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Effects of probiotic supplementation of a plant-based protein diet on intestinal microbial diversity, digestive enzyme activity, intestinal structure, and immunity in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

The aim of this study was to investigate the effects of intestinal microbial manipulation by dietary probiotic supplementation on digestive enzyme activity, immune-related gene transcription, intestinal structure alteration, and viability against pathogenic challenge in olive flounder. Similar-sized flounders (14.92 ± 0.21 g) were divided into three groups and supplemented with a control (without probiotic) or 1×10^8 CFU/g diet of each of *Bacillus* sp. SJ-10 (ProB) and *Lactobacillus plantarum* (ProL) for eight weeks. At the end of the feeding trial, the estimated intestinal microbial richness (Chao1) and diversity (Shannon) demonstrated a significant ($P < 0.05$) abundance in the ProB group (484.80 ± 88.75, 5.08 ± 0.17) compared to the ProL (285.32 ± 17.78, 4.54 ± 0.09) and control groups (263.23 ± 20.20, 4.30 ± 0.20). A similar alteration phenomenon was also found at the phylum level, with a higher abundance of Proteobacteria, Actinobacteria, and Acidobacteria. Trypsin and lipase activities were elevated in both the ProB and ProL groups compared to the control, but amylase was only higher in the ProB group. The expression levels of pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 were significantly higher in the ProB group than in the other two groups. There was a significant increase in transcription of IL-10 in both the ProB and ProL groups compared to the control. The length of villi and microvilli of probiotic-fed olive flounder was increased but was not significantly different from the control group. In an *in vivo* challenge experiment with *Streptococcus iniae* (1×10^8 CFU/mL), the survival rates of the ProB and ProL groups were 29.17% and 12.50%, respectively, when control mortality reached 100%. Therefore, intestinal microbiota manipulation by probiotic supplementation increased the richness of the bacterial population, digestive enzyme activity, intestinal immune gene transcription, and infectious disease protection in olive flounder.

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is an important fish species in the Korean aquaculture industry, with an annual production over the last five years of 36,944–45,759 tons, representing about 47–53% of the nation's total finfish production [1]. The culture of this carnivorous fish requires a high protein diet to fulfill the optimum 40–70% protein requirement [2]. Normally, 50–70% of the flounder diet consists of fish meal (FM) [3]. Although FM is a very important animal protein source, its production for the global aquaculture industry is no longer increasing and its price is increasing continuously [4]. The stagnation of FM production limits the further growth and profitability of the aquaculture industry [5]. Most FM alternatives are plant-originated protein sources, such as soybean, corn gluten, and canola meals [6–9]. However, plant protein is not as efficient as FM because some essential amino acids (*e.g.*, methionine and lysine) are lacking and the presence of anti-nutrient factors (*e.g.*, saponin, phytic acid, and lectin) reduce nutrient intake, digestion, absorption, and utilization [9–11].

The microbiota is a complex micro-ecosystem found in multicellular organisms, such as plants and animals [12]. The intestinal microbiota of fish contains a variety of microorganisms, such as bacteria, archaea, and fungi, which play an important role in their host's intestinal

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structure, enzyme secretion, intestinal immunity, pathogen eradication, metabolic homeostasis, and disease resistance [13–15]. Studies have shown that the manipulation of microbiota composition in the intestine of farmed fish can affect the host's physical and immunological health, and protect against infection [12,16]. The intestinal microbial community diversity composition is affected by intestinal structure, water quality, bacterial and viral infection, host feeding habit (herbivorous, carnivorous, or omnivorous), and, importantly, dietary supplementation [17–19].

Supplementation with live microorganisms (*i.e.*, probiotics) through dietary administration, can be applied to regulate the composition of the intestinal microbial community in farmed fish [16]. Intestinal bacterial manipulation has beneficial effects, such as increased immunity, digestibility, and infectious pathogenic bacterial disease resistance [10]. Previously we identified and quantified improved growth and feed utilization parameters, innate immunity, and disease resistance as a result of probiotic inoculated diet supplementation, where 30% of FM was replaced by soybean meal [10,20]. Probiotic supplementations also resulted in a higher survival against *Streptococcus iniae* infection, which has also been reported in other studies [10,21,22].

To understand the efficacy of a supplemented diet in olive flounder, it is very important to identify the changes that occur in the intestinal microbial community after probiotic supplementation. Manipulation by dietary probiotics affects the secretion of digestive enzymes, intestinal structure, and immunity, and pathogenic bacterial challenges in olive flounder have not been investigated yet. A knowledge of intestinal microbiota will improve our understanding of the relationship between intestinal microbiota and host health, nutritional absorption, and immune system activity [23,24].

The aim of this study was to understand the functions of probiotics in olive flounder intestines and to clarify the relationship between probiotic supplementation and changes in intestinal microbial diversity. Investigations of digestive enzyme activity, intestinal villus and microvillus length, and immune gene transcription were also conducted after probiotic supplementation in a 30% FM replaced diet.

2. Materials and methods

2.1. Probiotic bacteria and diet preparation

The composition of the basal diet is listed in Table 1. Among the feed ingredients, soybean meal, corn gluten meal, wheat gluten, and soy protein concentrate were used as plant protein sources.

Bacillus sp. SJ-10 (JCM 15709, KCCM 90078) was isolated form traditional Korean seafood in previous study [25]. *Lactobacillus plantarum* (KCCM 11322) [26] was purchased from the Korean Culture Center of Microorganisms (Seoul, South Korea).

Table 1
Composition of the basal diet.

