Thesis for the Degree of Master of Science

Effect of dietary inclusion of yacon, ginger and blueberry meals on the growth, body composition and serum chemistry of juvenile olive flounder (*Paralichthys olivaceus*) and challenge test against *Streptococcus iniae*

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by

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CONTENTS

List of Tables

List of Figures

배합사료내 야콘, 생강과 블루베리의 첨가가 넙치 (*Paralichthys olivaceus*) 치어의 성장, 체구성, 혈액성상 및 *Streptococcus iniae* 공격성에 미치는 영향

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요약

본 연구는 배합사료내 야콘(yacon), 생강(ginger), 블루베리(blueberry) 및 상업용 Probiotics (LF) (*Lactobacillus fermentum*)첨가제가 넙치 치어의 성장, 사료 이용성, 체구성, 혈액성상 및 *Streptococcus iniae* 공격성에 미치는 영향을 조사하였다. 총 600마리의 넙치를 15개의 50 L 유수식수조에 40마리씩 무작위로 각각 수용하였다. 첨가제가 포함되지 않은 대조구(CON) 사료와 대조구 사료 제조시 물 대신에 0.5%의 상업용 Probiotics (*L. fermentum*)가 포함된 LF 사료, 대조구 사료내 소맥분 대신에 1%의 야콘, 생강과 블루베리를 첨가한 YC, GG와 BB 사료를 각각 준비하였다. 총 5개의 실험사료를 준비하였으며, 각 사료는 3반복구를 두었다. 사육실험 기간은 총 8주간이며, 4주와 8주의 사육실험 종료후 인위적으로 *Streptococcus iniae*를 감염시켰으며 감염이후 8일간 누적폐사율을 관찰하였다. 사육실험 종료시 생존율, 체중 증가, 일일성장률, 체조성, 사료이용성 및 혈액성상학적 차이는

모든 실험구간에 유의적인 차이가 없었다. 실험사료를 4주간 공급한 후 *S. iniae* 세균 감염시 LF, YC, GG와 BB사료를 공급받은 넙치의 누적폐사율은 감염이후 48시간부터 관찰 종료시(감염이후 8일)까지 CON사료보다 유의적으로 낮은 누적폐사율을 보였다. 그리고 8주간 실험사료를 공급한 넙치의 *S. iniae* 세균 감염시 LF, YC, GG와 BB사료를 공급받은 넙치의 누적폐사율은 감염이후 96시간부터 관찰 종료시(감염이후 8일)까지 CON사료보다 유의적으로 낮은 누적폐사율을 보였다. 이러한 결과를 고려할 때 넙치용 배합사료내 야콘, 생강 및 블루베리의 첨가는 넙치의 성장에 영향을 미치지는 않지만, *S. iniae* 감염에 대한 효과적인 면역자극제였다. 따라서 넙치용 배합사료내 이러한 환경 친화적인 식물성 첨가제는 *S. iniae* 발병으로 인한 넙치의 폐사율을 감소시키는 효과를 기대할 수 있다.

KEY WORDS: Olive flounder (*Paralichthys olivaceus*), Yacon, Ginger, Blueberry, *Streptococcus iniae,* Challenge test

Effect of dietary inclusion of yacon, ginger and blueberry meals on the growth, body composition, serum chemistry of juvenile olive flounder (*Paralichthys olivaceus*) and challenge test against *Streptococcus iniae*

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Abstract

The effect of dietary inclusion of yacon (YC), ginger (GG) and blueberry (BB) meals on the growth, feed utilization, body composition and serum chemistry of juvenile olive flounder (*Paralichthys olivaceus*) and resistance to *Streptococcus iniae* compared to a commercial probiotic (LF) (*Lactobacillus fermentum*) was investigated. Six hundred fish were randomly distributed into 15 of 50-L flow-through tanks (40 fish per tank). Five experimental diets were prepared in triplicates. The control diet (CON) contained no additive. 0.5% LF was included into the CON diet instead of water based on the manufacture's recommendation. One percent of YC, GG and BB were included into the CON diet at the expense of wheat flour, referred to as the YC, GG and BB diets, respectively. Fish were fed with one of the experimental diets for 8 weeks. At the end of 4-week and 8-week feeding trials, fish were artificially infected with *S. iniae* and the cumulative mortality of fish was monitored for 8 days. No significant difference in survival, weight gain and specific growth rate of fish was observed at the end of 8-week feeding trial. None of feed utilization, serum chemistry and the whole body of fish was affected by the experimental diets. The cumulative mortality was significantly higher in olive flounder fed the CON diet compared to that of fish fed the YC, GG, BB and LF diets, starting at 48 h and 96 h until the end of 8-day post observation after *S. iniae* infection after 4-week and 8-week feeding trials, respectively. These results indicate that yacon, ginger and blueberry was the effective immunostimulants against *S. iniae* rather than growth promoter.

