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Evaluation of dietary fish meal analog with or without supplementation of natural feed additives as the substitute of fish meal in juvenile Japanese eel, *Anguilla japonica*

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We investigated the nine experimental diets containing fish meal (FM) and/or fish meal analog **(**FMA) as the major source of animal protein to determine the optimum FMA level as the substitute of FM protein in the diet of juvenile Japanese eel. In addition, two natural feed additives such as Song-Gang stone (SG) and Yucca meal (YM) were supplemented in the diet to evaluate their efficacy as the immunostimulants. The diets are as follows: 100% FM + 0% FMA in diet (FMA_0), 90% FM + 10% FMA in diet (FMA_{10}), 80% FM + 20% FMA in diet (FMA_{20}), 70% FM + 30% FMA in diet (FMA_{30}), 60% FM + 40% FMA in diet (FMA_{40}), FMA_0 + 0.4% SG (FMA_0SG), FMA_0 + 0.1% YM (FMA_0YM), FMA_{20} + 0.4% SG ($FMA_{20}SG$), and FMA_{20} + 0.1% YM ($FMA_{20}YM$). Nine groups of Japanese eel each with three replicates were distributed (initial weight of 9 ± 0.2 g) in rectangular tanks receiving flow through water. Each group of the treatment consisted with 15 fish and fed one of the diets for 8 weeks. At the end of the feeding trial, fish fed with the FMA_0 and FMA_{10} diets showed no significant differences in weight gain (WG), specific growth rate (SGR), feed efficiency (FE), and protein efficiency ratio (PER). Meanwhile, fish fed with FMA_{20}, FMA_{30}, and FMA_{40} diets showed significantly lower WG, SGR, FE, and PER than the fish fed with the FMA_0 (control) diet. In addition, there were no significant differences among fish fed with the SG- and YM-supplemented diet groups. However, lysozyme activities in fish fed with the FMA_{10}, FMA_{20}, FMA_{30}, and FMA_{40} were significantly lower than the fish fed with the FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. After 7 days of injection with *V. Anguillarum*, cumulative survival rates of fish fed with the FMA_0SG and FMA_0YM diets were significantly higher than the

FMA_0 diet group. The results revealed that the FMA could replace up to 10% of FM as a protein source in the diet of Japanese eel and both of the natural feed additives (SG and YM) could improve replacing rates of FMA from 10% to 20% without compromising growth and health status of fish.

KEYWORDS

fish meal, fish meal analog, feed additives, growth, hematology, innate immunity, challenge test, Japanese eel

Introduction

It has been predicted that world population would increase up to nearly 10 billion by 2050 (FAO, 2016). This means a 33% rise in demands for varieties of healthy and desirable animal protein sources. Whereas aquaculture is increasingly satisfying the growing demand, further progress of other animal protein production sectors does not seem to be feasible due to environmental and economic barriers (FAO, 2016; UNSD, 2016). However, aquaculture is also facing some major challenges associated with social, financial, and ecological sustainability such as the excesses use of fish meal (FM) due to increased production of aquafeeds (FAO, 2016; Moutinho et al., 2016). Therefore, lowering the inclusion level of FM in aquafeeds could significantly drop expenses and have meaningful effect on reducing the pressure of wild stock overexploitation (Naylor et al., 2009; Kokou et al., 2015).

FM is the most relevant and reliable protein source used extensively in commercial aquafeeds, especially for carnivorous aquatic species. FM is constituted with high-profile biochemical amino acids, high acceptability, and bioavailability (Zhou et al., 2004; Glencross et al., 2007; Kokou et al., 2015). Numerous research works have reported on the replacement of FM as the valuable ingredient in the diets of fish and shellfish through the use of less expensive alternatives such as bacteria (Aas et al., 2006), algae (Kiron, 2012), plants (Gatlin et al., 2007), invertebrates (Barrows and Frost, 2014), and by-products (Fowler, 1991; Tung and Alfaro, 2012). However, plant-derived protein sources such as soybean meal, wheat gluten meal, and corn gluten meal contain some anti-nutritional factors (ANFs) that negatively influence the digestibility of fish (Krogdahl et al., 2010; Oliva-Teles et al., 2015). Whereas, by-products from terrestrial animals are considered as potent alternatives for FM (Tacon, 1993; Moutinho et al., 2016). Fish meal analog (FMA) is a premix of animal by-products and plant ingredients including leather meal, poultry by-product, feather meal, blood meal, squid liver powder, tuna by-product, and soybean meal (Damusaru et al., 2019). With high crude protein, low ANFs (e.g., proteinase inhibitors, lectins, and phytic acid), acceptable palatability (because of tuna by-product and squid liver powder), and rich amino acid profile (by addition of methionine and lysine), FMA could be considered as a qualified candidate for substitution of FM (Jo et al., 2017; Damusaru et al., 2019). In a recent finding, we reported that 15.39% to 20% of dietary FM could replace with FMA in the diets of growing Japanese eel without affecting the growth and health status of the fish.

