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Effects of dietary multi-strain probiotics supplementation in a low fishmeal diet on growth performance, nutrient utilization, proximate composition, immune parameters, and gut microbiota of juvenile olive flounder (Paralichthys olivaceus)

Kai-Min Niu[a,b,1], Sanaz Khosravi[c,1], Damini Kothari[b], Woo-Do Lee[b], Jeong-Min Lim[b], Bong-Joo Lee[d], Kang-Woong Kim[e], Sang-Gu Lim[d], Sang-Min Lee[c,**], Soo-Ki Kim[b,*]

[a] *Institute of Biological Resource, Jiangxi Academy of Sciences, Nanchang, 330029, China*
[b] *Department of Animal Science and Technology, Konkuk University, Seoul, 05029, Republic of Korea*
[c] *Department of Marine Biotechnology, Gangneung Wonju National University, Gangneung, 25457, Republic of Korea*
[d] *Aquafeed Research Center, National Institute of Fisheries Science, Pohang, 37517, Republic of Korea*
[e] *Aquafeed Management Division, NIFS, Busan, 46083, Republic of Korea*

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ABSTRACT

A 12-week feeding trial was conducted to evaluate the effects of multi-strain probiotics (MSP) in a low fish meal (FM) diet on overall performance, gut microbiota, selected non-specific immune responses and antioxidant enzyme activities of olive flounder (*Paralichthys olivaceus*) juveniles. A total of 225 healthy olive flounders (initial mean body weight, 13.5 ± 0.01 g) were randomly separated into 3 groups of 75 fish, each group having three replicates of 25 fish; first group was fed with a FM-based control diet (Con), 2nd group was fed with a low-FM diet containing a blend of plant and animal protein meals replacing 30% of the FM protein (FM30), and 3rd group was fed with the FM30 diet supplemented with 10^8–10^9 CFU kg^{-1} of the MSP (Pro). With the exception of lipid retention, which was significantly lower in fish fed the FM30 diet compared to the other two treatments, no other statistically significant differences were recorded with respect to any of the other growth and nutrient utilization parameters. Myeloperoxidase and lysozyme activities of fish fed the Pro diet were much higher and significantly different than those of fish fed the FM30 diet. Glutathione peroxidase activity was significantly higher in Pro- than in Con-fed fish, which, in turn, was significantly higher than FM30-fed fish. Expression of immune-related genes including IL-1β, IL-6, and TNF-α was markedly upregulated in livers of the fish fed Pro diet compared to those fed the Con and FM30 diets. Furthermore, supplementation of MSP in FM30 diet enriched the *Lactobacillus* abundance in the fish gut as well as predictive gene functions in relation to lipid and carbohydrate metabolisms. These data suggested that the MSP could reduce the potential adverse effects of the low-FM diet and might be used as a healthy immunostimulant for olive flounder.

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is the most widely cultured carnivorous marine species in South Korea [1]. To attain sustainable flounder production, Korean researchers are searching for potential alternative fishmeal (FM) proteins for use in aquafeed due to price increases and limited FM production [2]. Extensive literatures have investigated the potential of plant and/or animal byproduct-based proteins as FM alternatives [3–5]. Soybean meal (SBM) is one of the most attractive FM replacer because of its high protein content, favorable amino acid profile, large scale production, and low price [6,7]. It has been applied as an effective FM alternative in feeds for various fish species including rainbow trout (*Oncorhynchus mykiss*) [8,9], Atlantic cod (*Gadus morhua* L.) [10], northern snakehead (*Channa argus* Cantor, 1842) [11], seabass (*Lateolabrax japonicus*) [12], and olive flounder [13]. However, high dietary SBM inclusion could impair the growth,

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[*] Corresponding author.
[**] Corresponding author.
E-mail addresses: smlee@gwnu.ac.kr (S.-M. Lee), sookikim@konkuk.ac.kr (S.-K. Kim).
[1] Kai-Min Niu and Sanaz Khosravi are as the co-first author.

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digestion, immune response, and homeostasis of gut microbiota in fish due to the presence of non-digestible carbohydrates and anti-nutritional factors in SBM [14–19]. Poultry by-product meal (PBM) is another well evaluated FM replacer in aquaculture [20–24] due to its high protein content, good digestibility, low carbohydrate content, and appropriate proper amino acid profile [5,25]. However, as a protein source in aquafeed, the PBM can be a reservoir of pathogenic bacteria and cause serious problems [26].

Fermentation, supplementary additives, proper processing, and some other treatments have been viewed as ways to reduce the aforementioned risks from the plant and/or animal-based proteins. As biofriendly additives, probiotics such as lactic acid bacteria, *Bacillus,* and *Saccharomyces* have commonly been used in aquaculture to improve growth performance, digestive ability, immune response, disease resistance, gut health, and well-being of aquatic animals [27–32]. Probiotics have been documented to elicit their beneficial effects through stimulating indigenous enzymes, producing vitamins and antimicrobial metabolites, competitively excluding enteric pathogens, and modulating mucosal and systemic immune functions [33]. Recently, the host-derived probiotics have been considered more suitable for use in farmed fish owing to their higher competitiveness with the resident microbes and tolerance to the gut environment, as well as quicker adhesion to the gut mucus [34]. Most studies have focused on the use of single probiotics, but that could be never suitable for all host species due to the differences in physiological and physico-chemical conditions of the host and/or surrounding environments [34]. Furthermore, it has been suggested that a combination of probiotic strains may complement or improve health benefits of an individual strain [35,36]. Surprisingly, however, despite the growing interest in utilization of multi-strain probiotics (MSP) as functional feed supplements, little information is yet available about the possible effect of their inclusion in low FM diets for farmed fish [37,38]. Therefore, the main objective of this study was to evaluate the supplemental effects of MSP in a low FM diet and to compare its efficiency with that of a FM-based diet for juvenile olive flounder.

2. Materials and methods

2.1. Preparation of multi-strain probiotic supplement

The multi-strain probiotic (MSP) was composed of *Bacillus licheniformis* SK3927, *Bacillus amyloliquefaciens* SK4079, and *Bacillus subtilis* SK4082, *Lactobacillus brevis* SK1751, *Lactobacillus plantarum* SK3494, and *Saccharomyces cerevisiae* SK3587. *B. licheniformis* SK3927, *B. amyloliquefaciens* SK4079, and *B. subtilis* SK4082 were isolated from the intestinal content of olive flounders. *L. brevis* SK1751 and *L. plantarum* SK3494 were isolated from sweet potato and *Artemisia princeps* var. *orientalis*, respectively. *S. cerevisiae* SK3587 was purchased from Bigbiogen Co. Ltd. (Anseong, Gyeonggi-do 616, South Korea). The *Bacillus* spp. and *Lactobacilus* spp. were respectively cultured in LB (Luria-Bertani, BD) and MRS (deMan, Rogosa and Sharpe, BD) media at 37 °C for 16 h. *Saccharomyces* was cultured in YM (Yeast and Mold, BD) medium at 37 °C for 16 h. All the probiotic strains were incubated in an incubator (Vision scientific, Seoul, South Korea) under dark, aerobic condition [39]. All the probiotic cells were, then, harvested by centrifugation (5000 g, 10 min, 4 °C), washed (PBS, pH 7), and immobilized in a complex carrier (SBM:Eggshell powder, 7:3 ratio) [40]. The multi-strain probiotic (MSP) powder was included into the prepared diet and used in the feeding experiment of olive flounders.