Ingredients	Percentage (%)	Ingredients	Percentage (%)
Brown FM	45.5	Threonine	0.3
Soybean meal	12.8	Methionine	0.3
Corn gluten meal	5.0	Lecithin	0.5
Wheat flour	14.7	Monocalcium phosphate	1.4
Wheat gluten	5.85	Mineral Mix[a]	1.0
Soy protein concentrate	5.85	Vitamin Mix[b]	1.0
Fish oil	4.7	Choline	0.5
Lysine	0.5	Cellulose	0.1

[a] Mineral premix (as mg kg^{-1} in diets): NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; $Ca(IO)_3$, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

[b] Vitamin premix (as mg kg^{-1} in diets): Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitate, 3000; Inositol, 150; Menadion, 6; Niacin, 150; Pyridoxine. HCl, 15; Rivoflavin, 30; Thiamine mononitrate, 15; dl-α-Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06.

The culture and supplementation of *Bacillus* sp. SJ-10 to the diet was performed as described by Hasan et al. [10,20]. Briefly, SJ-10 spore formation was induced in a sporulation medium (0.8% Bacto nutrient broth, 0.1% KCl, 0.025% MgSO4·7H2O, 0.0002% MnCl2·4H2O, 0.0055% CaCl2, 0.00003% FeSO4, pH 7.0) (Difco, USA) and confirmed by malachite green staining. The final concentration was confirmed by a serial dilution method on an LB (USB Corporation, USA) agar plate and spores were added to contain 1×10^8 CFU in each gram of the diet (ProB). *L. plantarum* was added to the diet using the spray method as described by Heo et al. [27,28]. Cultured L. *plantarum* in MRS (Difco, USA) agar was washed with phosphate-buffered saline and sprayed to contain 1×10^8 CFU in each gram of the diet every 3 days (ProL).

2.2. Maintenance of animals and feeding experiment

Juvenile olive flounders were obtained from a commercial hatchery (Won-Hong Susan, 34°47′35.6″N 128°34′20.7″E) and distributed to nine tanks (40L semi-recirculating seawater tank, 18 fish·$tank^{-1}$) for acclimatization for two weeks. During the acclimatization period, commercial feeds (Suhyupfeed Co., South Korea) were supplied. After two weeks, feeds were given at 2–2.5% of body weight up to apparent saturation twice in a day (9:00 and 17:00 h) for 8 weeks [10]. Stable aquatic environment parameters [water temperature (17.5 °C ± 0.5 °C), water flow (1.2 L/min), dissolved oxygen (7.0 mg/L), salinity (32 ± 1 ppt), and pH (7.4 ± 0.5)] were maintained.

2.3. Analysis of the intestinal microbiota

To investigate the effect of probiotics on the intestinal microbiota of olive flounder, after eight weeks of feeding, the whole intestines of randomly selected fish were sampled 24 h after the last feeding from each group. Total DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocol. The quality and quantity of DNA were measured on a NanoDrop Lite Spectrophotometer (Thermo Scientific, USA). Library construction and MiSeq sequencing (Illumina, USA) were conducted at Macrogen Inc. (Seoul, South Korea), with V3–V4 primers used to compare the V3 to V4 regions of 16S rRNA (V3–V4–F: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG, V3–V4-R: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C). The Quantitative Insights Into Microbial Ecology (QIIME) data analysis package was used to process the 16S rRNA data analysis. Fast Length Adjustment of SHort reads (FLASH) software was used to merge paired-end reads from original DNA fragments. Operational taxonomic units (OTUs) were assigned by performing three steps: raw read filtering and trimming, error-free read picking, and different distance cut-offs (97%).

2.4. Analysis of digestive enzymes

Three fish per tank were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, USA) and their intestines were sampled for an enzymatic activity assay. The samples were processed according to the procedure provided in each enzyme activity assay kit.

2.4.1. Amylase activity

Amylase (EC 3.2.1.1) activity was measured using an amylase activity colorimetric assay kit (BioVision, USA). Briefly, 100 mg of tissue was extracted by adding 0.5 of mL assay buffer and centrifuging for 10 min to separate the supernatant. A mixture of 50 μL supernatant and 100 μL reaction mix solution containing ethylenediamine-pNP-G7 as a substrate was prepared. Absorbance over time was measured at 405 nm using an ELISA multiplate reader (Sunrise, Tecan, Austria). One unit (U)

of amylase activity was defined as the amount of amylase that cleaved ethylenediamine-pNP-G7 to generate 1.0 μmol of nitrophenol per minute.

2.4.2. Trypsin activity

Trypsin (EC 3.4.21.4) activity was measured using a trypsin activity colorimetric assay kit (BioVision, USA). Tissue was extracted with four volumes of the assay buffer and was centrifuged for 10 min to obtain a clear extract. A mixture of 50 μL supernatant, 1 μL 50X chymotrypsin inhibitor solution, and 50 μL reaction mix solution (48 μL assay buffer, 2 μL *p*-nitroaniline as a trypsin substrate) was prepared. The mixture was incubated at room temperature, protected from light. Absorbance over time was measured at 405 nm. One unit (U) of trypsin activity was defined as the amount of trypsin that cleaved the substrate to yield 1.0 μmol of *p*-nitroaniline per minute.

2.4.3. Lipase activity

Lipase (EC 3.1.1.4) activity was measured using a lipase activity colorimetric assay kit II (BioVision, USA). Lipase substrate was added to 100 μL of tissue extracted with four volumes of assay buffer and measured at 412 nm. One unit (U) of lipase was defined as the amount of lipase that hydrolyzed the substrate and generated 1.0 μmol of TNB per minute.