KEY WORDS: Olive flounder (*Paralichthys olivaceus*), Yacon, Ginger, Blueberry, *Streptococcus iniae,* Challenge test

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is one of the most commercially important marine fish species for aquaculture in Eastern Asia, such as Korea, Japan and China (Cho et al. 2006). Its annual aquaculture production reached 41207 metric tons in Korea in 2017 (KOSIS 2018). However, frequent outbreak of disease and contamination of the surroundings have caused mortality of olive flounder through the year-around culture. Streptococcosis is a devastating disease in wild and farmed fish can be caused by a pathogen *Staphylococcus iniae*, which has been thought to be the primary reasons for less production and poor growth of fish (Colorni et al. 2002; Shin et al. 2007). To prevent or minimize loss of fish, fish farmers are likely to include synthetic antibiotics, such as ampicillin, streptomycin, doxycycline, nitrofurantoin and furazolidone into fish feed (Akinbowale et al. 2006; Chun & Jeong 1992; Darwish et al. 2005).

However, an inclusion of antibiotics into fish feed can cause several problems, such as fish residue of antibiotics, environmental pollution and food safety threats (Chevassus & Dorson 1990). The occurrence of antibiotic resistance has also been noted as a serious concern for fish culture (Chelossi et al. 2003; Naviner et al. 2007; Rigos & Troisi 2005). Therefore, their use in fish feed for human consumption is prohibited in some countries (Casewell et al. 2003) and Korean government also does not allow use of synthetic antibiotics in the production of fish feed for human consumption (Choi et al. 2010).

New natural sources of antibiotics can replace synthetic antibiotics in fish feed, which should be developed continuously to increase sustainable and eco-friendly aquaculture production. Recently, many researchers have been interested in the application of medicinal plants, such as yacon (*Smallanthus sonchifolius*), ginger (*Zingiber officinale*), garlic (*Allium sativum*), onion (*Allium cepa*), blueberry (*Vaccinium corymbosum*), bermuda grass (*Cynodon dactylon*), long pepper (*Piper longum*), green tea (*Camellia sinensis*), stonebreaker (*Phyllanthus niruri*), coat buttons (*Tridax procumbens*), medicated leaven (*Massa medicata fermentata*), hawthorne (*Crataegi fructus*), virgate wormwood (*Artemisia capillaris*) and *Cnidium officiale* as a growth promoter and/or substitute for antibiotics in fish diets (Cho et al. 2007; Cho & Lee 2012; Gabor et al. 2010; Ji et al. 2007a, b; Kim et al. 2014; Kim et al. 2017; Lee et al. 2016; Punitha et al. 2008; Shalaby et al. 2006). Therefore, development of an alternative source for antibiotics is one of the best options to improve immune responses of fish and maintain antibiotics-free aquaculture systems.

Yacon (YC) is an important economic species grown for its juicy tuberous root and potentially has antioxidative, antiinflammatory and antimicrobial properties (Campos et al. 2012; Ohyama et al. 1990), particularly known as an abundant source of β-(2→1) fructo-oligosaccharides (Goto et al. 1995). In addition, prebiotic effect of YC enhances immune system and improves resistance to infections and allergic reactions (Delgado et al. 2013). Antimicrobial activity of YC leaves extract against devastating pathogen *S. aureus* has been reported (Choi et al. 2010).

Ginger (GG) has been reported as an antibiotic substitute since GG extract possess antimicrobial, antioxidant and anticancer properties (Onyeagba et al. 2004; Sebiomo et al. 2011; Weil 2005; White 2007; Yusof et al. 2002). Gingerol is an active volatile oil in GG, being responsible for its pungent flavor (Longe et al. 2005). Talpur et al. (2013) demonstrated that dietary inclusion of GG improved growth, strengthen the non-specific immunity and reduce susceptibility to *Vibrio harveyi* of Asian sea bass (*Lateolabrax japonicus*). Highest antibacterial activity of GG extract compared to 3 antibiotics (chloramphenicol, ampicillin and tetracycline) against *S. aureus* and *S. pyogenes* have also been reported (Sebiomo et al. 2011). Akintobi et al. (2013) showed that water extract of GG was highly active against *Salmonella typhi* and slightly active against *S. aureus* and *Proteus mirabilis,* and concluded that GG extract could be used as substitutes for antibiotics.

