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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How to cite
Yin, L.L., Cho, S.H., Park, S.C., Kwon, M.G. (2021). 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. Turkish Journal of Fisheries and Aquatic Sciences, 21, 265-274. http://doi.org/10.4194/1303-2712-v21_6_01

Article History
Received 17 December 2020
Accepted 03 March 2021
First Online 04 March 2021

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Keywords
Phytic-additive
Olive flounder (Paralichthys olivaceus)
Vibrio anguillarum
Immunostimulants

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*) 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* 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, terpinoids, 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 sonchifolius) 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 & Rajaram, 2012). It contains gingerol, gingerdiol and gingerdione, which have health promoting effect (Simonovska & 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 compared to the control diet containing no additive against S. iniae in rockfish (Sebastes schlegelii) 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 (Karuppiah & 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 Sobaity sea bream (Sparidentex hasta), rainbow trout (Oncorhynchus mykiss) and Asian sea bass (Lates calcarfer) 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 4th and 8th 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®) 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⁻¹ tank⁻¹. 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 (Whatman filter
paper) prior to extraction with abs.

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

| Ingredients (% DM) | CON | YCE | GGE | SUP |
|-------------------|-----|-----|-----|-----|
| 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   | 1   |     |
| Ethanol extracts of ginger***** | 1   | 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® (a commercial probiotic, Lactobacillus fermentum) was purchased from Chang-Jo Biotech 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 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
and HSI = Liver weight × 100/fish weight.
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°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°C for 24 h, and ash was determined using a muffle furnace at 550°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°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 Software.

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 (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.

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 a | 38.3±0.16 | 0.81±0.003 | 1.62±0.006 b |
| YCE                | 26.0±0.33                | 0.88±0.003 | 1.78±0.007 b | 37.6±0.12 | 0.80±0.000 | 1.65±0.003 a |
| GGE                | 26.4±0.06                | 0.88±0.001 | 1.82±0.003 a | 38.1±0.31 | 0.80±0.000 | 1.65±0.003 a |
| SUP                | 25.8±0.20                | 0.89±0.002 | 1.79±0.004 b | 37.5±0.24 | 0.81±0.003 | 1.63±0.003 a |

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

*Feed efficiency ratio (FER) = Weight gain of fish/food 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 length³.

*****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 arc sine-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.
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. baikalensis* 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. baikalensis* 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*.

Figure 1. Kaplan-Meier survival curves of juvenile olive flounder infected with *Vibrio anguillarum* after the 8-week feeding trial (means of triplicate ± 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).
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 4th day after infection 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 damselae*, *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 flavuatin 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.

**Funding Information**

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (No. 2020R1A2C1009903). This work was also supported by a grant National Institute of Fisheries Sciences (NIFS), Republic of Korea (R2020060).

**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.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (No. 2020R1A2C100903). This work was also supported by a grant National Institute of Fisheries Sciences (NIFS), Republic of Korea (R2020060).

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