, 2007a) and plasma chemistry (Cho *et al.*, 2013) of olive flounder fed the diets containing medicinal herb mixture (*Massa medicata fermentata: Crataegi fructus: Artemisia capillaris: Cnidium officinale* = 2:2:1:1) and *S. baicalensis* extract have been reported. Unlike these studies, however, dietary inclusion of various phyto-additives affected the chemical composition (Hwang *et al.*, 2013; Kim *et al.*, 2016) and plasma chemistry of fish (Cho *et al.*, 2007; Nya & Austin, 2009; Talpur *et al.*, 2013; Jahanjoo *et al.*, 2018).

Higher survival of olive flounder fed the YCE and GGE diets after experimental infection of *V. anguillarum*

at the end of 7-day post observation compared to that of fish fed the CON and SUP diets indicated that administration of the ethanol extracts of yacon and ginger were more resistant against *V. anguillarum* than the commercial probiotic (Super lacto®), supported by Kim *et al.* (2014b)'s study showing that the dietary inclusion of *S. fusiforme* and *E. cava* powder did not affect the growth performance and feed utilization of olive flounder, but achieved higher disease resistance against *E. tarda* compared to that of fish fed the control diet and the diet containing 0.5% oxytetracycline at the 4<sup>th</sup> day after injection although none of the experimental group showed any difference in mortality in the end of 15-day challenge test. Antibacterial activity of ginger essential oil (1:1 = pure oil: dimethyl sulfoxide) against major bacterial pathogens (*Photobacterium damsela*, *Lactococcus garvieae*, *S. iniae* and *S. parauberis*) of olive flounder have been reported, which are resistant to one or more commonly used the synthetic antibiotic, such as ampicillin, tetracycline, chloramphenicol and erythromycin (Hossain *et al.*, 2019). Kim *et al.* (2019) also reported that yacon and ginger played as immunostimulants for olive flounder and achieved comparable survival to fish fed a diet containing 0.5% probiotic of *L. fermentum* after *S. iniae* infection during the 8-day post observation after the 8-week feeding trial.

Unlike this study, however, administration of yacon and ginger improved not only growth performance, but also immune responses of different species of fish infected with different pathogens (Nya & Austin, 2009; Talpur *et al.*, 2013; Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018; Jahanjoo *et al.*, 2018). In considering the results (Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018) that oral administration of yacon and ginger not only improved growth performance and feed utilization, but also disease resistance of rockfish and the results of Kim *et al.* (2019)'s study and this study showing that inclusion of their ethanol extractions in diets did not affect either growth performance or feed utilization, but just improved disease resistance of olive flounder. Although we had not compared dietary inclusion effect of ethanol extraction of yacon and ginger on rockfish, yacon and ginger seemed to play as both growth promoter and immunostimulants for rockfish, but only immunostimulants for olive flounder. Desirable effect of dietary inclusion of yacon and ginger appeared to have priority in the experimental fish species tested rather than their extraction method. The biological compounds, such as fluctuanin exhibiting the strongest antibacterial activity against *Bacillus subtilis* (Lin *et al.*, 2003) and chlorogenic acid having a strong antioxidant property (Sugahara *et al.*, 2015) in yacon, and zingiberene having immunoenhancing activity in ginger (Zakaria-Rungkat *et al.*, 2003) seemed to play as immunostimulants for olive flounder in this study.

Improved growth performance and immunological activity of Nile tilapia (*O. niloticus*) fed the diet containing 1.5% blueberry syrup compared to the

control diet and the diet containing 0.002% antibiotic (Flormis Aqua®) against *Plesiomonas shigelloides* have been reported (Yilmaz, 2019). Talpur *et al.* (2013) explained that oral administration of various concentrations (0.1, 0.2, 0.3, 0.5 and 1%) of ginger increased survival of Asian sea bass infected with *V. harveyi* and modulated plasma lysozyme activity, phagocytic activity of the head kidney macrophages and serum anti-protease activity. Disease resistant capacity of fish fed the diets containing yacon and ginger was also reported against artificial infection of different pathogenic bacteria (Sebiomo *et al.*, 2010; Kim *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2018). Therefore, relatively lower cumulative mortality of olive flounder fed the YCE and GGE diets compared to fish fed the CON and SUP diets at the end of 7-day post observation in this study can be related with the presence of different biologically active compounds, which accelerate immune activity and inhibit pathogenic aggregation, supported by several other studies showing that ginger improved the phagocytic and lysozyme activity of fish (Nya & Austin, 2009; Talpur *et al.*, 2013; Jahanjoo *et al.*, 2018), and yacon showed efficacy at occurrence of various pathogenic bacteria (Kim *et al.*, 2007a, 2016; Lee *et al.*, 2016)

Although the growth, feed utilization (FER and PR), chemical composition and plasma chemistry of olive flounder were not affected by dietary inclusion of the ethanol extracts of yacon and ginger in the 8-week feeding trial, they effectively lowered the cumulative mortality of olive flounder infected with *V. anguillarum* compared to the CON diet or the diet containing commercial probiotic (Super lacto®). Therefore, the ethanol extracts of yacon and ginger seem to play as natural immunostimulant against *V. anguillarum* in olive flounder. Further research is needed to determine the effect of different doses of the ethanol extracts of yacon and ginger on growth performance and immune response of olive flounder against other pathogens.

## Ethical Statements

Not applicable.

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## Author Contribution

Lei Li Yin ran the feeding trial and analyzed the samples. Sung Hwoan Cho designed the study and prepared the draft. Seung-Chun Park supplied extracts of additives. Mun-Gyeong Kwon supplied pathogen. All authors have read and approved the manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

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