Blueberry (BB) is recognized as the best sources of phenolics and flavonoids, especially anthocyanins (howard et al. 2003; Naczk et al. 2006; Riihinen et al. 2008) and also have antioxidant and antimicrobial properties (Deng et al. 2014; Lee et al. 2016; Papandreou et al. 2009; Vizzotto et al. 2013). Li et al. (2013) demonstrated the fruits, pomace, and leaves of rabbiteye BB were good sources of polyphenols and natural antioxidants.

Herbs and spices are very important and useful feed additives as therapeutic agent against many pathogenic infections (Gull et al. 2012). Therefore, YC, GG and BB seem to have high potential as a substitute for antibiotics and improve growth performances and immune responses of fish.

Nowaday's probiotics are widely used in aquaculture practices for increasing disease resistance and growth and feed efficiency of olive flounder (Kim et al. 2012; Kim et al. 2013). Probiotics (*Lactobacillus fermentum*) are recently developed and commercially available in Korea. Peran et al. (2007) demonstrated that *Lactobacillus fermentum* can exert beneficial immunomodulatory properties in inflammatory bowel disease in Wistar rat. Balcázar et al. (2008) reported that the adhesion (*Aeromonas hydrophila, A. salmonicida, Yersinia ruckeri, Vibrio anguillarum*) of all rainbow trout pathogens to intestinal mucus was reduced by *L.*

fermentum. Cha et al. (2013) showed that *B. subtilis* has positive effect on growth performance and innate immunity of olive flounder among three *Bacillus* spp. (*B. subtilis*, *B. pumilus* and *B. licheniformis*) tested and resistance against pathogen of *S. iniae*. *Lactococcus* is another potential probiotics. Heo et al. (2013) concluded that *L. lactis* subsp. *lactis* I2 increased growth of olive flounder compared to the untreated group and has potential as an alternative source for antibiotics for the prevention of streptococcosis in aquaculture.

This study was, therefore, performed to determine effect of dietary inclusion of phyto-additives (YC, GG and BB) on the growth, body composition, serum chemistry and challenge test of juvenile olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* compared to commercial probiotics.

2. Materials and Methods

2.1 Fish and the experimental conditions

Juvenile olive flounder were purchased from a private hatchery (Pohang City, Gyeongsangbuk-do, Korea) and acclimated to the experimental conditions for 2 week before starting the feeding trial. Fish were hand-fed with commercial extruded pellet (WooSung Feed Co. LTD, Daejeon City, Korea) twice a day during the acclimation period.

Six hundred juvenile flounder (an initial body weight of 5.4 g) were randomly distributed into 15 of 50-L flow-through tanks (40 fish per tank; water volume: 40L). The sand-filtered seawater was supplied throughout the feeding trial at temperature ranging from 10.6 to 23.8 ℃ (mean ± SD: 18.2 ± 3.07 ℃) and flow rate was 1.44-L/min/tank. Proper aeration was supplied to each tank, and the photoperiod followed natural condition. Dead fish were removed daily and the bottoms of the tanks were cleaned every day. After the 8-week feeding trial, all surviving fish from each tank were collectively weighed to evaluate weight gain.

2.2 Preparation of the experimental diets

Five experimental diets were prepared in triplicates (Table 1). Sixty percent fish and fermented soybean meals and wheat four were used as the protein and carbohydrate sources, respectively, however, 4% squid liver and 2% soybean oils as lipid sources, in the control (CON) diet without additive. 0.5% *Lactobacillus*

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

	Experimental diets				
	CON	LF[1]	YC[2]	GG[2]	BB[2]
Ingredients (%, DM)					
Fish meal[3]	60	60	60	60	60
Fermented soybean meal[4]	7.5	7.5	7.5	7.5	7.5
Wheat flour	24	24	23	23	23
Lactobacillus fermentum (LF)		0.5			
Phyto-additives (YC, GG, BB)			1	1	1
Squid liver oil	4	4	4	4	4
Soybean oil	2	2	2	2	2
Vitamin premix[5]	1	1	1	1	1
Mineral premix[6]	1	1	1	1	1
Choline	0.5	0.5	0.5	0.5	0.5
Nutrients (%)					
Dry matter	96.6	96.5	96.8	97.1	95.3
Crude protein	51.2	51.0	51.2	51.0	51.3
Crude lipid	10.7	10.9	10.7	10.5	10.7
Ash	12.0	11.9	11.9	12.0	11.6

[1]*Lactobacillus fermentum* (LF) was purchased from Chang-Jo Biotec Co Ltd. (Jeju, Korea), which was an aqueous type was included into the experimental diets instead of the same amount of water.