2.2. Diet preparation

A practical fish meal (FM) based diet was designed with 50% crude protein and 8% fat [41], using steam dried sardine and anchovy FM as the main protein sources, and regarded as a control diet (Con). A low-FM diet formulated to be isonitrogenous and isolipidic with the Con diet in which 30% of FM protein was replaced by combined plant and animal origins including wheat gluten, soy protein concentrate, tankage meal and poultry by-product meal and labeled as FM30 (Table 1). The FM30 was supplemented with five different species of bacteria and one species of yeast in equal proportion as a multi-strain probiotic (MSP) at the concentration of 10^8–10^9 CFU kg^{-1} diet and labeled as Pro. Each experimental diet was prepared by mixing all the dry ingredients using a 30 L upright DAEYUNG mixer (NVM-14, Daeyung Bakery Machinery Co. Ltd., Seoul, South Korea). Fish oil and double-distilled water were then blended into the mixture and the resulting moist mash was passed through a meat chopper machine (SMC-32, SL Co., Incheon, South Korea) fitted with a 3 mm die. The moist strands were then crushed into pellets of a desired size, collected on aluminum trays, dried in a forced air oven (SI-2400, SIN IL Drying Machine Co. Ltd., Daegu, South Korea) at 25 °C overnight, and kept at 4 °C until used.

Table 1
Formulation and proximate composition of the FM-based (Con) and low-FM (FM30) diets (% dry matter).

Ingredients	Experimental diets	
	Con	FM30
Sardine fish meal	32.50	22.75
Anchovy fish meal	32.50	22.75
Soybean meal	8.00	8.00
Corn gluten meal	2.00	2.00
Wheat flour	19.0	17.6
Wheat gluten	0.00	3.50
Soy protein concentrate	0.00	5.25
Tankage meal	0.00	5.25
Poultry by-product meal	0.00	4.00
Fish oil	3.00	4.20
Mineral Mix[a]	1.00	1.00
Vitamin Mix[b]	1.00	1.00
Lecithin	0.50	0.50
Choline	0.50	0.50
Taurine	0.00	0.60
Monocalcium phosphate	0.00	0.60
Analyzed proximate composition		
Crude protein	50.5 ± 0.01	51.1 ± 0.01
Crude lipid	8.7 ± 0.01	9.8 ± 0.04
Ash	10.1 ± 0.01	9.6 ± 0.07

[a] Vitamin premix contained the following amount which were diluted in cellulose (g kg^{-1} premix): L-ascorbic acid, 121.2; DL-(tocopheryl acetate), 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myoinositol, 18.8; D-biotin, 0.27; folic acid, 0.68; *p*-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; and cyanocobalamin, 0.003.

[b] Mineral premix contained the following ingredients (g kg^{-1} premix): $MgSO_4 \cdot 7\ H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4 \cdot 7\ H_2O$, 20.0; Ca–lactate, 365.5; 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; and $CoCl_2 \cdot 6H_2O$, 1.0.

2.3. Experimental fish and feeding conditions

Juvenile olive flounder were purchased from a private hatchery (Namhae, South Korea) and transported to fish holding facilities at the GWNU Marine Biology Center (Gangneung, South Korea). The feeding experiment followed the guidelines of Animal Care and Use Committee of GWNU and was approved as GWNU-2017-10. The fish were acclimatized in an open system of circular polyvinyl tanks of about 5,000-L capacity, supplied with a continuous flow of natural, aerated seawater, and fed with a commercial diet (Daehan Feed Co., Incheon, South Korea; 52% crude protein and 8% lipid) for 2 weeks. After the acclimation, 25 juveniles (Initial mean body weight, 13.5 ± 0.01 g) were randomly captured, weighted as a group, and stocked into separate 150-L polyvinyl circular tanks, continuously supplied with filtered natural seawater at a flow rate of 2.5 L min^{-1}. During the feeding trial, fish were held under ambient water temperature (increasing from

18.7 °C to 21.2 °C) and simulated natural photoperiod (light: dark; 14: 10 h) condition. The dissolved oxygen level was maintained above 7.8 mg L^{-1} using supplemental aeration provided by a regenerative blower and air stones. Salinity (33.1 ± 0.5 ppt) and pH (7.6 ± 0.3) of the influent seawater were monitored weekly. Each experimental diet was assigned to three tanks in a completely randomized design. Fish were hand-fed twice a day (9:00 and 17:00 h) until apparent satiation for a period of 12 weeks. The uneaten feed was siphoned out, oven dried and weighed to calculate the feed intake level. Fish were fasted for 18 h prior to weighing or sampling to minimize handling stress.

2.4. Sample collection

At the end of the feeding trial, all surviving fish in each tank were counted and weighed to the nearest 0.01 g for calculation of the survival rate and performance indices. Total body length of each individual fish was measured to the nearest 0.1 mm. A random sample of 20 and 3 fish (per tank) were taken at the beginning and the end of the experiment, respectively, and stored at −43 °C for subsequent whole-body proximate composition analyses.

Weight gain (WG, %) = [(final body weight − initial body weight)/initial body weight] × 100.

Specific growth rate (SGR, %/day) = [(ln final body weight − ln initial body weight)/days] × 100.

Daily feed intake (DFI, %) = (feed intake × 100)/[(initial body weight + final body weight + dead fish weight) × days/2] [41].

Feed efficiency (FE, %) = (wet weight gain/feed intake) × 100.

Protein efficiency ratio (PER) = wet weight gain/total protein given.

Feed conversion ratio (FCR) = feed intake/wet weight gain.

Protein retention (PR, %) = [(final body weight × final carcass protein content) – (initial body weight × initial carcass protein content)/protein intake] × 100.

Lipid retention (LR, %) = [(final body weight × final carcass lipid content) – (initial body weight × initial carcass lipid content)/lipid intake] × 100.

Condition Factor (CF, %) = [weight of fish/(length of fish)^3] × 100.

Hepatosomatic index (HSI, %) = (weight of liver/weight of fish) × 100.

Viscerasomatic index (VSI, %) = (weight of viscera/weight of fish) × 100.

Survival (%) = (final number of fish/initial number of fish) × 100.

Blood samples were collected from the caudal veins of three anaesthetized (200 mg L^{-1} 2-phenoxyethanol) fish per tank (nine fish/dietary treatment) using non-heparinized syringes. Serum samples were then separated by centrifugation at 5000 × g for 10 min using a high-speed refrigerated micro-centrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, South Korea) and immediately stored at −80 °C for the analyses of total immunoglobulin (TIg) level and enzyme activities including lysozyme, myeloperoxidase (MPO), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities.

Immediately after blood collection, viscera, liver, and midgut were collected and quickly dissected out. Viscera and liver samples were weighed to determine the viscerosomatic (VSI) and hepatosomatic (HSI) indices, respectively. Further, the liver samples were also used to determine gene expression regarding inflammation. The contents of the midgut were squeezed out and stored in liquid nitrogen for further intestinal microbiota analysis. Fillet samples were subsequently taken from the same fish and stored at −80 °C until analyzed for proximate composition.