2.5. Analysis of immune-related gene expression

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to identify the effect of probiotic-supplemented feed on the transcription of immune-related genes in the intestine. Intestine samples were collected from nine fish per group (3 fish per tank). Total RNA was prepared using a GeneAll Hybrid-R RNA purification kit (GeneAll Biotechnology, South Korea). To remove the residual DNA, a Riboclear plus kit (GeneAll Biotechnology, South Korea) was used. Total RNA concentration and purity were determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific, USA), and first-strand cDNA obtained from 1 μg of RNA was synthesized using a PrimeScript 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's protocol. qRT-PCR was performed using TB Green Premix Ex Taq (Takara, Japan). The gene-specific primers used in this study are presented in Table 2 β-Actin was used as a reference gene, and its relative quantification was analyzed with the thermal cycler dice software (V5.0x) installed in the thermal cycler dice (Model TP700/760, Takara, Japan), using the $2^{-\Delta\Delta CT}$ method.

Table 2
Gene specific primers, product size and gene bank accession number of the gene used in this study.

Gene	Sense	Oligonucleotide Sequence (5′ to 3′)	Product size (bp)	Access No.
β-actin	F	CATCAGGGAGTGATGGTGGGTA	107	HQ386788.1
	R	ATACCGTGCTCGATGGGGTACT		
TNF-α	F	CAGCAGCGTCACTGCAGAGTTA	120	AB040448.1
	R	GTTACCACCTCACCCCACCATT		
IL-10	F	AGCGAACGATGACCTAGACACG	114	KF025662.1
	R	ACCGTGCTCAGGTAGAAGTCCA		
IL-6	F	CAGTGCCAACTTCAGCAAGGAG	130	DQ267937.1
	R	GTGATATCTGGCGTGCAAGAGG		
IL-1β	F	CATCACCACTGTCTGCTGGAAA	122	KF025662.1
	R	GCTACTCAACAACGCCACCTTG		
CD4-1	F	AGGTGCCAGTGAGGTGGTTTAT	112	AB716323.1
	R	GCCGTCCTGTTTACCAAAACTC		
CD4-2	F	CTCTGTTTCATGCCAAGGTGTC	109	AB716324.1
	R	CTTGCAGGTAAACATCCCACTG		
IFN-γ	F	GAATTGCACCGTCGACTACAAG	108	AB435093.1
	R	GGATGTGTGATCCTCTGACCTG		
CD-18	F	TACCATGGTGACTTCTGCGAGT	117	KR998307.1
	R	CCTCGTAGCCTGTGTGACAATC		
CD-83	F	CTGTGAGGTGGTACAGGGTCAG	113	KY354513.1
	R	CCACCTCTCTGTCCAGACCATA		

2.6. Intestinal histology

Intestinal histology was evaluated as described by Abid et al. [29]. The intestinal tissues from olive flounder (3 fish/group) were fixed in 10% neutral buffered formalin. The dehydration process used a graded series of ethanol and a solid wax block was made using paraffin. The embedded flounder intestine was sectioned at 5-μm intervals with a microtome, and stained with hematoxylin and eosin. The length of the villi and microvilli of the muscular layer were observed with a light microscope.

2.7. Challenge with S. iniae

After eight weeks, 24 fish from each group were anesthetized with 2-phenoxyethanol and injected intraperitoneally with 100 μL (1×10^8 CFU/mL) of *S. iniae* (KCTC 3657), which was purchased from the Korean Collection for Type Cultures (Seoul, South Korea). The number of dead fish was recorded every 6 h. Swabs from dead fish gills, kidney and liver were collected and spread on BHI agar plate (Difco, USA) to confirm streptococcosis. The relative percentage of survival (RPS) was calculated using the following equation: RPS = 100–100 × (test mortality/control mortality).

2.8. Statistical analysis

All data were analyzed by a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) followed by Duncan's multiple range test. Statistical significance was accepted at a P-value < 0.05 unless otherwise noted.

3. Results

3.1. Diversity analysis

An average of 75,744 high-quality reads (average length 453 bp) from the control, ProB, and ProL groups were obtained, resulting in 259 ± 19.31, 477 ± 87.71, and 312 ± 36.72 bacterial OTUs, respectively. Table 3 shows the Chao1 and ACE index values for estimating richness and the Shannon and Simpson index values for estimating diversity. The richness and diversity of the groups followed the order ProB > ProL > control. The ProB group had significantly higher levels of richness and diversity than the other groups.

3.2. Beta diversity analysis

The beta diversity based on weighted unifrac metrics was analyzed using a principal coordinate analysis. The control and ProL groups were located closer together, indicating their similarity, whereas the ProB group was less similar, indicating a difference in intestinal microbiota composition (Fig. 1).

3.3. Taxonomic composition analysis

At the phylum level, Firmicutes were the most abundant in all groups, followed by Bacteroidetes. The relative abundances of the *Proteobacteria* (7.85 ± 2.48%), *Actinobacteria* (5.00 ± 2.25%) and *Acidobacteria* (0.25 ± 0.06%) in the ProB group were significantly higher than in the other groups (Fig. 2). At the genus level, *Aerococcus* and *Bacteroides* were abundant in all groups. The abundance of *Bacillus* followed the order ProB (0.11 ± 0.03%) > ProL (0.01 ± 0.01%) > control (0%), and was significantly higher in the ProB group than in the other groups. The abundance of *Lactobacillus* followed the order ProL (1.19 ± 0.09%) > ProB (0.67 ± 0.15%) > control (0.24 ± 0.04%), and was significantly

Table 3
Alpha diversity of intestinal microbiota of olive flounder.