[2]YC (yacon), [2]GG (ginger) and [2]BB (blueberry) were purchased from Tojongherb Co Ltd. (Seoul, Korea).

[3]Fish meal was purchased from Abank Co Ltd. (Seoul, Korea).

[4]Fermented soybean meal was supplied by CJ CheilJedang Corp. (Seoul, Korea).

[5]Vitamin premix contained the following amount which were diluted in cellulose (g/kg mix): 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-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[6]Mineral premix contained the following ingredients (g/kg mix): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

fermentum (LF) was included into the CON diet instead of water based on manufacture's recommendation. One percent of YC, GG and BB were included into the CON diet at the expense of wheat flour, referred to as the YC, GG and BB diets, respectively. The ingredients of the experimental diets were well mixed with water at a ratio of 3:1 and pelletized by laboratory pellet extruder.

The experimental diets were dried at room temperature overnight and stored in –20°C until use. All experimental diets were prepared to satisfy dietary nutrient requirements for olive flounder (Kim et al. 2002; Lee et al. 2000, 2002). Each diet were hand-fed to satiation twice a day (09:00 and 17:00 h) for 7 day a week, for the 8-week feeding trial.

2.3 Chemical analysis of the whole body and serum of olive flounder

All remaining olive flounder (≥ 3 fish) from each tank after 4^{th} (10 fish) and 8^{th} week (20 fish) challenge test were frozen, homogenized and then used for chemical analysis of the whole body of fish. Chemical analysis of the experimental diets and whole body of olive flounder was done according to AOAC (1990). Crude protein was determined by the Kjeldahl method (Auto Kjeldahl System, Buchi B-324/435/412, Switzerland). Crude lipid was determined using an ether-extraction method (Soxtec TM 2043 Fat Extraction System, Foss Tecator, Sweden), 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.

For serum analysis, olive flounder were starved for 24 h at the end of 8-week feeding trial. After that, fish were anesthetized with Ethyl aminobenzoate at a concentration of 50 ppm and blood samples were taken by heparinized syringe from the caudal vein of five randomly chosen olive flounder from each tank. Serum was collected after centrifugation (7,000 rpm. 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).

2.4 Challenge test

Healthy and disease-free olive flounder were chosen from each tank for challenge test after the 4-week and 8-week feeding trial and then stocked into 12, 50-L tanks. The water was static. At the end of the 4- and 8-week feeding trial, 10 and 20 sampled olive flounder from each tank were injected intraperitoneally with 0.1 mL culture suspension of pathogenic *S. iniae* (FP5024) and the concentration was 7.8×10^6 and 8.2×10^6 CFU/L. The cumulative mortality of fish was monitored for the following 8 days after pathogen injection. Dead fish were removed every 6 h for the first 4 days and every 12 h for the rest post monitoring period. Fish were starved during 8-day post observation after challenge test.

2.5 Statistical analysis

The data were subjected to one-way ANOVA and Duncan`s multiple range test (Duncan 1955) to determine the significant differences among the means of treatments by using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). All percentage data were arcsine-transformed prior to statistical analysis.

3. Result

Survival, weight gain and SGR of olive flounder fed the experimental diets for 8 weeks are given in Table 2. Survival of fish ranging from 96.7 to 100 % and SGR ranging from 2.63 to 2.64 %/day were not significantly ($P > 0.05$) different among the experimental diets. Olive flounder fed all experimental diets showed the similar weight gain of 18.3 g and was not significantly ($P > 0.05$) different.

Feed consumption, FE, PER, PR and condition factor (CF) of olive flounder fed the experimental diets for 8 weeks are presented in Table 3. Feed consumption ranging from 22.7 to 23.2 g, FE ranging from 0.96 to 0.98, PER ranging from 1.54 to 1.56, PR ranging from 32.5 to 32.9 and CF ranging from 0.79 to 0.80 were not significantly ($P > 0.05$) affected by the experimental diets.

The proximates of the whole body of olive flounder at the end of the 8-week feeding trial are given in Table 4. Moisture content ranging from 70.3 to 70.4%, crude protein ranging from 20.0 to 20.3%, crude lipid ranging from 2.7 to 2.9% and ash content ranging from 4.2 to 4.3% were not significantly ($P > 0.05$) affected by the experimental diets.