2.5. Chemical composition analyses

Chemical composition of the experimental diets, whole-body and fillet samples were measured following the standard methods of the Association of Official Analytical Chemists [42]. Moisture content was measured by oven drying at 105 °C and ash content by combustion using a Thermolyne™ muffle furnace (Thermo Scientific, Asheville, NC, USA) at 600 °C for 4 h. Crude protein content was determined using an automatic Kjeldahl System (Buchi, Flawil, Switzerland). Crude lipid content was estimated gravimetrically by extraction with petroleum ether, using a Soxhlet extractor (VELP Scientifica, Milano, Italy). An oxygen bomb calorimeter (PARR 6100, Moline, IL, USA) was used to measure gross energy content of the experimental diets.

2.6. Selected non-specific immune parameters and antioxidant enzyme activity assays

MPO activity assay was performed according to Quade and Roth's description [43]. Lysozyme activity was determined according to the method of Hultmark et al. [44] using a sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) as a substrate. One unit of lysozyme activity was defined as the amount of enzyme required to decrease a 0.001 absorption value at 450 nm per min. TIg level was determined following the polyethylene glycol precipitation procedure described by Anderson and Siwicki [45]. The superoxide anion radical scavenging activity of the serum samples were assessed using an SOD assay kit (Sigma 19160, St. Louis, MO, USA) that quenches the rate of inhibition of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) reduction by the superoxide anion released after xanthine oxidation with xanthine oxidase. One unit of SOD activity was defined as the quantity of enzyme needed to cause 50% inhibition of the rate of WST-1 reduction. GPx activity was measured by quantifying the oxidation rate of the reduced glutathione (GSH) to the oxidized glutathione (GSSG), on the basis of the catalytic action of the enzyme, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase and NADPH, using a colorimetric assay Kit (BioVision Inc., Milpitas, CA, USA). One unit of GPx activity was defined as the quantity of enzyme catalyzing the oxidation of 1.0 μmol of NADPH per minute.

2.7. Inflammatory cytokine gene expression

The liver samples were ground into powder in liquid nitrogen. Total mRNA of the liver powder was extracted by adding 1 mL of TRIzol reagent (15596-018, Life Technologies, USA) to 0.1 g of the tissue powder, followed by homogenization using a homogenizer (IKA T10 basic, Seoul, South Korea). The upper aqueous phase was transferred into a new 1.5 ml micro-tube after centrifugation (12,000 g, 4 °C, 10 min). 200 μL of chloroform was added and vortexed, then the solution was incubated at room temperature for 2 min. After centrifugation (12,000 g, 4 °C, 15 min), the upper aqueous layer was transferred into a new 1.5 ml micro-tube, following the addition of 1.5 mL isopropanol. The tube was incubated at room temperature for 10 min, then the RNA was precipitated following centrifugation (12,000 g, 4 °C, 15 min). The supernatant was removed, thereafter the RNA was washed with 1 mL of 75% and 100% ethanol, respectively. The mRNA pellets was vacuum dried for 15 min and dissolved into DEPC (diethyl pyrocarbonate, Sigma-Aldrich, Korea) water with heat at 60 °C for 10 min. The total mRNA concentration was measured using NanoDrop 1000 (Thermo Scientific, Seoul, Korea). The mRNA integrity was evaluated using an RNA Nano 6000 Assay Kit with an Agilent Bioanalyzer 2100 system (Agilent Technologies, Richardson, TX, USA) and the RIN above 6.5 was used for the cDNA synthesis. The cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, Foster City, California 94404, USA) by adding 1 μg of RNA with 20 μL of reaction volume. Quantitative real-time PCR (qRT-PCR) was conducted using a CFX Connect™ Real-Time System (Bio-Rad, Seoul, South Korea) with IQ™ SYBR Green Supermix reagents (Bio-Rad, Seoul, South Korea). The following PCR conditions were used: 95 °C for 3 min and 40 cycles at 95 °C for 10 s, 51–61 °C for 30 s, and 72 °C for 30 s. The melting curve was confirmed to verify the reaction specificity during the amplification

Table 2
The primer used in the study.

Primer	Direction	Sequence	Temperature	Reference
GAPDH	Forward	ATGCTGGTGCCCACTATGT	60.8 °C	46
GAPDH	Reverse	ACCTGGTGCTCGGTGTATG		
IL-1B	Forward	AGTGAGATGGTGCGATTTCTGTTCT	63.9 °C	In the study
IL-1B	Reverse	TGTTGTCTTGCTCTGATGTGCTGAT		
IL-6	Forward	TCC ACC CAT CCA TCC CTC TT	62.5 °C	In the study
IL-6	Reverse	GCG CAG TAG ATT GCC GTA GA		
TNF-α	Forward	GTAGAGACGAGGAAGACGAGGAGAC	58.5 °C	In the study
TNF-α	Reverse	CGCCTGGCTGTAGACGAAGTAGA		

process. The qRT-PCR quantification was determined using the Ct value as the PCR threshold cycle number at the end point. The relative gene expression of IL-1b, IL-6 and TNF-α was normalized to reference houekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) [46] using the ΔΔCT method with the CFX Manager™ software (Bio-Rad, version 3.1, USA). The primers of IL-1b, IL-6 and TNF-α genes used in this study were designed using primer 6 software (Primer6 Software Distribution Website) and are shown in Table 2.

2.8. Intestinal microbiota

2.8.1. Genomic DNA extraction and sequencing

The 0.2 g of midgut content was collected and the genomic DNA was extracted using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instruction. Concentration and purity of the genomic DNA were measured using PicoGreen and NanoDrop1000 (Thermo Fisher Scientific, USA). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using primers of 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) [47]. Subsequently, specific primers with barcode were then subjected to 300 bp paired-end sequencing on an Illumina MiSeq platform (Illumina Inc., San Diego, USA) in Macrogen, Ltd. (Seoul, South Korea). In brief, the PCR was conducted with the following conditions: 95 °C for 3 min followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min and held at 4 °C. The replicate amplicons were pooled and purified using AMPure beads (Beckman Coulter, Inc., Brea, CA, USA).

2.8.2. Sequence analysis

The MiSeq raw data was changed to FASTQ format using MiSeq Reporter after sequencing finished. The Phix sequences were removed by Burrows-Wheeler Aligner (BWA) [48]. The paired-end sequences in FASTQ format were processed using FLASH-1.2.11 to produce high quality sequences about length of 440–460 bp with 120–160 bp overlap region [49]. After that, the low quality sequences, treated as sequencing error, ambiguous sequences, and chimera sequences, were removed by CD-HIT-OTU [50]. The sequences with ≥ 97% similarity were clustered and assigned to operational taxonomy units (OTU). Each representative sequence was subjected to EBI (European Bioinformatics Institute) reference database and compared with the 16S rDNA sequences of registered type strains in StrainInfo (http://www.straininfo.net/). The taxonomic assignment was determined based on the highest sequence similarity of the subject organisms using BLASTN (v2.4.0) without consideration of the query coverage and identified with a value less than 85% [50]. The diversity analysis of the operational taxonomic unit (OTU) data was performed using QIIME (v1.8) [51]. Alpha diversity analysis (within sample) was determined by the Shannon Index, Simpson index, Chao 1, and rarefaction curve. Beta diversity analysis (among samples) was determined by PCoA (principal coordinate analysis) plot and UPGMA (unweighted pair-group mean average) tree based on the value of Weighted UniFrac distance. Alpha and beta diversity analysis were used to evaluate the microbial diversity and abundance of fish intestinal samples.