Feed additives are familiar as supplemental and nutritional/non-nutritional ingredients that added to the formulated diets to increase physical characteristics of feed and/or to enhance biological performance of aquatic animals (Bai et al., 2015). A variety of feed additives such as feeding stimulants and palatability enhancers, antimicrobial agents, antioxidants, binders, pigmentation agents, organic acids, enzymes, immunostimulants, and hormones are used in aquafeeds (NRC, 2011). Song-Gang stone (SG) powder is a natural mineral compound that could found in Republic of Korea. It consists mainly of SiO_2, Al_2O_3, K_2O, Na_2O, Fe_2O_3, and other trace elements (Won et al., 2017). The SG is considered as an immunostimulant that could enhance performance and health status of fish (Choi et al., 2004; Won et al., 2017). Yucca meal (YM) is a naturally manufactured powder derived from a flowering plant known as *Yucca schidigera* (Cheeke et al., 2006; Njagi et al., 2017). YM can act as an antioxidant, antistress, and antimicrobial agent that can enhance growth, immunity, and appetite of target species (Kelly and Kohler, 2003; Piacente et al., 2005; Gaber, 2006; Citarasu, 2010; Güroy et al., 2014; Amoah et al., 2017; Bae et al., 2020). Both of the SG and YM additives show their potentials as immunostimulants in fish in terms of improving chemical composition of diet and boost the nutritional quality of aquafeeds, which ultimately improved the growth and immune performance in fish (Won et al., 2017; Bae et al., 2020).

Japanese eel, *Anguilla japonica*, is an expensive and important freshwater fish species with high medicinal value cultured in several Asian countries especially in China, Japan, and Republic of Korea (Jo et al., 2017; Damusaru et al., 2019). The species is already enlisted as endangered by the International Union for Conservation of Nature and Natural Resources (Jacoby and Gollock, 2014), meaning that special

attention should be directed its aquaculture and nutrition. Therefore, as the price of FM increasing and supply is squeezing around the world, it is imperative to find alternatives of FM for highly carnivorous aquaculture fish species like Japanese eel. For this, the aim of the present study was to evaluate the influence of FM replacement by graded levels of FMA with or without YM and SG supplementations in terms of growth, whole-body proximate composition, hematological indexes, and non-specific immune responses of juvenile Japanese eel. In addition, we also conducted a challenge test with *Vibrio anguillarum*, a globally distributed bacterial pathogen that causes severe hemorrhagic disease in aquaculture farms including Japanese eel (Frans et al., 2011).

Materials and methods

Ethical statement

The experiment was followed under the guidelines of Institutional Animal Care and Use Committee Regulations, No. 554, issued by the Pukyong National University, Busan, Republic of Korea. Every effort was taken to minimize fish suffering.

Fish meal analog

FMA consisted of 15% leather meal (The Feed Co., Seoul, Republic of Korea), 15% poultry by-product meal (Cherry Buro Co., Chungcheongbuk-do, Republic of Korea), 25% feather meal (Cherry Buro Co., Chungcheongbuk-do, Republic of Korea), 2% tuna by-product (Woonam Fish Co., Seoul, Republic of Korea), 24% blood meal (The Feed Co., Seoul, Republic of Korea), 1% squid liver powder (The Feed Co., Seoul, Republic of Korea), 15% soybean meal (The Feed Co., Seoul, Republic of Korea), 2% lysine (The Feed Co., Seoul, Republic of Korea), and 1% methionine (The Feed Co., Seoul, Republic of Korea). Here, lysine and methionine were supplemented in the FMA based on our previous findings (Damusaru et al., 2019).

Experimental design and diets

A total of nine experimental diets were formulated (Table 1) to replace FM with FMA at 0% (FMA_0), 10% (FMA_{10}), 20% (FMA_{20}), 30% (FMA_{30}), 40% (FMA_{40}), FMA_0 + SG (FMA_0SG), FMA_0 + YM (FMA_0YM), FMA_{20} + 0.4% SG ($FMA_{20}SG_{0.4}$), and FMA_{20} + 0.1% YM ($FMA_{20}YM_{0.1}$). The supplementation of SG, SG (Davistone Co. Ltd., Busan, Republic of Korea) and YM, and YM (as De-Odorase,

TABLE 1 Formulation and proximate composition of the experimental diets in juvenile Japanese eel.

Ingredients	Diets (%)								
	FMA_0	FMA_{10}	FMA_{20}	FMA_{30}	FMA_{40}	FMA_0SG	FMA_0YM	$FMA_{20}SG$	$FMA_{20}YM$
Fish meal	65.0	58.5	52.0	45.5	39.0	65.0	65.0	52.0	52.0
FMA	0.00	6.50	13.0	19.5	26.0	0.00	0.00	13.0	13.0
Wheat gluten meal	7.90	7.90	7.90	7.90	7.90	7.90	7.90	7.90	7.90
Corn starch	18.6	18.7	18.9	19.1	19.3	18.6	18.6	18.9	18.9
Chicken oil	0.70	0.60	0.40	0.20	0.00	0.70	0.70	0.40	0.40
Soybean oil	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
Fish oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Cellulose	0.40	0.40	0.40	0.40	0.40	0.00	0.30	0.00	0.30
Song-gang stone[1]	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.40	0.00
Yucca meal[2]	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.10
Vitamin premix[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[4]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Proximate analysis (% of As-is basis)									
Moisture %	10.2	9.40	9.30	9.40	9.16	8.53	8.99	8.54	9.05
Crude protein %	50.2	50.1	50.5	50.3	50.6	50.6	50.2	50.7	50.3
Crude lipid %	7.70	7.50	8.50	8.10	7.80	8.48	8.40	8.00	7.97
Crude ash %	12.1	12.0	11.7	11.5	12.9	12.9	12.7	12.3	11.5