The serum chemistry (total protein, total cholesterol, GOT, GPT and triglyceride) of olive flounder at the end of the 8-week feeding trial are shown in the Table 5. The serum total protein ranging from 4.08 to 4.58 g/dL, total cholesterol ranging from 184.3 to 224.3 mg/dL, GOT ranging from 44.3 to 73.7 IU/L and triglyceride ranging from 203.7 to 351.0 mg/dL were not significantly ($P > 0.05$) affected by the experimental diets.

Table 2. Survival (%), weight gain (g/fish) and specific growth rate (SGR) of olive flounder fed the experimental diets containing the various phyto-additives for 8 weeks

Experimental diets	Initial weight (g/fish)	Final weight (g/fish)	Survival (%)	Weight gain (g/fish)	SGR[1] (%/day)
CON	5.4 ± 0.01[a]	23.7 ± 0.06[a]	96.7 ± 0.00[a]	18.3 ± 0.05[a]	2.63 ± 0.002[a]
LF	5.4 ± 0.01[a]	23.7 ± 0.09[a]	98.9 ± 1.11[a]	18.3 ± 0.08[a]	2.63 ± 0.004[a]
YC	5.4 ± 0.01[a]	23.7 ± 0.14[a]	100.0 ± 0.00[a]	18.3 ± 0.15[a]	2.64 ± 0.013[a]
GG	5.4 ± 0.01[a]	23.7 ± 0.11[a]	100.0 ± 0.00[a]	18.3 ± 0.12[a]	2.63 ± 0.010[a]
BB	5.4 ± 0.01[a]	23.7 ± 0.09[a]	98.9 ± 1.11[a]	18.3 ± 0.09[a]	2.63 ± 0.008[a]

Values (means of triplicate ± SE) in the same column sharing the same superscript letter are not significantly different ($P > 0.05$).

[1]SGR (%/day) = (Ln final weight of fish - Ln initial weight of fish)×100/days of feeding trial.

Table 3. Feed consumption, feed efficiency (FE), protein efficiency ratio (PER), protein retention (PR) and condition factor (CF) of olive flounder fed the experimental diets containing the various phyto-additives for 8 weeks

Experimental diets	Feed consumption (g/fish)	FE[1]	PER[2]	PR[3]	CF[4]
CON	23.2 ± 0.08^a	0.97 ± 0.003^a	1.54 ± 0.006^a	32.7 ± 0.18^a	0.80 ± 0.002^a
LF	23.0 ± 0.18^a	0.97 ± 0.002^a	1.56 ± 0.009^a	32.6 ± 0.17^a	0.79 ± 0.002^a
YC	22.9 ± 0.07^a	0.97 ± 0.004^a	1.56 ± 0.009^a	32.5 ± 0.19^a	0.79 ± 0.000^a
GG	23.0 ± 0.05^a	0.96 ± 0.004^a	1.56 ± 0.008^a	32.7 ± 0.18^a	0.80 ± 0.002^a
BB	22.7 ± 0.23^a	0.98 ± 0.009^a	1.56 ± 0.024^a	32.9 ± 0.48^a	0.80 ± 0.001^a

Values (means of triplicate ± SE) in the same column sharing the same superscript letter are not significantly different ($P > 0.05$).

[1]Feed efficiency (FE) = Weight gain of fish/feed consumed

[2]Protein efficiency ratio (PER) = Weight gain of fish/protein consumed.

[3]Protein retention (PR) = Protein gain×100/protein consumed.

[4]Condition factor (CF) = Fish weight×100/total length^3.

Table 4. Proximate analysis (%, wet weight basis) of the whole olive flounder fed the experimental diets containing the various phyto-additives for 8 weeks

Experimental diets	Moisture	Crude protein	Crude lipid	Ash
CON	70.3 ± 0.09[a]	20.3 ± 0.06[a]	2.9 ± 0.03[a]	4.2 ± 0.03[a]
LF	70.4 ± 0.07[a]	20.1 ± 0.05[a]	2.8 ± 0.01[a]	4.3 ± 0.02[a]
YC	70.3 ± 0.10[a]	20.0 ± 0.02[a]	2.8 ± 0.07[a]	4.4 ± 0.09[a]
GG	70.3 ± 0.05[a]	20.1 ± 0.01[a]	2.8 ± 0.02[a]	4.2 ± 0.03[a]
BB	70.3 ± 0.13[a]	20.1 ± 0.04[a]	2.7 ± 0.02[a]	4.3 ± 0.00[a]

Values (means of triplicate ± SE) in the same column sharing the same superscript letter are not significantly different ($P > 0.05$).