2.8.3. Predicted molecular functions based on 16S rRNA data using PICRUSt

The molecular functions of intestinal metagenomes were predicted using the pick closed reference otu.py QIIME (1.8.4) script with a 97% sequence similarity threshold based on the Greengenes database (version 13.5). The resultant biom-formatted OTU table was then used to generate inferred metagenomic data by a software package of Phylogenic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) with the default parameters [52]. Predicted functional pathways were exported using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database after the first normalization of OTU abundance to the predicted 16S rRNA copy numbers.

2.9. Statistical analysis

Effects of dietary inclusion of probiotic mixture on fish performance and immune parameters were statistically analyzed by one-way analysis of variance (ANOVA), using SPSS program version 21 (SPSS Inc., Chicago, IL, USA). Tukey's HSD multiple range test was used to identify statistically significant differences ($P < 0.05$) in the mean responses to dietary treatments. Data were examined for normal distribution (Shapiro-Wilks's test) and homogeneity of variances (Levene's test). Data are presented as mean ± standard error (SE.) of the triplicate groups. Differential abundance of the bacterial components was assessed using canonical correspondence analysis in R software (V 3.1.2). ANOVA with Tukey's HSD multiple range test at 95% confidence interval was used to determine the statistical difference of the identified bacterial functional pathways, based on the relative abundance of the three dietary groups.

2.10. Key resources table

Resource	Source	Identifier
Chemical		
chloroform		
diethyl pyrocarbonate		
ethanol		
Petroleum ether		
iScriptTM cDNA Synthesis Kit		
isopropanol		
DEPC (diethyl pyrocarbonate)		
PowerSoil DNA isolation kit		
RNA Nano 6000 assay kit		
SOD assay kit		
Trizol reagent; Glutathione peroxidase; SYBR Green Supermix reagents		
Medium		
BD	N/A	LB, YM, MRS
Probiotic		

Bacillus	SK3927; SK4079; SK4082	Bacillus licheniformis; Bacillus amyloliquefaciens; Bacillus subtilis
Lactobacillus	SK1751; SK3494	Lactobacillus brevis; Lactobacillus plantarum
Yeast -	SK3587	Saccharomyces cerevisiae

3. Results

3.1. Growth performance, biometric parameters and proximate composition

The results of the present study have shown that fish growth performance, feed efficiency, and survival rate were not affected by the dietary treatments (Table 3). Dietary inclusion of the MSP supplement in the low FM diet (FM30) resulted in significant improvement of lipid retention and made it comparable to that of the Con group. The results of proximate analyses revealed no significant alterations in whole-body or fillet composition (Table 4).

3.2. Selected non-specific immune parameters, antioxidant enzyme activities, and immune-related gene expression

The results of selected immune parameters and antioxidant enzyme activities of juvenile olive flounder are shown in Table 5. Serum MPO and lysozyme activities were significantly increased in fish fed the Pro diet as compared with fish fed the FM30 diet. Serum GPx activity in fish fed the Pro diet was significantly higher than that of fish fed the Con diet, which was itself significantly higher than that found in the FM30-fed fish. A similar, but not statistically significant, trend was observed for serum TIg levels, with fish fed the Pro diet having the highest TIg values as compared with the other two dietary treatments. However, the incorporation of probiotics in the FM30 diet had no defined impact on serum SOD activity. In addition, the gene expression of pro-inflammatory cytokines such as IL-1b, IL-6 and TNF-α were significantly increased in the liver of fish fed the Pro diet as compared with those of the fish fed the Con and FM30 diets (Fig. 1).

Table 3
Growth performance, feed utilization and biometric parameters of juvenile olive flounder fed the three experimental diets for 12 weeks.

Items	Experimental diets		
	Con	FM30	Pro
IBW[a] (g)	13.5 ± 0.01	13.5 ± 0.01	13.5 ± 0.03
FBW[b] (g)	119 ± 2	116 ± 4	115 ± 5
WG[c] (%)	782 ± 11	753 ± 32	759 ± 42
SGR[d] (%/day)	2.59 ± 0.02	2.55 ± 0.04	2.56 ± 0.06
DFI[e] (%)	1.51 ± 0.06	1.45 ± 0.07	1.41 ± 0.02
FE[f] (%)	123 ± 4	124 ± 6	131 ± 2
PER[g] (%)	2.20 ± 0.08	2.23 ± 0.11	2.29 ± 0.04
FCR[h] (%)	0.82 ± 0.03	0.81 ± 0.03	0.77 ± 0.01
PR[i] (%)	35.3 ± 0.8	36.7 ± 2.2	37.9 ± 1.4
LR[j] (%)	51.8 ± 1.3^{b}	35.2 ± 2.1^{a}	51.9 ± 1.8^{b}
CF[k] (%)	1.01 ± 0.02	1.03 ± 0.03	1.05 ± 0.03
HSI[l] (%)	1.81 ± 0.03	1.53 ± 0.10	1.50 ± 0.08
VSI[m] (%)	4.25 ± 0.11	4.01 ± 0.04	4.18 ± 0.07
Survival (%)	88.0 ± 2.3	86.7 ± 2.7	82.7 ± 5.8

Values are mean of triplicate groups and presented as mean ± S.E. Values with different superscripts in the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[a] Initial body weight.
[b] Final mean body weight.
[c] wt gain.
[d] Specific growth rate.
[e] Daily feed intake.
[f] Feed efficiency.
[g] Protein efficiency ratio.
[h] Feed conversion ratio.
[i] Protein Retention.
[j] Lipid Retention.
[k] Condition factor.
[l] Hepatosomatic index.
[m] Viscerasomatic index.

Table 4
Whole-body and muscle proximate composition of juvenile olive flounder fed the three experimental diets for 12 weeks.

Items	Experimental diets		
	Con	FM30	Pro
Whole-body (% ww)			
Moisture	75.9 ± 2.2	71.0 ± 4.6	75.7 ± 0.7
Protein	16.6 ± 0.2	16.7 ± 0.3	17.6 ± 0.6
Lipid	4.11 ± 0.52	3.04 ± 0.34	3.38 ± 0.35
Ash	3.48 ± 0.02	3.35 ± 0.08	3.12 ± 0.05
Muscle (% ww)			
Moisture	76.0 ± 0.2	79.7 ± 3.6	76.1 ± 0.4
Protein	21.7 ± 0.6	21.6 ± 0.7	22.8 ± 0.7
Lipid	1.24 ± 0.09	0.54 ± 0.07	0.66 ± 0.28
Ash	1.57 ± 0.09	1.48 ± 0.03	1.38 ± 0.04

Values are mean of triplicate groups and presented as mean ± S.E. Values with different superscripts in the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

Table 5
Selected non-specific immune parameters and antioxidant enzyme activities of juvenile olive flounder fed the three experimental diets for 12 weeks.