[1]Davistone Co. Ltd., South Korea.
[2]De-Odorase®, Alltech Korea, Seocho-Gu, Seoul, South Korea.
[3]Contains (as mg/kg in diets): ascorbic acid, 300; dl-calcium panthothenate, 150; choline bitatrate, 3,000; inositol, 150; menadione, 6; niacin, 150; pyridoxine·HCl, 15; riboflavin, 30; thiamine mononitrate, 15; dl-α-tocopherol acetate, 201; retinyl acetate, 6; biotin, 1.5; folic acid, 5.4; B_{12}, 0.06.
[4]Contains (as mg/kg in diets): NaCl, 437; $MgSO_4 \cdot 7H_2O$, 1,380; $NaH_2P_4 \cdot 2H_2O$, 878; $Ca(H_2PO_4) \cdot 2H_2O$, 1,367; KH_2PO_4, 2,414; $ZnSO_4 \cdot 7H_2O$, 226; Fe-citrate, 299; Ca-lactate, 3,004; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

Alltech Korea, Seoul, Republic of Korea) was based on our recent studies reported (Won et al., 2017; Amoah et al., 2017; Bae et al., 2020). The experimental diets were iso-nitrogenic at 50% crude protein. The FM, wheat gluten meal, and FMA were main sources of protein in the experimental diets. Soybean oil, fish oil, and chicken oil were main sources of lipid. Dietary corn starch was mainly used as energy source, and cellulose was used as binder in the diets. At first, all dry ingredients were mixed using a mechanical mixing machine (HYVM-1214, Hanyoung Food Machinery, Republic of Korea) thoroughly. Then, filtered fresh water (300 ml kg^{-1} diet) was added simultaneously with fish oil, soybean oil, and chicken oil in the diets. The experimental diets were pelleted using a screw-type pelleting machine (SFD-GT, Shinsung, Republic of Korea) and stored at −20°C until use.

Experimental fish and feeding trial

Juvenile Japanese eel *Anguilla japonica* were purchased from Nonsan fish farm (Nonsan city, Republic Korea) and distributed in 250-L fiberglass tanks belongs to Feed & Foods Nutrition Research Center (FFNRC, Pukyong National University, Busan, Republic of Korea). The fish were adapted to the experimental environment for 4 weeks and fed with the control diet two times per day. After this step, 405 fish with average initial body weight of 9 ± 0.2 g (mean ± SD) were randomly distributed into the 27 glass aquarium tanks (15 fish per tank). The flow of filtered freshwater was maintained in the glass tanks (40-liter capacity) at a rate of 2 L/min from the semi-circulating central tank during the experimental period. The 50% of freshwater in the experimental tanks was daily renewed through clean water that was continuous filtrated and aerated to ensure the high saturation of dissolved oxygen (10 ppm) level. A photoperiod of 12-h light:12-h dark was used throughout the experimental period. The water heater was used to maintain the water temperature at 27.0 ± 0.5°C during the experimental period. Fish were fed with one of the nine experimental diets two times per day (10:00 and 18:00) in triplicate at the rate of 1.5% to 2% of wet body weight for 8 weeks. Fish tanks were daily siphoned at 15:00 pm to clean up the fecal matters and uneaten feeds. Mortality of all treatment groups was checked every day during the experimental period. Dead fish were immediately removed from the tanks and weighed to keep record, if there any died. Total fish weight in each tank was recorded at the end of the fourth week and at the end of the eight week, and the amount of feeds fed to the fish was calculated accordingly. Before each sampling, feeding was stopped and the fish were starved for 24 h to prevent any intervention of additional measurement of ingested feeds by the fish during fish weight sampling as well as to reduce stress in fish.

Growth parameters

At the end of the 8-week feeding trial, weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival rate (SR) of experimental treatments were calculated. Three fish per tank randomly were collected and the weight and length of fish were measured to obtain condition factor (CF), and then, the fish were dissected to measure weight of different organs for organosomatic indices such as weight of visceral mass for viscerosomatic index (VSI) and weight of liver for hepatosomatic index (HSI) in relation to the fish weight. Equations of each variable are as follows:

$$WG(\%) = [final\ weight\ (g) - initial\ weight(g)] \times 100/initial\ weight(g)$$

$$SGR(\%/day) = 100 \times [ln\ final\ weight(g) - ln\ initial\quad weight(g)]/days$$

$$FE\ (\%) = (final\ weight(g) - initial\ weight(g)) \times 100/feed\ ration(g)$$

$$PER = wet\ weight\ gain(g)/protein\ intake(g)$$

$$Survival(\%) = 100 \times (final\ number\ of\ fish/initial\ number\ of\ fish)$$