Table 5. Serum chemistry of olive flounder fed the experimental diet containing the various phyto-additives for 8 weeks

Experimental diets	Total protein (g/dL)	Total cholesterol (mg/dL)	GOT (IU/L)	GPT (IU/L)	Triglyceride (mg/dL)
CON	4.43 ± 0.218[a]	224.3 ± 7.80[a]	44.3 ± 5.61[a]	2.3 ± 0.67[a]	203.7 ± 18.41[a]
LF	4.55 ± 0.478[a]	184.3 ± 23.07[a]	56.3 ± 10.17[a]	2.3 ± 0.33[a]	351.0 ± 43.62[a]
YC	4.22 ± 0.581[a]	206.3 ± 11.05[a]	52.3 ± 6.74[a]	2.7 ± 0.33[a]	319.7 ± 45.12[a]
GG	4.08 ± 0.510[a]	191.3 ± 17.65[a]	68.0 ± 6.51[a]	2.3 ± 0.33[a]	215.7 ± 14.52[a]
BB	4.58 ± 0.309[a]	194.0 ± 23.18[a]	73.7 ± 6.39[a]	3.0 ± 0.58[a]	224.0 ± 37.54[a]

Values (means of triplicate ± SE) in the same row sharing a common superscript are not significantly different ($P > 0.05$).

Olive flounder were infected with *S. iniae* after 4- and 8-week feeding trials and the cumulative mortality were not significantly ($P > 0.05$) affected by the experimental diets during 36 h post observation after *S. iniae* infection (Figs. 1 and 2, respectively). In challenge test after 4-week feeding trial, significantly ($P < 0.05$) greater cumulative mortality was observed in olive flounder fed the CON diet compared to all other diets at 42 h until the end of the rest of observation periods after *S. iniae* infection. The lowest cumulative mortality was observed in olive flounder fed the GG and LF diets. The highest cumulative mortality (96.7%) was recorded in olive flounder fed the CON diet at the end of 8-day post observation after *S. iniae* infection.

In challenge test after 8-week feeding trial, significantly ($P < 0.05$) higher cumulative mortality was observed in olive flounder fed the CON diet compared to other diets (LF, YC, GG and BB diets) at 96 h post observation until the end of post monitoring periods. The dead olive flounder exhibited typical disease symptoms caused by *S. iniae* (dark body color, muscle bleeding, erratic swimming behavior, lethargy, external and internal bleeding). The highest (95%) cumulative mortality was observed in olive flounder fed the CON diet at the end of 8-day post observation, respectively.