Items	Experimental diets		
	Con	FM30	Pro
MPO[1]	1.96 ± 0.25^{ab}	1.63 ± 0.37^{a}	3.08 ± 0.25^{b}
SOD[2]	97.6 ± 0.7	95.9 ± 1.9	96.5 ± 1.2
Lysozyme[3]	627 ± 136^{ab}	441 ± 64^{a}	819 ± 57^{b}
TIg[4]	28.6 ± 2.9	21.2 ± 2.9	31.5 ± 5.8
GPx[5]	92.9 ± 5.8^{b}	57.8 ± 4.2^{a}	171.3 ± 7.0^{c}

Values are mean of triplicate groups and presented as mean ± S.E. Values with different superscripts in the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Myeloperoxidase level (absorbance); [2]Superoxide dismutase (% inhibition); [3]Lysozyme (U mL^{-1}); [4]Total immunoglobulin (mg mL^{-1}); [5]Glutathione peroxidase activity (mU mL^{-1})

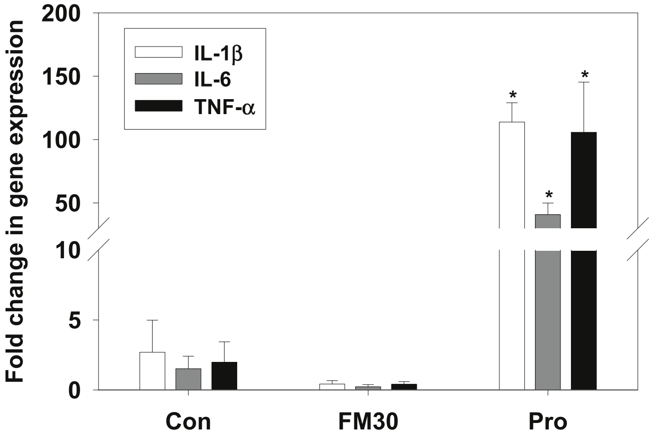

Fig. 1. Fold change expression of immune-related genes in liver of juvenile olive flounder fed the three experimental diets for 12 weeks. Stars (*) indicate significant differences at $P < 0.05$.

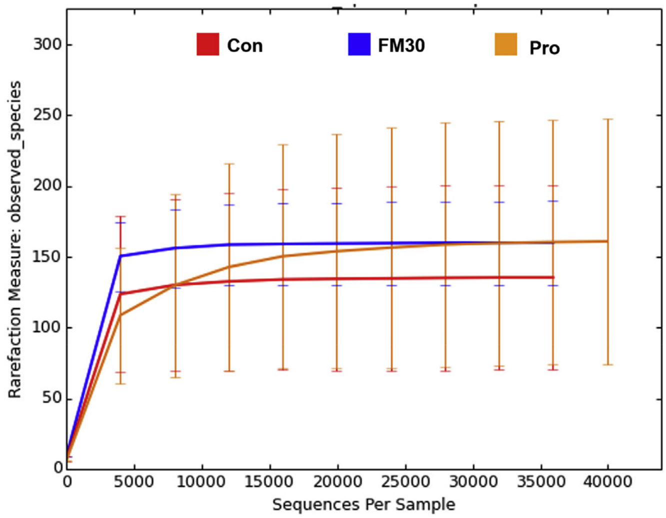

Fig. 2. Rarefaction curves (observed species) of the gut microbial community of the juvenile olive flounder fed the three experimental diets for 12 weeks.

3.3. Gut microbial diversity analysis

3.3.1. Sequencing and diversity analysis

For gut microbiota analysis, a total of 885,948 sequence reads (450 bp/read) were obtained from gut contents of nine fish fed with the Con (n = 3), FM30 (n = 3), and Pro (Pro, n = 3) diets. There were 357,997 remaining reads after removing low-quality and chimera reads, and among these, median reads of 39,677 ± 2,812, 38,917 ± 1,953, and 40,739 ± 1,052 were valid in Con, FM30, and Pro groups, respectively. These sequences were thereafter assigned to 801 OTUs based on 97% similarity using the QIIME pipeline and plotted in a rarefaction curve (Fig. 2). Following the increase of sequencing reads, the observed species were increased in all the dietary treatments until reached 130–150 species. No significant differences in the OUT number, Chao1 (community richness), and Simpson diversity were observed among the dietary treatments, while the values of the Shannon index significantly decreased in the fish fed the Pro diet as compared with the other two diets ($P < 0.05$) (Table 6). The UPGMA tree showed that the gut microbial composition in the Pro-fed fish varied slightly and presented close phylogenetic distance with the fish fed the Con diet (Fig. 3A). Similarly, when each diet group was considered independently, the samples from the Pro diet were more clustered together with fish fed the Con diet as compared with fish fed the FM30 as shown by PcoA analysis (Fig. 3B).

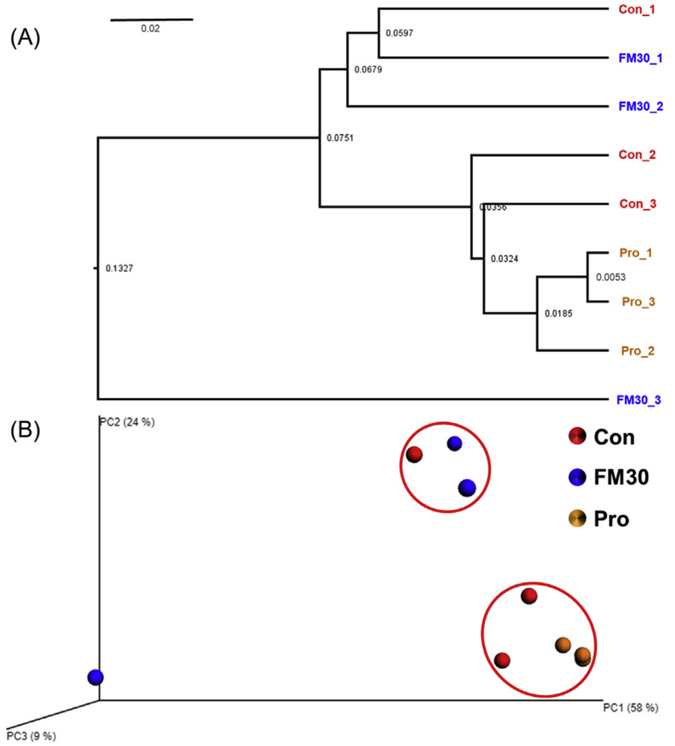

Fig. 3. Microbiome similarity of juvenile olive flounder fed the three experimental diets for 12 weeks. (A) UPGMA clustering of samples based on microbial community. (B) Principal coordinate analysis (PCoA) plot based on the unweighted UniFrac distance matrix.