$$CF = \left[wet\ body\ weight(g)/total\ body\ length^3(cm)\right] \times 100$$

$$VSI(\%) = [wet\ weight\ of\ viscera\ (g)/wet\ body\ weight(g)] \times 100$$

$$HSI(\%) = [wet\ weight\ of\ liver(g)/wet\ body\ weight(g)] \times 100$$

Proximate composition analysis

The proximate compositions of diets and fish were determined according to standard procedures of AOAC (2000). For this, three additional fish per tank were collected according to the experimental group to analyze the whole-body proximate composition. The fresh fish samples were minced and stored in −20°C according to the dietary treatments until further analyses. Samples of the diets and minced fish were dried to a constant weight (1 g for each sample) at 105°C to determine the moisture contents in experimental diets and fish. For the analysis of ash contents, samples (1 g for each sample) were incinerated in a muffle furnace at 550°C for 3 h. For crude lipid analysis, samples (1 g for each sample) were extracted by ethyl-ether to

obtain fat contents using a Soxhlet extraction unit (Soxtec system 1046, Tecator AB, Foss, Hoganas, Sweden). The crude protein contents in the diets and fish samples were analyzed using the Kjeldahl method (N × 6.25) after the determination of nitrogen (N) content through acid digestion, distillation, and titration of the samples.

Serum biochemical and non-specific immune responses analyses

Blood samples were collected from the same fish (three fish per tank) after measuring weight and length of fish but before dissecting the fish for organosomatic indices. The whole blood was withdrawn from the randomly collected three fish per tank and then pooled for the analyses of serum biochemical parameters and non-specific immune responses. For this, fish from the each tank were anesthetized with ethylene glycol phenyl ether (200 mg/L), and blood samples were collected through the puncturing of caudal vein using 1 ml of non-heparinized syringe. Serum was separated from the blood by centrifugation at 5,000 × *g* for 10 min, and then, the transparent supernatant (serum) was stored at −70°C freezer for the biochemical analysis such as glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), glucose, and total protein (TP) and non-specific immune response enzymes such as lysozyme and superoxide dismutase (SOD) activities.

For the analysis of serum biochemical parameters in fish (0.1 ml for each sample), different kits for GPT, GOT, TP, and glucose analyses (Fuji Photo Film Ltd., Tokyo, Japan) were used through an automatic biochemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan) according to the manufacturer's protocols.

For the analyses of non-specific immune responses in fish, turbidometric assay was used according to Hultmark et al. (1980) for serum lysozyme activity with slight modifications. Briefly, serum samples (0.2 ml each) were added to bacterial solution, *Micrococcus lysodeikticus* (0.75 mg/ml) with 0.1 M sodium phosphate buffer (pH 6.4) based on the dietary treatments and according to the manufacturers' protocols. The enzymatic reactions were taken place in a 96-well microplate at 20°C and an absorbance at 570 nm between 0 and 30 min using a microplate reader (TECAN M200 Plate Reader, Tecan Trading AG, Männedorf, Switzerland). A reduction in the absorbance of 0.001/min was considered as one unit of lysozyme activity. The lysozyme activity was analyzed using the following formula:

$$Units/ml = (\Delta A570/min \times 1,000)/ml\ enzyme\ in\ reaction\ mixture$$

Furthermore, SOD activity of serum was determined by the percentage inhibitory reaction of enzyme with WST-1 (water-soluble tetrazolium dye) substrate and xanthine oxidase using a SOD assay kit (Enzo ADI-900-157, Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm, and each point was monitored after incubation of the samples for 20 min at 37°C (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) using the microplate reader (TECAN M200 Plate Reader, Tecan Trading AG, Männedorf, Switzerland). The inhibition percentage was normalized by milligram (mg) protein and presented as SOD enzyme activity unit.

Challenge test against *Vibrio anguillarum*

At the end of 8-week feeding trial, a 7-day long bacterial challenge test was conducted to check the efficacy of experimental diets against disease resistance in fish infected against the pathogenic bacterium, *Vibrio anguillarum*. The pathogenic bacterium, *V. anguillarum* (KCCM 40293, Korean Culture Center of Microorganisms, Seoul, Republic of Korea), was obtained from the Department of Biotechnology, Pukyong National University, Busan, Korea. The bacteria originally sourced from infected Japanese eel with *Vibrio anguillarum* was cultured in tryptic soy broth (tryptic soy agar, Sigma-Aldrich, USA) at 26°C for 24 h and stored with 20% glycerol at −80°C for further use in challenge test. For the challenge test program, fish were re-accommodated in the experimental tanks according to the diet groups, and five fish for each tank were intraperitoneally (i.p.) injected with 0.1 ml per fish at a suitable concentration of 5×10^7 CFU/ml of the pathogenic bacterium based on the study of Lee et al. (2018). The water temperature in the experimental tanks was maintained at 27 ± 0.5°C throughout the challenge test. The water in the experimental tanks was unchanged, and no water circulation is provided in the tanks during challenge test. Everyday mortality of fish was checked and recorded accordingly. If there were any dead fish, then they were immediately removed from the tanks until 7 days. The pathological samples such as skin mucus, liver, gill, and kidney from immediately removed dead fish were analyzed by an API 20 E pathogenic bacteria identification kit (BioMerieux, Durham, NC, USA) to ensure the pathogenicity of *V. anguillarum* in dead fish.

Statistical analysis

The mean values of the fish tanks (n = 3) were subjected to statistical analysis. The Shapiro–Wilk and O'Brien tests were used for normality and homogeneity of variance, respectively. Data were statistically presented using one-way analysis of variance (ANOVA) to verify the effects of the experimental treatments. For the comparison of the experimental treatment means, a Fisher's protected least significant difference (LSD) *post hoc* test was used to elucidate the significant effects of the dietary treatments. The dietary effects were based on the significant level

at $P< 0.05$. The statistical analyses were performed using a SAS version 9.1 analytical software (SAS Institute, Cary, NC, USA). According to Kaplan and Meier (1958), a survivability curve for the challenge test was used to represent the cumulative survival rate in fish fed with the experimental diets.