Diets	Sampling depth	Richness estimate		Diversity estimators		Good coverage
	Reads	Chao1	ACE	Shannon	Simpson	
Control	74294	263.23 ± 20.20	270.50 ± 21.84	4.30 ± 0.20	0.97 ± 0.01	0.99 ± 0.01
ProB	75888	484.80 ± 88.75	495.83 ± 90.43	5.08 ± 0.17	0.99 ± 0.01	0.99 ± 0.01
ProL	77050	318.65 ± 40.78	326.90 ± 43.58	4.51 ± 0.09	0.97 ± 0.01	0.99 ± 0.01

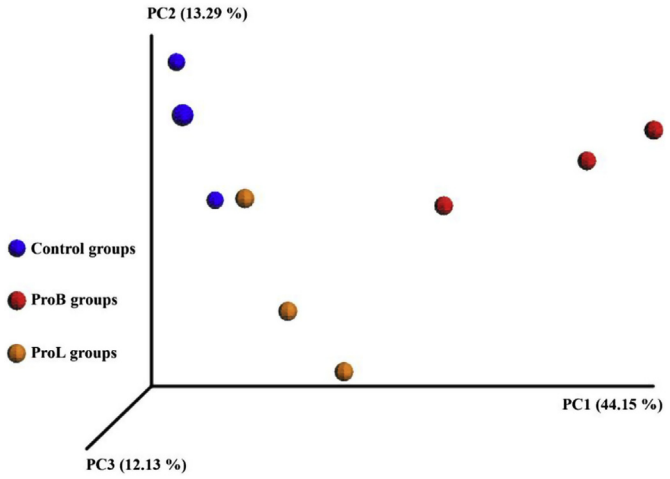

Fig. 1. Principal coordinate analysis based on the weighted unifrac metrics of bacterial operational taxonomic units between the different diets.

higher in the ProL group than in the other groups (Fig. 3). The heatmap analysis of olive flounder intestine microbiota abundance at the genus level demonstrated that the ProB group was clearly different from the other groups, whereas the ProL and control groups were relatively similar (Fig. 4).

3.4. Digestive enzyme activity

The activity of digestive enzymes in olive flounder intestine is shown in Fig. 5. The activity of the ProB group was significantly higher than that of the other groups for all measured factors. Lipase and trypsin activity were significantly higher in the ProL (123.06 ± 18.63, 3.70 ± 0.58 mU/g) group than in the control group (65.16 ± 11.66, 2.30 ± 0.17 mU/g). Amylase activity was higher, albeit non-significantly, in the ProL group (5.98 ± 1.67 mU/g) than in the control group (3.72 ± 0.93 mU/g).

3.5. Immune responses of the intestine

The relative expressions of immune-related genes in the intestine are shown in Fig. 6. The ProB group had significantly altered expression of pro-inflammatory cytokines. Tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 expression were increased by about 2.23, 3.16, and 1.54 times in the ProB group compared with the control group. The ProL group had lower relative expression levels of pro-inflammatory cytokines compared to the control group. The relative expression levels of TNF-α, IL-1β, and IL-6 were 0.81, 0.67, and 0.99-fold, respectively, those of the control group. There were significantly higher expression levels of IL-10 in both the ProB (4.20-fold) and ProL groups (3.15-fold) than in the control group. There were no significant differences in the expression levels of CD4-1 and CD4-2 (the two subtypes of T cells), CD18 (which is expressed on myeloid cells and natural killer cells), and the mature dendritic cell marker CD83.

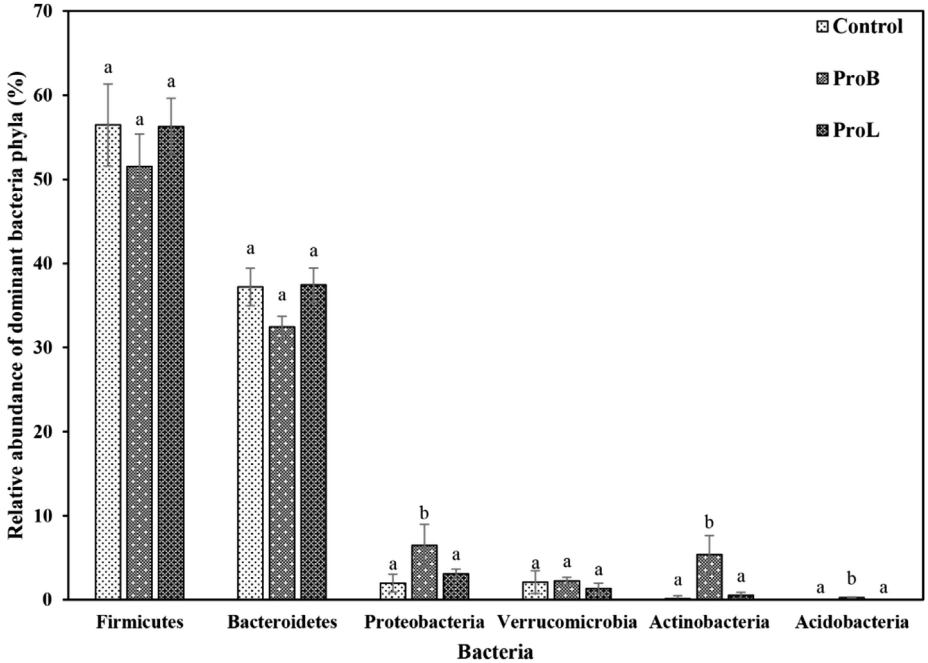

Fig. 2. Composition and relative abundance of the intestinal bacterial communities of olive flounder with different diets at the phylum level. Data represent the mean ± standard deviation; means that do not share the same letter differ significantly ($P < 0.05$).