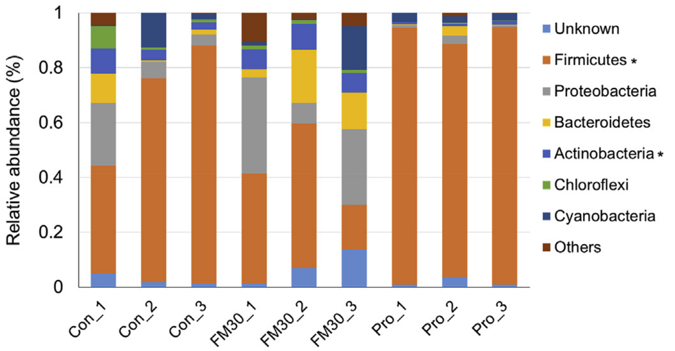

Fig. 4. Relative abundance (%) of predominant bacterial phyla in the gut microbiota of the juvenile olive flounder fed the three experimental diets for 12 weeks. Sequences that could not be classified into any known group were labeled as “Unknown”. Stars (*) indicate significant differences at $P < 0.05$.

3.3.2. Dominant gut microbiota and its dietary variances

Six core phyla were identified in the gut microbiota of the nine fish, in the order of abundance, as Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, and Cyanobacteria (Fig. 4). Of these phyla, the relative abundance of Firmicutes in the gut microbes was significantly influenced by the dietary treatments ($P < 0.05$) as the relative percentages of 90.9 ± 3.0%, 36.5 ± 10.6%, and 66.7 ± 14.2% in the Pro, FM30, and Con diets, respectively. Furthermore, the relative abundance of Actinobacteria in the gut microbes was significantly reduced in the fish fed the Pro diet (1.0 ± 0.2%) as compared with the Con (9.2 ± 2.0%) and FM30 (7.4 ± 0.8%) diets ($P < 0.05$). The dietary effects on the relative abundance of the dominant gut microbes were further analyzed at the genus and species level (Table 7). At genus level, 27 dominant genera were identified in the gut microbiota of fish. The inclusion of probiotics has significantly increased the relative abundance of *Lactobacillus, Marinilactibacillus,* and *Globicatella* and decreased the relative abundance of *Bifidobacterium* and *Streptomyces* in the gut microbiota of fish as compared with the Con and FM30 diets ($P < 0.05$). *Lactobacillus* and *Bacillus* were the most representative

Table 6
Alpha diversity of the gut microbial community of the juvenile olive flounder fed the three experimental diets for 12 weeks.

Indices	Experimental diets		
	Con	FM30	Pro
OTUs	135.7 ± 46.2	160.0 ± 20.8	161.0 ± 61.0
Chao1	136.0 ± 46.1	160.0 ± 20.8	162.8 ± 60.6
Shannon	4.8 ± 0.8^{ab}	6.2 ± 0.1^{a}	3.7 ± 0.3^{b}
Simpson	0.9 ± 0.1	1.0 ± 0.0	0.8 ± 0.0

Values are presented as mean ± S.E. Values with different superscripts in the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

Table 7
Relative abundance (%) of predominant bacterial genera and species in the gut microbiota of the juvenile olive flounder fed the three experimental diets for 12 weeks.

Taxonomy[1]	Experimental diets		
	Con	FM30	Pro
Genus			
Bacillus (F)	29.12 ± 16.06	2.50 ± 1.16	34.83 ± 1.34
Lactobacillus (F)	12.11 ± 4.46^{a}	7.07 ± 3.07^{a}	34.03 ± 1.58^{b}
Aerosakkonema (Cya)	3.79 ± 3.16	0.69 ± 0.21	2.64 ± 0.30
Streptococcus (F)	3.63 ± 1.12	3.73 ± 1.59	1.10 ± 0.20
Ancalomicrobium (P)	3.25 ± 2.79	0.77 ± 0.38	0.11 ± 0.06
Paraclostridium (F)	2.65 ± 1.31	2.50 ± 1.03	0.45 ± 0.13
Staphylococcus (F)	2.04 ± 1.31	3.58 ± 1.55	0.52 ± 0.05
Levilinea (Ch)	1.97 ± 1.27	0.70 ± 0.35	0.09 ± 0.06
Aquabacterium (P)	1.95 ± 1.74	0.30 ± 0.17	0.27 ± 0.22
Aerococcus (F)	1.94 ± 1.62	0.00 ± 0.00	3.29 ± 0.26
Enterococcus (F)	1.87 ± 1.44	0.02 ± 0.01	2.79 ± 0.28
Corynebacterium (A)	1.83 ± 1.26	1.58 ± 0.49	0.11 ± 0.04
Lentimicrobium (B)	1.59 ± 1.26	0.00 ± 0.00	0.15 ± 0.12
Lactococcus (F)	1.29 ± 0.60	2.15 ± 0.96	0.64 ± 0.09
Clostridium (F)	1.27 ± 0.94	1.14 ± 0.41	0.09 ± 0.02
Marinilactibacillus (F)	1.24 ± 0.73^{a}	0.00 ± 0.00^{a}	7.29 ± 0.85^{b}
Peptostreptococcus (F)	1.19 ± 0.52	1.68 ± 0.91	0.45 ± 0.26
Weissella (F)	1.17 ± 0.87	3.80 ± 1.97	1.86 ± 0.11
Peptoniphilus (F)	1.00 ± 0.58	1.43 ± 0.64	0.26 ± 0.06
Loriellopsis (Cy)	0.84 ± 0.52	5.12 ± 4.94	0.03 ± 0.02
Klebsiella (P)	0.81 ± 0.26	0.61 ± 0.22	0.05 ± 0.01
Parabacteroides (B)	0.79 ± 0.45	0.73 ± 0.42	0.01 ± 0.01
Bacteroides (B)	0.45 ± 0.23	2.05 ± 1.50	0.06 ± 0.03
Globicatella (F)	0.31 ± 0.20^{a}	0.00 ± 0.00^{a}	1.30 ± 0.11^{b}
Bifidobacterium (A)	0.22 ± 0.15^{a}	0.69 ± 0.12^{b}	0.06 ± 0.03^{a}
Streptomyces (A)	0.18 ± 0.10^{ab}	0.29 ± 0.02^{a}	0.06 ± 0.00^{b}
Pseudomonas (P)	0.03 ± 0.02	0.14 ± 0.10	0.02 ± 0.01
Species			
B. subtilis (F)	26.98 ± 16.06	1.92 ± 1.28	31.04 ± 2.52
L. brevis (F)	6.37 ± 4.13^{a}	1.06 ± 0.63^{a}	32.38 ± 1.59^{b}
L. ultunensis (F)	3.65 ± 1.48	3.58 ± 1.65	0.87 ± 0.10
B. flexus (F)	1.75 ± 1.49	0.00 ± 0.00	2.74 ± 0.28
L. kimchicus (F)	0.76 ± 0.53	0.73 ± 0.39	0.24 ± 0.05
L. aviaries (F)	0.64 ± 0.08	0.95 ± 0.68	0.18 ± 0.05
L. plantarum (F)	0.05 ± 0.04^{a}	0.06 ± 0.05^{a}	0.28 ± 0.06^{b}

Values are presented as mean ± S.E. Values with different superscripts in the same row are significantly different ($P < 0.05$).