Results

Growth performance

Growth performance data of juvenile Japanese eel, *Anguilla japonica*, fed with the dietary treatments for 8 weeks are presented in Table 2. At the end of the feeding trial, WG, SGR, FE, and PER of fish fed with the FMA_0 were significantly higher than fish fed with the FMA_{20} diet. However, there were no significant differences among fish fed with the FMA_0, FMA_{10}, FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. Moreover, no significant differences were observed in WG and SGR of fish fed with the FMA_{10}, FMA_{20}, $FMA_{20}SG$, and $FMA_{20}YM$ diets. HSI of fish fed with the FMA_0 diet was significantly higher than fish fed with the FMA_{20} and FMA_{40} diets. However, there were no significant differences in HSI among fish fed with the FMA_0, FMA_{10}, FMA_{30}, FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$. VSI of fish fed with the FMA_0 diet was significantly higher than fish fed with the FMA_{40} diet. However, there were no significant differences in VSI among fish fed with the FMA_0, FMA_{10}, FMA_{20}, FMA_{30}, FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. There were no significant differences in CF and survival among fish fed with all the experimental diets.

Whole-body proximate analysis

Whole-body proximate compositions of juvenile Japanese eel fed with the experimental diets are presented in Table 3. There were no significant differences ($P< 0.05$) in whole-body crude protein, crude lipid, moisture, and ash contents among fish fed with all the experimental diets after the feeding trial.

Non-specific immune responses

Non-specific immune responses of juvenile Japanese eel fed with the experimental diets are summarized in Table 4. Lysozyme activities in fish fed with the FMA_{10}, FMA_{20}, FMA_{30}, and FMA_{40} diets were significantly lower than fish fed with the FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. However, there were no significant differences in lysozyme activities among the fish fed with the FMA_0, FMA_{10}, FMA_{20}, FMA_{30}, and FMA_{40} diets as well as among the fish fed with the FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. There were no significant differences ($P< 0.05$) in SOD activities among fish fed with all the experimental diets

Hematological indices

Serum biochemical parameters of juvenile Japanese eel fed with the experimental diets are summarized in Table 5. There were no significant differences ($P< 0.05$) in serum glucose, TP, GOT, and GPT among fish fed with all the experimental diets at the end of 8 weeks feeding trial.

TABLE 2 Growth performance of juvenile Japanese eel fed with the experimental diets for 8 weeks[1].

Item	Diets									Pooled SEM[10]
	FMA_0	FMA_{10}	FMA_{20}	FMA_{30}	FMA_{40}	FMA_0SG	FMA_0YM	$FMA_{20}SG$	$FMA_{20}YM$	
WG[2]	120^{a}	113^{ab}	107^{b}	94.4^{c}	78.1^{d}	124^{a}	121^{a}	118^{ab}	118^{ab}	2.89
SGR[3]	1.88^{a}	1.80^{ab}	1.73^{b}	1.58^{c}	1.37^{d}	1.92^{a}	1.89^{ab}	1.85^{ab}	1.86^{ab}	0.03
FE[4]	90.0^{a}	87.5^{ab}	81.0^{b}	72.4^{c}	53.7^{d}	93.3^{a}	93.5^{a}	90.1^{a}	89.1^{a}	2.53
PER[4]	1.79^{a}	1.75^{ab}	1.60^{b}	1.44^{c}	1.06^{d}	1.84^{a}	1.86^{a}	1.78^{a}	1.77^{a}	0.05
HSI[6]	1.31^{a}	1.18^{ab}	1.01^{b}	1.08^{ab}	0.95^{b}	1.04^{ab}	1.13^{ab}	1.19^{ab}	1.08^{ab}	0.03
VSI[7]	1.13^{a}	1.03^{ab}	1.06^{ab}	0.97^{ab}	0.82^{b}	1.06^{ab}	1.05^{ab}	1.10^{a}	1.04^{ab}	0.03
CF[8]	0.09	0.08	0.10	0.10	0.09	0.10	0.10	0.09	0.09	0.01
Sur[9]	75.6	84.4	84.4	82.2	73.3	84.4	75.6	86.7	77.8	1.55

[1]Values are means from triplicate groups of fish (n = 3), where values in each row with different superscripts are significantly different ($p< 0.05$).
[2]Weight gain (WG) = (final weight -initial weight) ×100/initial weight.
[3]Specific growth rate (SGR, %) = (ln final weight − ln initial weight) × 100/day.
[4]Feed efficiency (FE, %) = wet WG (g) × 100/dry feed intake (g).
[5]Protein efficiency ratio (PER) = wet weight gain/protein intake.
[6]Hepatosomatic index (HSI, %) = (liver weight/body weight) × 100.
[7]Viscerosomatic index (VSI, %) = (visceral weight/body weight) × 100.
[8]Condition factor (CF) = [fish weight (g)/fish length $(cm)^3$] ×100.
[9]Survival (%) = (final number of fish/initial number of fish) ×100.
[10]Pooled standard error of mean = SD/√n.