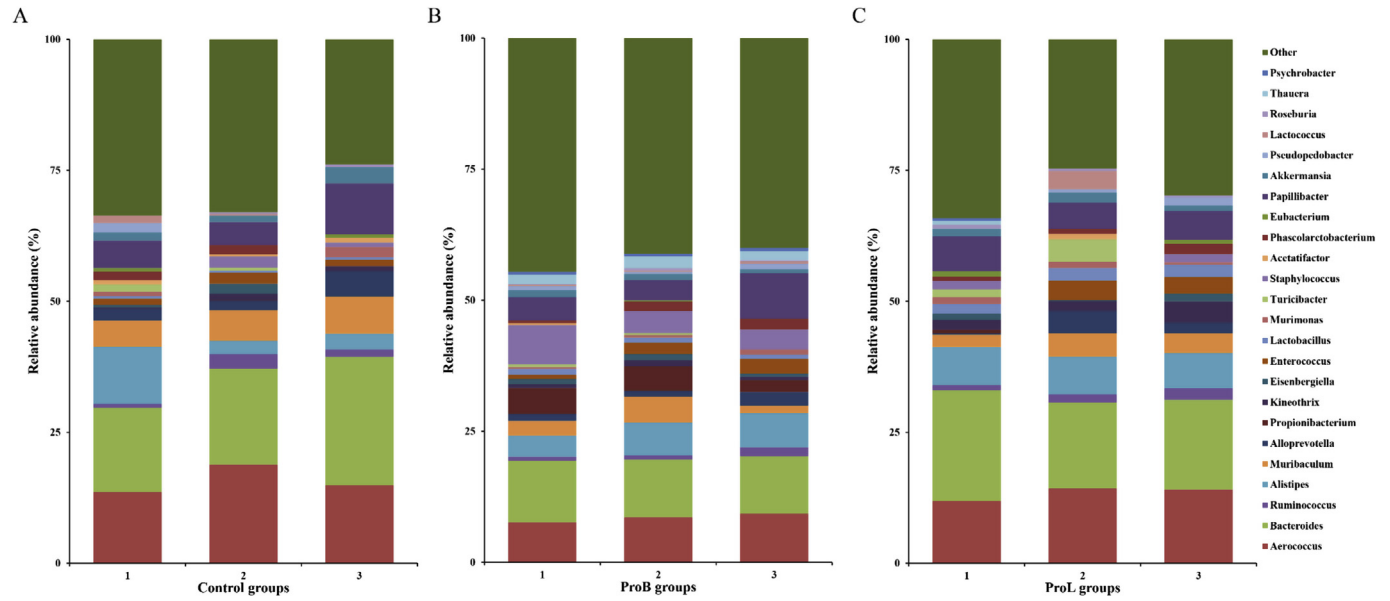

Fig. 3. Composition and relative abundance of the intestinal bacterial communities of olive flounder with different diets at the genus level. Results from three randomly selected olive flounders from the control (A), ProB (B), and ProL (C) groups.

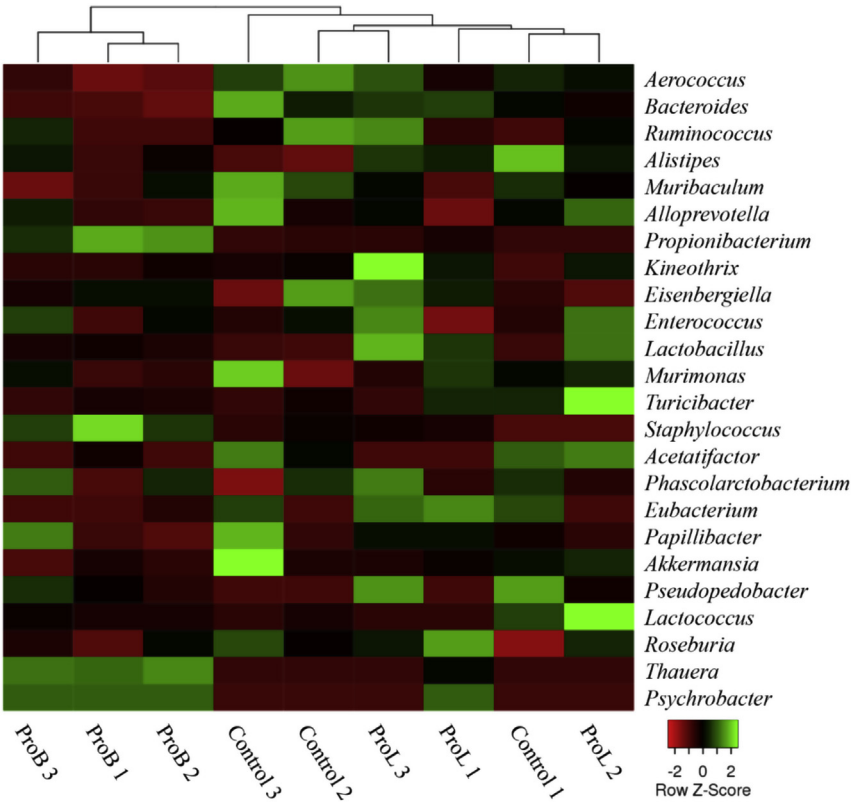

Fig. 4. Heatmap analysis of the genus abundance within the olive flounder intestinal microbiota following random sampling from each group. Green represents the more abundant genus in the corresponding sample and red represents the less abundant genus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.6. Intestinal histology

The villus and microvillus length of olive flounder intestines were examined by histological examination (Fig. 7). The mean villus lengths of the control, ProB, and ProL groups were 695.24 ± 91.84, 795.24 ± 21.82, and 685.71 ± 75.59 μm, respectively. The microvillus lengths were 1.51 ± 0.06, 1.59 ± 0.08, and 1.59 ± 0.05 μm, respectively. There was no significant difference in the villus and microvillus lengths in olive flounder intestines between the groups.