[1] Taxonomy (Phylum): (A), Actinobacteria; (B), Bacteroidetes; (Ch), Chloroflexi; Cyanobacteria; (P), Proteobacteria; (F), Firmicutes.

genus in the fish gut bacteria and were further analyzed at the species level. A significantly higher relative abundance of *L. brevis* and *L. plantarum* was observed in fish fed the Pro diet as compared with the other two diets ($P < 0.05$). *B. subtilis* remained as the most predominant species in the fish gut bacteria not being affected by the dietary treatments.

3.3.3. Functional analysis by PICRUSt

The presumptive functions of the fish gut bacteria were analyzed based on metagenome prediction by PICRUSt. A total of 35 gene functional pathways were identified, and they displayed almost similar gene functions (at level 2) among the dietary treatments, expect the nervous system, nucleotide metabolism, biosynthesis of other secondary metabolites, and translation functions (Fig. S1). The dietary effects on metabolism related gene functions at level 3 were further analyzed, and significantly modulated gene functions are presented in Fig. 5. The gene functions related to carbohydrate, lipid, terpenoids, and polyketides metabolisms were enhanced in fish fed the Pro diet, while amino acid, other secondary metabolites, cofactors, and vitamins related gene functions decreased with the inclusion of MSP.

4. Discussion

In the present study, the low-FM diet (FM30), composed of various plant and animal proteins, with or without MSP inclusion, has been shown to support flounder performance equivalent to a typical FM-based diet (Con). Kikuchi [53] demonstrated that approximately up to 45% of FM can be replaced by a mixture of SBM, corn gluten meal, blood meal and blue mussel meat without compromising the performances and chemical composition of juvenile olive founder. Comparable results have also been found in the same species when fed with a blend of fermented SBM and squid by-product, supporting that the performance of fish was not impaired up to the inclusion of 36% of alternative proteins in the basal diet [54]. Indeed, it has been noted that using mixtures of alternative plant and animal proteins that may complement one another in terms of biological value is usually a more adequate and efficient strategy for replacing FM, without compromising fish performance, than using individual protein sources which might be deficient in an essential nutrient [54,55]. In this study, however, the strategic combination of plant proteins with terrestrial animal by-products did not seem to be effective in preventing dietary-induced alterations in normal regulation of lipid uptake and metabolism in fish, since flounders fed the FM30 diet had significantly lower lipid retention than those fed the Con and Pro diets. The reduced body fat retention in fish fed diets containing alternative proteins replacing FM has been related to impaired digestion and absorption of fat [3,56,57]. There is also some evidences suggesting that the quality of dietary protein sources can influence the regulation of lipid metabolism in fish [57–60]. Recently, Diógenes et al. [58] observed a linear decrease in the activities of hepatic lipogenic enzyme (G6PD, EC 1.1.1.49) with the increasing levels of FM replacement by corn distillers dried grains with solubles, accompanied by a reduction in the body fat content of turbot (*Scophthalmus maximus*, L.). Similarly in European seabass (*Dicentrarchus labrax*), the replacement of FM by soy protein concentrate or corn gluten meal depressed the activities of enzymes involved in lipogenesis, a situation which was suggested to be related to the lower biological value of the alternative proteins or a decline in the dietary FM level [59]. In turbot, Xu et al. [60] proposed that a soybean-based diet was responsible for reduced postprandial influx of free amino acids, when compared to fish receiving an FM-based diet, resulting in reduced expression of key enzymes involved in lipogenesis. They hypothesized that the down-regulation of lipogenic gene expression in fish fed the soy protein diet could be related to the combined effects of hypoactivated rapamycin (TOR) signaling and a hyperactivated amino acid response (AAR) pathway, leading to a lower lipid retention. It is therefore likely that the low retention of fat observed in the present study reflects the inefficient lipid uptake and synthesis in fish fed the FM30 diet. Herein, to reinforce this idea, it should be noted that partial replacement of FM by the alternative protein blend did not affect growth rates and feed intake of flounders, and, therefore the reduction observed with respect to lipid retention is mainly attributable to the decreased body lipid content per se. However, it is evident that retention of lipid in fish fed the FM30 diet significantly improved with dietary MSP supplementation, and it was comparable to those of the FM-fed fish. Improved nutrient digestibility and retention in fish fed probiotic diets has been reported by several authors [61–63]. These studies have suggested that probiotics might exert their beneficial effects by stimulating digestive enzyme production, modifying the gastrointestinal structure and microbiota and/or serving as a source of biogenics, which are known for their ability to modulate several functions of the host [62,64,65]. Nevertheless, as our observation of lipid retention response to MSP supplementation of a low-FM diet seems to be the first such report for fish, it entails further investigation.

In the present study, the dietary effects of a low-FM diet with or without MSP inclusion have also been evaluated on selected immune parameters and antioxidant enzyme activities. MPO is a peroxidase enzyme capable of producing oxidative metabolites with potent