TABLE 3 Whole-body proximate composition of juvenile Japanese eel fed with the experimental diets for 8 weeks (% dry matter basis)[1].

Content (%)	Diets									Pooled SEM[2]
	FMA_0	FMA_{10}	FMA_{20}	FMA_{30}	FMA_{40}	FMA_0SG	FMA_0YM	$FMA_{20}SG$	$FMA_{20}YM$	
Moisture	69.8	71.2	69.0	69.4	69.1	69.5	69.3	69.5	69.7	0.25
Crude protein	19.0	18.2	18.8	19.2	19.5	19.1	18.9	19.7	18.7	0.12
Crude lipid	8.45	8.00	9.51	8.74	8.56	8.60	9.56	7.64	8.15	0.32
Ash	2.59	2.71	2.45	2.55	2.78	2.48	2.69	2.79	2.68	0.05

[1]Values are means from triplicate groups of fish (n = 3), where values in each row with different superscripts are significantly different ($p< 0.05$).
[2]Pooled standard error of mean = SD/√n.

TABLE 4 Non-specific immune responses in juvenile Japanese eel fed with the experimental diets for 8 weeks[1].

Activity	Diets									Pooled SEM[3]
	FMA_0	FMA_{10}	FMA_{20}	FMA_{30}	FMA_{40}	FMA_0SG	FMA_0YM	$FMA_{20}SG$	$FMA_{20}YM$	
SOD[2] (% of inhibition)	67.0	68.1	67.5	66.2	65.0	66.5	66.5	67.5	67.8	0.35
Lysozyme (U/ml)	0.62^{bc}	0.60^{c}	0.77^{c}	0.66^{c}	0.64^{c}	0.94^{a}	1.05^{a}	0.90^{ab}	0.91^{a}	0.33

[1]Values are means from triplicate groups of fish (n = 3), where values in each row with different superscripts are significantly different ($p< 0.05$).
[2]SOD, superoxide dismutase.
[3]Pooled standard error of mean = SD/√n.

TABLE 5 Serum biochemical parameters in juvenile Japanese eel the experimental diets for 8 weeks[1].

Content (%)	Diets									Pooled SEM[4]
	FMA_0	FMA_{10}	FMA_{20}	FMA_{30}	FMA_{40}	FMA_0SG	FMA_0YM	$FMA_{20}SG$	$FMA_{20}YM$	
Glucose (mg/dl)	98.7	97.7	90.3	86.0	91.0	101	90.7	91.7	92.7	2.16
Total protein (g/dl)	4.33	4.20	4.27	4.70	4.77	4.43	4.53	4.40	4.10	0.11
GOT (U/l)[2]	113	117	116	119	121	115	115	115	114	1.69
GPT (U/l)[3]	7.67	9.33	7.33	8.00	7.67	7.67	7.67	8.00	6.67	0.21

[1]Values are means from triplicate groups of fish (n = 3), where values in each row with different superscripts are significantly different ($p< 0.05$).
[2]GOT, glutamic oxaloacetic transaminase.
[3]GPT, glutamic pyruvic transaminase.
[4]Pooled standard error of mean = SD/√n.

Challenge test

Cumulative survival rate of juvenile Japanese eel challenged with *V. Anguillarum* for 7 days is shown in **Figure 1**. During the challenge test, the first mortality in fish was recorded on the second day, and it was prominent after the third day of intraperitoneal injection in fish. At the end of 7 days of challenge test program, cumulative survival rates of fish fed with the FMA_0SG and FMA_0YM diets were significantly higher than fish fed with the FMA_0 diet ($P< 0.05$). However, cumulative survival rate of fish fed with the $FMA_{20}SG$, $FMA_{20}YM$, and FMA_0 diets showed no significant differences ($P< 0.05$).

Discussion

The high survival rate recorded during this study could be attributed to the high acceptability of the experimental diets by the juvenile Japanese eel. In the present study, WG, SGR, and FE of fish fed with 20%, 30%, and 40% of FMA were significantly lower than those of control (FMA_0) group. Wang et al. (2013) reported similar findings while replacing FM by dietary poultry by-product meal for Japanese sea bass, *Lateolabrax japonicas*. However, there is scarce information on the replacement of FM by FMA in the diets of any type of fish species. In a study, it was demonstrated that inclusion of 25% of feather meal in the diet of

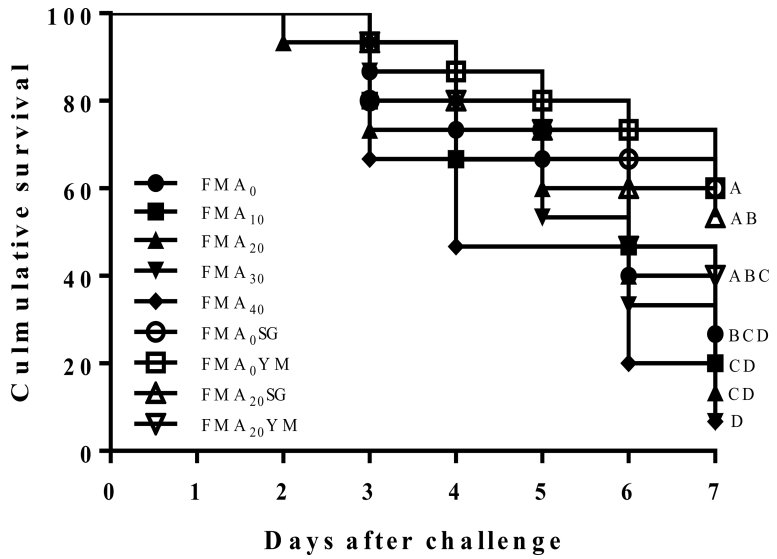

FIGURE 1
Cumulative survival rate after challenged with *V. anguillarum* for 7 days in Japanese eel fed with the nine experimental diets after 8 weeks.