3.7. Survival after S. iniae challenge

The highest survival rate of olive flounder after a *S. iniae* challenge was observed in the ProB group (Fig. 8). Moreover, at 9 days post-challenge, the RPS of the ProB group (44.44%) was higher than that of the ProL group (27.78%). Significant differences were only apparent between the ProB and control groups.

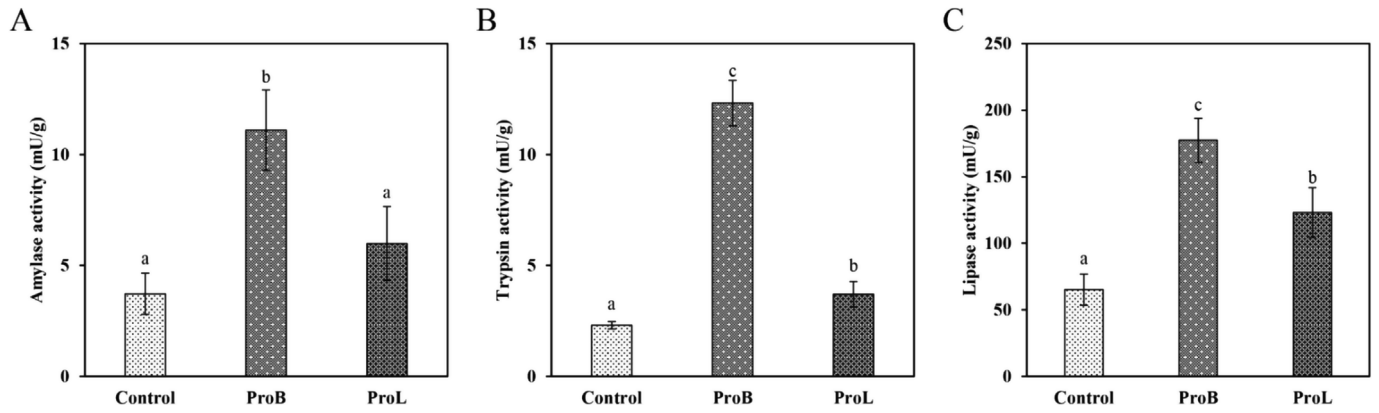

Fig. 5. Comparison of the measured amylase (A), trypsin (B), and lipase (C) activities between the three groups. Data represent the mean ± standard deviation; means that do not share the same letter differ significantly ($P < 0.05$), respectively.

4. Discussion

It has been previously reported that a diet supplemented with probiotics increases growth and disease resistance, and stimulates immune responses in farmed fish [10]. Colonized probiotics in the host's intestine prevent the adhesion of pathogens and produce a variety of enzymes that help to increase host digestion, resulting in better feed utilization [30]. Antimicrobial peptides produced by some probiotics also remove infectious pathogens [31]. In addition to these direct effects, the dietary intake of probiotics can have some complex effects due to changes in the microbial community in fish intestine. Host gut microbiota and their interaction with the administrated probiotics is very important. Satisfactory interactions provide host immunity benefits, which was the main focus of this study.

There have been few studies of the intestinal microbiota in olive flounder; therefore, our understanding of the relationship between intestinal microbiota and fish health is limited. A comparison of the intestinal microbial diversity of wild and farmed olive flounder was reported from a study that adopted a culture-dependent approach [32]. However, fish intestinal microbiota is not easily cultivable and in some species its efficiency is less than 0.1% [33]. Therefore, intestinal microbiota analysis using culture-independent technologies, such as next-generation sequencing (NGS), is necessary to quantify the relationships between intestinal microbiota and host health.

The intestinal microbiota plays an important role in digestion, absorption, metabolism, and defense against pathogens [34–36]. In this study, the diversity of intestinal microbiota clearly differed between the three test groups. Interestingly, although only one strain was provided in each treatment diet, the probiotic-supplemented group had a higher microbial diversity than the unsupplemented group. Similar results have been reported for other commercially cultured species, *e.g.*, Nile tilapia (*Oreochromis niloticus*) [37], whiteleg shrimp (*Litopenaeus vannamei*) [38,39], and sea cucumber (*Apostichopus japonicas*) [36]. Wang et al. reported that probiotic supplements could change the intestinal environment (*e.g.*, pH), providing a more favorable environment for microorganisms derived from seawater to colonize the intestine [36]. Due to these kinds of changes in microbial diversity, a host supplied with a probiotic is more likely to maintain a good physical and immunological health status [40].

At the phylum level, *Firmicutes* were the most abundant in all groups. A similar result was reported by Ramírez et al. [24]. According to Ramírez et al., *Firmicutes* (61.2 ± 28.4%) were the most abundant phylum in aquaculture fine flounder (*Paralichthys adspersus*). On the other hand, the most abundant phylum in wild fine flounder was *Proteobacteria*. This difference may be due to sample from different habitat (wild or aquaculture). *Proteobacteria* and *Actinobacteria* abundance were increased by a probiotic-inoculated diet. *Proteobacteria* include many pathogenic bacteria, such as *Vibrio* and *Aeromonas*. However, at the genus level, the *Proteobacteria* were found to be *Thauera* and *Psychrobacter* rather than *Vibrio* or *Aeromonas*, and there have been no reports that these bacteria cause diseases in olive flounder. Ramírez et al. reported the relative abundance of *Proteobacteria* and *Actinobacteria* in wild fine flounder compared to cultured fish [24]. The *Psychrobacter* that were identified belonged to the family *Moraxellaceae* in the *Proteobacteria* phylum, which inhibit pathogenic *Vibrio* spp. and produce enzymes useful for nutrient utilization [41,42]. In the present study, *Psychrobacter* were most abundant in the ProB group and were absent in the control group. *Actinobacteria* are more abundant in herbivorous fish and are known to produce secondary metabolites, such as antibiotics [43–46]. *Actinobacteria* abundance increased following probiotic