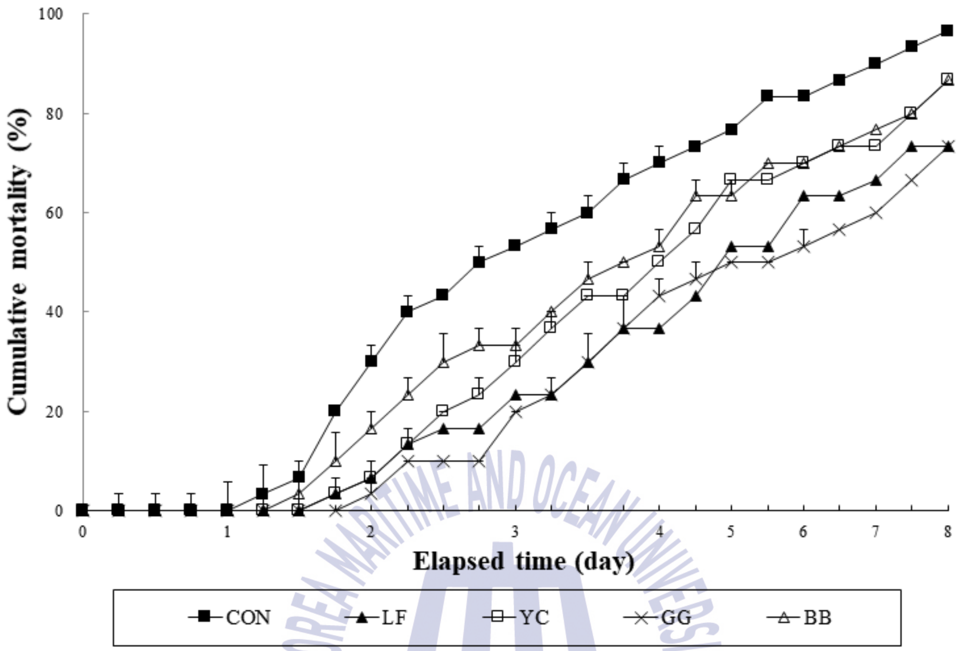

Fig. 2. Cumulative mortality (%) of olive flounder fed the experimental diets containing commercial probiotics, *Lactobacillus fermentum* (LF) and the various phyto-additives for 4 weeks, and then infected by gram-positive *Streptococcus iniae* (means of triplicate ± SE).

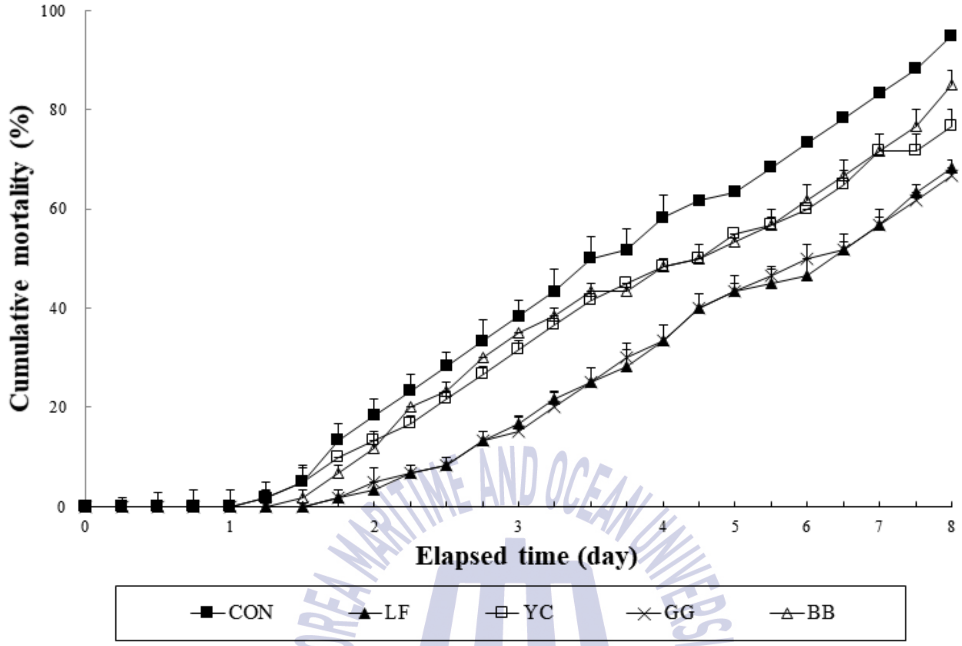

Fig. 2. Cumulative mortality (%) of olive flounder fed the experimental diets containing commercial probiotics, *Lactobacillus fermentum* (LF) and the various phyto-additives for 8 weeks, and then infected by gram-positive *Streptococcus iniae* (means of triplicate ± SE).

4. Discussion

Frequent occurrence of different bacterial, viral and fungal infections is a rising concern not only in Eastern Asia, such as Korea, China and Japan, but also in the worldwide and causes huge economic losses (Akinbowale et al. 2006; Darwish et al. 2005; Lee et al. 2016; Lunder et al. 2000; Nguyen & Kanai 1999; Toranzo et al. 2005). The pathogen along with poor environmental conditions on farms, unbalanced nutrition, generation of toxins, and genetic factors affected fish mortality (Kautsky et al. 2000). In recent decades, fish farmers have focused on the use of the chemical additives, especially antibiotics for controlling bacterial infections in aquaculture, which generate threat to the public health, environment and non-targeted organisms. Excessive use of antibiotics also can reduce drug efficacy by inducing antibiotic resistance (Alderman & Hastings 1998; Chelossi et al. 2003; Naviner et al. 2007; Park et al. 2009; Rigos & Troisi 2005).