juvenile tench, *Tinca tinca*, significantly lowered the growth performance (total length, total weight, SGR, and FCR) compared with the control treatment without feather meal (González-Rodríguez et al., 2016). One of the main ingredients of FMA is feather meal; thus, our results are in consistent with the findings by González-Rodríguez et al. (2016). It is reported that animal protein sources such as meat and bone meal, feather meal, blood meal, and poultry by-product meal can replace FM up to 50% in salmonid feeds (Lee et al., 2001). Fowler (1991) found that a practical diet consist of 20% of poultry by-product meal could reduce 50% of FM in the diet of chinook salmon without hampering the growth and FE in fish. In another study, MAB (mixture of animal by-product; mixture of leather meal, meat and bone meal, feather meal, squid liver powder, poultry by-product meal, and blood meal) was used as a fishmeal replacer in the diet of juvenile rainbow trout, and lower WG, SGR, FE, and PER were reported for fish fed with 20% of FM replacement level (Lee et al., 2001). To the best of our knowledge, the results are the first to use six animal protein sources in combined form as FMA to replace FM in the diet of a fish species (Lee et al., 2001). Feather meal as an alternative animal protein source was found to be slight replacer for FM in the diets for coho salmon (Higgs et al., 1979), chinook salmon (Fowler, 1991), and Nile tilapia (Bishop et al., 1995). Blood meal was also evaluated as a FM replacer in the diets for rainbow trout (Luzier et al., 1995), eel (Lee and Bai, 1997a), and tilapia (Lee and Bai, 1997b). Ma et al. (2014) indicated that more than 21% of FM can be replaced by poultry by-product meal in the diets for golden pompano. Likewise, the present study demonstrated that the dietary FMA with addition of additives produced similar responses in terms of growth increment, feed utilization, and immunity in juvenile Japanese eel. In this study, the results demonstrated a more positive response of juvenile Japanese eel to dietary SG and YM supplementations compared with the control diet. Fish fed with the FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets exhibited significantly higher WG, SGR, FE, and PER compared with fish fed with the FMA_{20} diet but they were not significantly different among FMA_0, FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diet groups. The results indicate the potential of the supplementation of natural feed additives in replacing FM in the diet of juvenile Japanese eel. In consistent with the present study, growth enhancement by Yucca extract additives have been reported previously in several fish species such as channel catfish, tilapia, and striped catfish (Kelly and Kohler, 2003; Gaber, 2006; Güroy et al., 2014). Francis et al. (2001) reported that supplementation of *Quillaja* saponin at 150 mg/kg diet can increase the growth performance of Nile tilapia. Likewise, it is reported that dietary additive, *Quillaja* saponin, at the same inclusion level (150 mg/kg) could increase 18% of average weight of common carp. In another study, Güroy et al. (2014) found that a diet containing 0.15% of *Yucca schidigera* extract could improve the zootechnical parameters in striped catfish (*Pangasianodon hypophthalmus*). Moreover, supplementation of Yucca in the diet of Nile tilapia could increase the feed utilization, apparent protein digestibility coefficient, and whole-body protein content of the fish (Gaber, 2006). The observations were similar to the findings by Won et al. (2017); Amoah et al. (2017), and Bae et al. (2020) with yellow loess, SG, and YM in rainbow trout, Amur catfish, and olive flounder fish. It is widely reported that dietary plant extract

together with minerals can enhance the growth performance in fish through lipid metabolism by the breakdown of fatty acids and production of energy (Ji et al., 2007). According to our results, more than 10% replacement of FM resulted in reduction of growth, which is probably due to the inferior quality of the animal protein sources, where leather meal and squid liver powder were found to be effective in the diets of freshwater fish species (Higgs et al., 1979). Animal by-products are important source of minerals especially calcium (Ca), iron (Fe), and manganese (Mn) that may exert the antagonistic behavior of Ca with other minerals during the mineral absorption in fish (Sugiura et al., 2000). When FM is replaced with FMA that constitute with blended ingredients from animal by-products, it is difficult to interpret the results in nutrient metabolism due to the presence of minerals in the diets (Jamil et al., 2007). Consequently, the findings of the present study suggest up to 10% of FM can be replaced by FMA with the addition of YM and SG as natural mineral additives (Won et al., 2017) in the diets of juvenile Japanese eel diets, which ultimately improved the growth and feed utilization in fish.