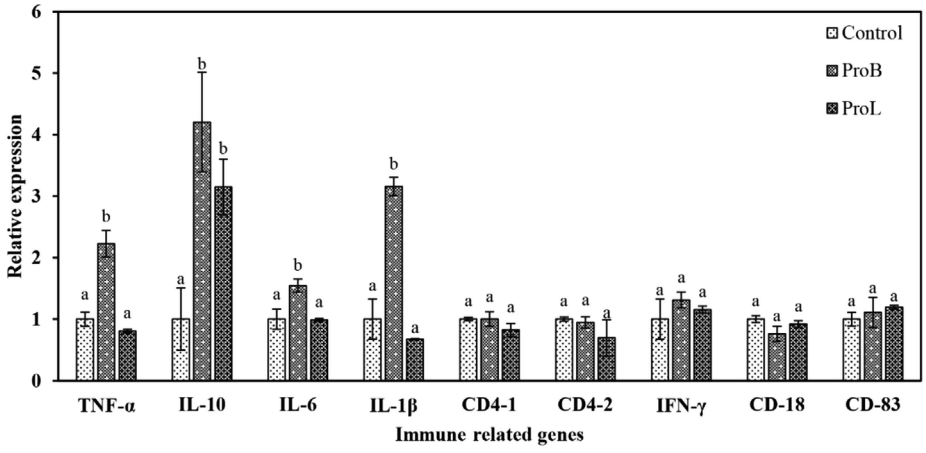

Fig. 6. Profiles of immune-related gene expression in the olive flounder intestine. The expression of these genes in olive flounder was measured by quantitative reverse transcription polymerase chain reaction after eight weeks feeding for control, ProB, and ProL groups. Levels of cytokine expression were quantified relative to β-actin transcription. Data represent the mean ± standard deviation; means that do not share the same letter differ significantly, respectively.

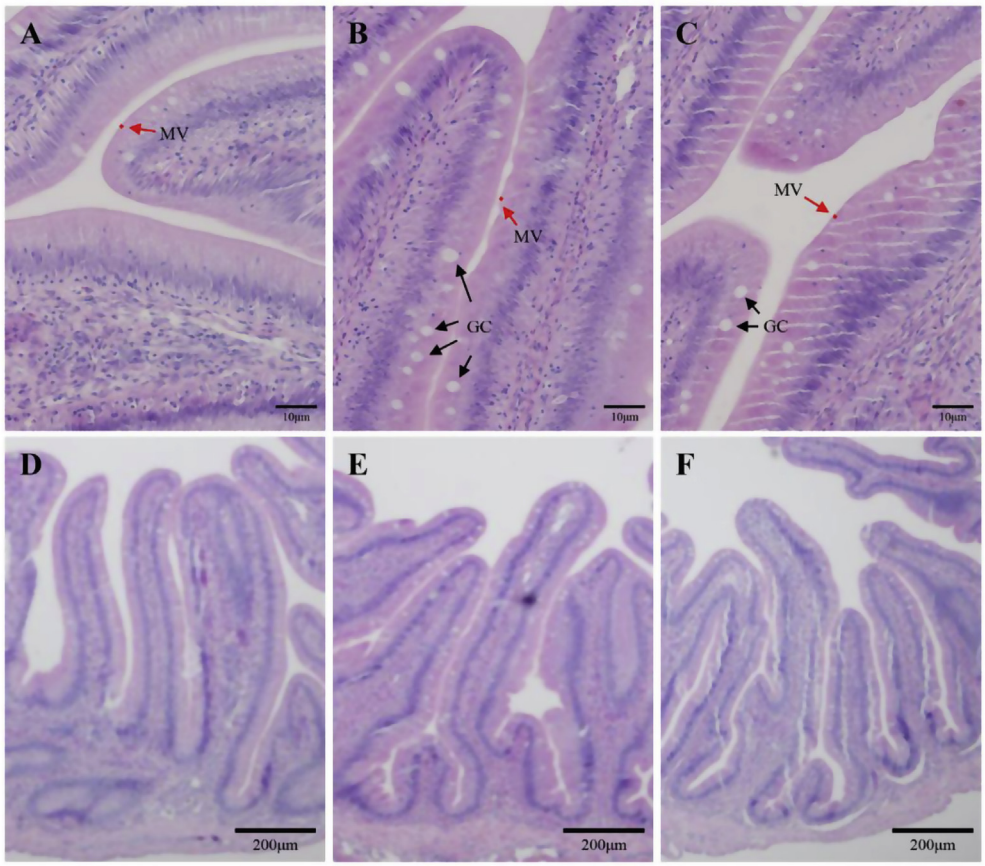

Fig. 7. Microphotographs of microvilli (A, B, and C) and villi (D, E, and F) in the intestines of olive flounder. Posterior intestinal histopathology of olive flounder fed the control (A and D), ProB (B and E), and ProL (C and F) diets estimated by light microscope photography. MV: microvilli; GC: goblet cell. Light microscopy staining: hematoxylin and eosin. Scale bar: 10 μm (A, B, and C), 200 μm (D, E, and F).