Dietary inclusion effect of phyto-additives (YC, GG and BB) on either growth performance of fish or feed utilization was not observed in this study. Similarly, weight gain and SGR of olive flounder were not affected by dietary additives (Cho & Lee 2012; Cho et al. 2013; Choi et al. 2004; Kim et al. 2005). Unlike this study, however, Lee et al. (2016) reported that an inclusion of phyto-additives (GG, YC, BB) in juvenile rockfish (*Sebastes schlegeli*) diet achieved improved growth performance, feed utilization and resistance against to *S. iniae* compared to a commercial antioxidant, ethoxyquin, commonly used in commercial fish diet. Kim et al. (2016) reported the greatest weight gain and SGR of rockfish fed the YC supplemented diet. Dietary inclusion effect of phyto-additives on growth performance of fish and feed utilization seemed to be fish-specific. Talpur et al. (2013) showed that GG diet improved the SGR, feed conversion ratio and weight gain of Asian sea bass. Weight gain of rainbow trout and sea bass fed the diet containing various levels of garlic and GG was proportional to the amount of additives included in the diet (Nya & Austin 2009a, b; Talpur 2014; Talpur et al. 2013). Therefore, use of environment-friend and natural products can be considered as the best option to enhance growth and immunity of fish and replace antibiotic use in aquaculture.

Proximate and serum composition of olive flounder was not affected by the experimental diet in the present study. Different studies showed that dietary inclusion effect of oriental herbs improved growth and muscle quality of olive flounder and lowered plasma cholesterol of fish (Kim et al. 1998; Kim et al. 2000; Lee et al. 1998). Cho et al. (2007) reported that dietary inclusion of green tea was effective in lowering serum low-density lipoprotein cholesterol (LDL) cholesterol and GPT in olive flounder when different sources of green tea were treated. Cho & Lee (2012) also showed that olive flounder fed the diet containing onion improved lysozyme activity and lowered mortality of fish against *Edwardsiella tarda*. Ji et al. (2007a) demonstrated that olive flounder achieved higher weight and carcass unsaturated fatty acid content and lower carcass saturated fatty acid content than fish fed control diet including no additive when a herbal mixture of *Massa medicata fementata*, *Crataegi fructus*, *Artemisia capillaris* and *Cnidium officiale* (2:2:1:1) was added into diet. Bioactive compound saponin present in GG is capable of lowering the blood cholesterol, improving hyperlipidemia and possible antimicrobial properties to resist the infection of foreign pathogens (Otunola et al. 2010; Talpur et al. 2013).

Significant reduction in cumulative mortality of olive flounder fed the experimental diets containing various phyto-additives was the most attractive result in the present study. Olive flounder fed the CON diet showed the highest cumulative mortality compared to all other diets at 48 h and 96 h, respectively until the end of 8-day post observation after *S. iniae* infection after 4-week and 8-week feeding. Typical symptoms of dead fish caused by *S. iniae* were also observed in olive flounder in this study. The lowest cumulative mortality was observed in olive flounder fed the GG and LF diets after 4- (Fig. 1) and 8-week feeding trial (Fig. 2), respectively. The highest cumulative mortality of olive flounder fed the CON diet compared to all other diets in this study probably indicated that phyto-additives and commercial probiotics used were effective to lower mortality of olive flounder at occurrence of *S. iniae*. Similarly, Kim et al. (2017) reported that dietary inclusion of YC, GG and BB effectively lowered cumulative mortality of olive flounder infected with *E. tarda*. The bioactive compounds polyphenols, flavonoids, tannins and saponins found in GG, YC and BB may be linked with the lower cumulative mortality in olive flounder fed the YC, GG and BB diets in this study compared to the CON diet. Because these phyto-additives are natural substance and do not affect fish, humans or the environment, they can be utilized as an alternative source for antibiotics without retarding the growth, body composition and immunity of fish.

Phyto-additives can enhance immunity by macrophage activation in fish (Jorgensen et al. 1993), affect the blood cells (Nya & Austin 2009a, b; Sahu et al. 2007; Talpur & Ikhwanuddin 2012, 2013), increase lysozyme, phagocytic and complement activity and increase plasma protein (globulin and albumin), which has a strong innate responses in fish (Cho & Lee 2012; Engstad et al. 1992; Wu et al. 2010; Yuan et al. 2007). Dietary supplementation of fermented garlic powder can enhance the non-specific immune responses and disease resistance in olive flounder against *V. anguillarum* and *S. iniae* (Kim et al. 2014).

5. Conclusion

In conclusion, dietary inclusion of YC, GG and BB for 4 and 8 weeks effectively lowered cumulative mortality of olive flounder at occurrence of *S. iniae*. A 4-week oral administration of various phyto-additives (YC, GG and BB) was long enough to induce their desirable effect of lowering mortality of fish at occurrence of *S. iniae*. More researches are needed before practical application of these phyto-additives in olive flounder feed.

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