CF, HSI, and VSI are important measurement tools in terms of assessing food value in an animal and its health status and general welfare (Ighwela et al., 2014). In the present study, there were significant differences in HSI and VSI between fish fed with the control and FMA_{40} diet groups, which can be attributed to the reduced growth, feed utilization, and health status in fish at higher replacement level of FM with FMA in diet. Abdel-Warith et al. (2001) reported the alterations of liver tissues in terms of degeneration in liver structures in African catfish fed with high inclusion levels of poultry by-product meal in relation to FM in the diets. Therefore, it has utmost importance to examine the liver and gastrointestinal tract (GIT) of fish in replacing FM with the rendered animal protein ingredients in the fish diets. In this study, insignificant and high survival rate of fish might be due to the high acceptability of the experimental diets by the juvenile Japanese eel.

Whole-body proximate composition in fish fed with the experimental diets was not significantly affected after chemical analyses. Gaber (2006) reported higher body protein and low body lipid levels in Nile tilapia fed Yucca-supplemented diets in comparison with the basal diet. In a related study, Yilmaz et al. (2012) also found lower crude lipid levels in sea bass, *Dicentrarchus labrax*, fed with the herbal extracts compared with the control diet. According to these studies, plant extracts could reduce the crude lipid contents in cultured fish. However, the observations were on contrary to our results and the reason could be related to different diet compositions and culture conditions. In agreement with the present study, Damusaru et al. (2019) did not find any significant effect of different levels of FMA replacing FM on proximate composition of growing Japanese eel.

Non-specific immune response is a primary or basic defense system in fish which also plays an important function in the specific or secondary immune system and body homeostasis through a system of receptor proteins (Halver and Hardy, 2002; Uribe et al., 2011; Reverter et al., 2014; Srivastava and Pandey, 2015). Lysozyme is produced mainly by macrophages in response to microbial components and many other immune stimulants (Siwicki and Anderson, 1993; Ringø et al., 2012), and it is a preferred marker of the immune response due to its close association with leucocytes (Kiron, 2012). On the other hand, SOD is a metallo-enzyme that catalyzes the dismutation of the superoxide radical (O_2) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Radi et al., 1985), providing an important defense against oxidative damage. In the present study, no significant differences for serum SOD enzyme were observed in fish fed with the experimental diets. However, serum lysozyme activities of fish fed with the diets consisting of SG and YM were significantly higher than other diets, which indicate the enhanced immunity function in fish due to the supplementation of natural minerals (SG and YM) in the diets. Improved immunity has been reported in cultured fish and shellfish fed with several feed additives such as mushroom extract (Chelladurai and Maran, 2019), yellow loess (Won et al., 2017), SG (Won et al., 2017; Bae et al., 2020), and YM (Njagi et al., 2017; Bae et al., 2020). Ji et al. (2015) demonstrated that 80% replacement of FM by silkworm pupae meal in the diet of juvenile Jian carp (*Cyprinus carpio*) significantly decreased hepatic SOD activities. These results are partly comparable with our results showing the beneficial effects of SG and YM for improving non-specific immune responses.

Hematological and serum parameters are good indicators of the health status of an organism, and they can vary with season, temperature, and nutritional status (Blaxhall, 1972). Bae et al. (2020) suggested that any unhealthy condition caused by poor nutrition could affect the hematological characteristics of fish. Glucose is one of the stress indicators in fish. Stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish to cope with the energy demand of a stressor (Aedo et al., 2019). Total plasma protein is considered a strong innate response in fish (Reverter et al., 2014), and measurements can reflect nutritional status (Acharya and Mohanty, 2014). It is the protein component of the blood, which increases with starvation or any other stress. Plasma GOT and GPT, on the other hand, are proteins or enzymes found mainly in liver cells and elevated levels in the blood indicate an inflammation or damage of liver cells (Giannini et al., 2005). In the present study, no significant differences were recorded in all serum parameters in juvenile Japanese eel fed with the experimental diets, indicating good health status of the fish. In the present study, challenge test supports the results of the immune responses such as lysozyme activities in fish fed with the experimental diets.

Conclusions

In summary, the results indicated that WG, SGR, FE, and PER were significantly different in fish fed with the FMA_0

(control diet) diet compared with fish fed with the FMA_{20}, FMA_{30}, and FMA_{40} diets. Whereas no significant differences were observed among fish fed with the FMA_0, FMA_{10}, FMA_0SG, FMA_0YM, $FMA_{20}SG$, and $FMA_{20}YM$ diets. Whole-body proximate composition of fish did not show significant differences among treatment groups. Fish fed with the diets consisting of SG and YM showed a higher lysozyme activity than all other diets that replaced FM with FMA. In addition, there were no significant differences in the hematological indexes among fish fed with the experimental diets. Therefore, on the basis of the results for WG, SGR, FE, PER, lysozyme, SOD, and hematological indexes, FMA could replace up to 10% of FM in the diets of juvenile Japanese eel. However, considering the natural minerals as additives, supplementation of dietary SG and YM in FMA could replace up to 20% of FM in the diets of juvenile Japanese eel.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee Regulations, No. 554, issued by the Pukyong National University, Busan, South Korea.

Author contributions

JB has planned the experiment; determined the growth, somatic indices, non-specific immune enzyme activities, and hematology; and drafted the final article. SgL did supervision, statistical analyses, and final drafting of the manuscript. MM analyzed the statistical data and finalized the manuscript. AH, WC, and SnL determined the growth, somatic indices, non-specific immune enzyme activities, and hematology. TM helped in final drafting and checking grammar of the manuscript. S-KK and SB critically supervised and helped in experimental planning with the addition of manuscript drafting and finalizing. The authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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