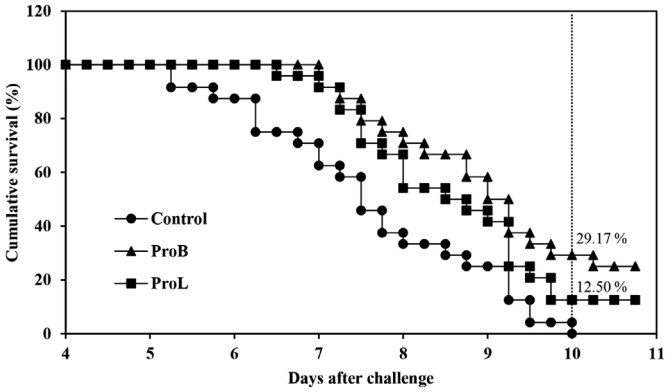

Fig. 8. Cumulative survival rates of olive flounder in each diet challenged by the injection of *Streptococcus iniae* (1×10^8 CFU/mL). Means (24 fish/group) were compared at identical times.

supplementation, especially in the ProB group, which may indicate a better adaptability to a plant protein-inoculated diet compared to an animal protein diet (fishmeal). At the genus level, the added probiotic bacteria were significantly different compared to the other groups, but accounted for a small percentage of the overall diversity. Therefore, the probiotics supplied to the olive flounder may have affected the overall diversity rather than having a direct effect on the host.

After eight weeks of feeding trial, the weight gain of ProB (183.88 ± 6.33%) and ProL (180.67 ± 4.69%) was significantly higher compared to the control group (170.08 ± 5.88%), which was similar to the previous reports [10,26]. Bacteria in the intestine of fish can produce various enzymes, such as protease, amylase, and lipase, for digestion [47]. Increased digestive enzymatic activity following probiotic addition can improve feed utilization parameters by increasing the availability of absorbable nutrients [48]. Soybean meal as a replacement for FM decreases trypsin activity in Atlantic salmon (*Salmo salar*) [49], and both protease and amylase activity in juvenile tilapia (*Oreochromis niloticus*) [50] compared to the original diet. In this study, the probiotic diet was found to increase the activity of intestinal digestive enzymes in olive flounder. In particular, the ProB group had a significantly higher digestive activity than the control group when FM was replaced by soybean meal. Therefore, the probiotic diet is an important factor that can increase the growth rate by altering the digestive enzymes of olive flounder in a diet containing soybean meal.

Cytokines play an important role in the immune system [51]. Probiotic supplementation mostly increases the transcription levels of pro-inflammatory cytokines in fish [10]. TNF-α, IL-6, and IL-1β are well-studied pro-inflammatory factors and are commonly used as reference genes to identify the immunomodulation mechanism [37,51]. In this study, TNF-α, IL-6, and IL-1β had higher expressions in the ProB group compared to the other groups. Liu et al. reported that the adhesive and non-adhesive characteristics of probiotics to the intestinal wall may be responsible for this difference [51]. These results provide clear evidence of increased probiotic associated constituents, which are known as microbial-associated molecular patterns, such as peptidoglycan and flagella in the ProB group [52]. Increased expression of these pro-inflammatory cytokines is associated with a higher immunological status, which has already been reported in fishes [53–56]. Previously, non-adhesive *Bacillus subtilis* C-1302 and adhesive *Lactobacillus delbrueckii* were shown to upregulate pro-inflammatory cytokine expression to significant [57] and non-significant [58] levels, respectively, compared to the control, which was an identical result to that of the present study. IL-10, an anti-inflammatory cytokine, plays a key role in preventing host tissue damage and maintaining the homeostasis of host immunity by limiting the activities of invading pathogens [59]. IL-10 was upregulated in both of the treatment diets that were supplemented with

probiotics, indicating that these supplemented groups had a better immune balance [59]. These results will contribute to efforts to increase the resistance of fish against pathogens.

The structure of the intestine, such as the lengths of villi, microvilli, and intestine, and thickness of the epithelium layer, increases the absorption area for the digestion and absorption of nutrients [60–62]. Increased villi and microvilli lengths following the provision of feed additive supplements have been reported [10,60]. Live and heat-killed probiotic and synbiotic supplementation increased the microvillus length in olive flounder to non-significant and significant levels, respectively [10,56]. Similarly, the addition of probiotics increased the length of villi and microvilli numerically in this study, but there was no statistical difference compared to the control group. Therefore, increased digestion and absorption of nutrients by probiotics may be a result of microbial diversity and digestive enzyme activity modulation rather than an alteration in intestinal structure.

Streptococcus iniae is a pathogenic bacterium that causes flounder death in a very short period of time [10]. Studies have reported that the increased cellular and humoral activities of an innate immune system increases protection against streptococcosis in olive flounder after dietary administration of probiotics and prebiotics [10,21,56,63,64]. It has also been reported that bacteriocins such as nisin, produced by lactic acid bacteria, protect the host from *S. iniae* infection [26]. Disease resistance due to increased intestinal microbial diversity and intestinal immunity of olive flounder have not yet been reported. However, in this study, the increases in the abundances of *Actinobacteria*, which produce secondary metabolites such as antibiotics, and *Psychrobacter*, which inhibit pathogenic bacteria, were able to protect the host from infection.

5. Conclusions

The investigation of intestinal microbial diversity using culture-independent techniques will provide a better understanding of digestibility and immune enhancement due to probiotic dietary supplements. In this study, intestinal microbial diversity, digestive enzyme activity, intestinal immune-related gene expression, and the alteration of intestinal structure in olive flounder clearly differed according to the exact probiotic supplementation provided. Dietary probiotic administration increased intestinal microbial diversity, digestive enzyme activity, and resistance to pathogens in olive flounder. This study has improved our understanding of the relationship between intestinal microbiota and olive flounder health. The study was conducted after eight weeks of probiotic supply. However, further research on intestinal microbial diversity modulation by probiotic supplementation should be conducted over different time periods to confirm the course of intestinal microbial changes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.056.

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