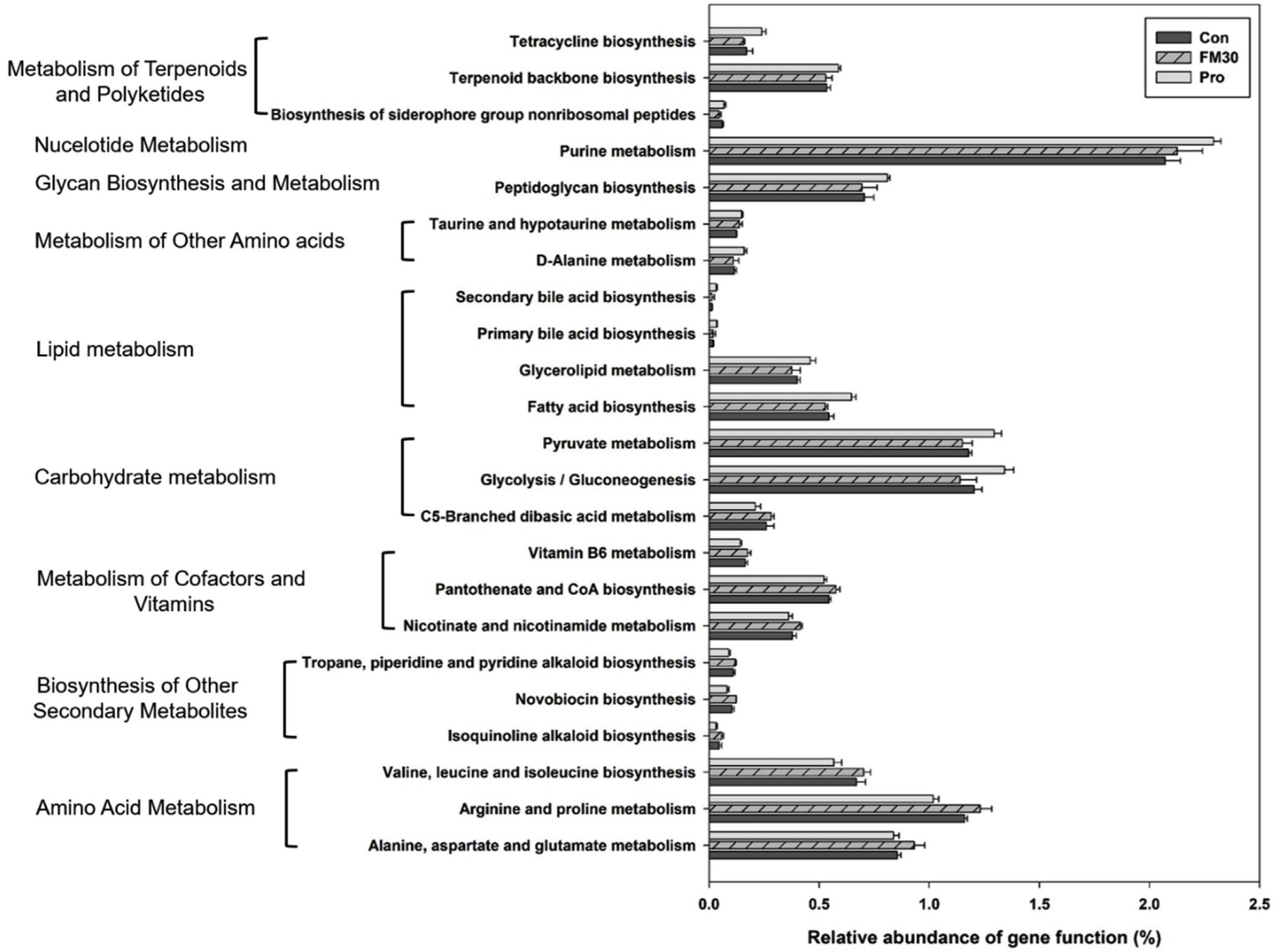

Fig. 5. Presumptive metabolism functions of the gut microbiota of the juvenile olive flounder fed the three experimental diets for 12 weeks based on relative abundance (Significant differences in gene categories at level 3, $P < 0.05$).

antimicrobial properties, like hypohalous acids (HClO), during phagocytosis [66,67]. Lysozyme is another important antimicrobial enzyme well-known for its ability to destroy bacterial peptidoglycan by cleaving the glycoside bond between N-acetylmuramic acid and acetylglucosamine, thereby contributing to innate host defense against infectious disease [68,69]. Results of the present study indicated that the dietary MSP supplementation remarkably improved serum MPO and lysozyme activities in juvenile flounders fed the FM30 diet, which were even higher than those found in fish fed the Con diet. Similarly, several studies have demonstrated that dietary supplementation of probiotics, as either single or mixed strains, had a marked immunopotentiating effect on both cell-mediated and humoral immune responses of olive flounder [30,36,70] and other fish species [29,35,71,72]. Probiotics have been suggested to exert their beneficial impact on the host immune system by modulating gut microbiota, producing antimicrobial compounds like bacteriocins, short chain fatty acids, etc. and interacting with phagocytes and other immunocompetent cells which effectively protect the fish against infectious pathogens [73,74]. Previous studies have also suggested the notion that dietary probiotic supplementation could help upregulating host anti-oxidative defense system, by detoxifying free radicals and enhancing antioxidant capacity, thereby offering protection against oxidative damage [75–77]. Our results lent further credence to this notion where MSP supplemented in the flounder diet a caused significant increment in the activity of GPx, which is a vital component of the antioxidant defense system and plays a crucial role in redox homeostasis [78]. As well balanced redox homeostasis is essential for the maintenance and efficient operation of the animal's immune system, the enhanced antioxidant enzyme activity observed here, in treated-flounders, may further indicate the effectiveness of the dietary mixed probiotics in promoting the immune responses of fish compared to the untreated groups. Indeed, the probiotic-induced immune enhancement in the present study seems to have occurred at the transcription level, as also reported by several other researchers [30,36,79,80]. The MSP treatment used in this study was able to remarkably up-regulate the expression of hepatic genes encoding the pro-inflammatory cytokines, including interleukin-1 (IL-1β), IL-6, and tumor necrosis factor α (TNF-α), which have all been shown to play a key role in the initiation, regulation, and perpetuation of fish innate immune response [81]. These results are consistent with the observations of Hasan et al. [80], who reported that dietary *Bacillus sp.* SJ-10 significantly enhanced expression of pro-inflammatory cytokine genes in liver, kidney, gill, and spleen of juvenile olive flounder, resulting in improved immune response and decreased susceptibility to streptococcosis. On the other hand, our data have also shown that dietary administration of the MSP can effectively prevent or ameliorate the adverse effects of a low-FM diet (FM30) on immune and oxidative status of flounders. Similarly, Dawood et al. [37] noted the positive effect of dietary heat-killed *Lactobacillus plantarum* inclusion on the innate immune response and stress resistance of amberjack (*Seriola dumerili*) juveniles fed a low-FM diet. Nevertheless, information about this aspect is rather limited and deserves further investigation.

The stability of gut microbial communities is one of the important factors affecting the overall health of marine fish [82]. Herein, the alteration of protein sources and MSP inclusion in the diet affected the colonization and development of gut microbiota in the intestine of juvenile olive flounder. In the present study, four indices, namely OTUs, Chao 1, Shannon and Simpson, were opted to evaluate the α-diversity of the gut bacterial community. OTUs and Chao 1 are abundance-based estimators of species richness, while Shannon and Simpson are used to describe the species richness and evenness [83]. Only the Shannon index significantly decreased in the Pro treatment group as compared with the FM30 treatment, implying that the MSP supplement is likely to have impact more on the bacterial species evenness than on the species richness. *Bacillus* spp. as a supplement in the diet for *Totaba macdonaldi* has also shown effects of decreasing the Shannon index based-gut bacterial diversity, but without affecting the Chao1 index-based species richness [31]. In Atlantic salmon (*Salmo salar*), Gupta et al. [84] observed that *Lactobacillus* feeding did not affect the gut bacterial diversity associated species richness and evenness. In addition, the β-diversity of the bacterial community showed a distant relationship among fish individuals fed with the FM30 diet and Pro diet in terms of the PCoA and phylogenetic tree data [84]. These are consistent with the PCoA-based results showing differently clustered bacterial patterns between the basal diet and the probiotic supplement diet [31,84]. Coupled with the taxonomic information at the phyla, genus and species levels, a shift in the gut microbial composition was further uncovered. This study presented Firmicutes and Proteobacteria as the most abundant phyla in farmed olive flounder, and which are also found in many other fish species [31,84–86] as well as in wild olive flounder [87]. The probiotic supplementation significantly increased the relative abundance of Firmictues and decreased the Actinobacteria abundance, which might have resulted from the intestinal colonization of the used MSP. The enriched abundance of *Lactobacillus*, *Marinilactibacillus*, and *Globicatella* (belonging to the Firmicutes) in the fish gut are considered to be the main reason for the enriched Firmicutes. Furthermore, *Lactobacillus* mainly presented as *L. brevis* and *L. plantarum*; especially, *L. brevis* counted as the most dominant species in fish fed the Pro diet. *L. plantarum* has been used for olive flounder to enhance the growth, feed efficiency, immune response, and disease resistance [36,70,88]. *L. brevis* was reported to enhance the immunity of hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂) [89] and produce bacteriocin [90]. *Marinilactibacillus* is a marine lactic acid bacterium showing production of lactate, formate, acetate, and ethanol as well as anti-listerial activity [91]. The functional property of *Globicatella* has not yet been known in aquaculture, although one particular *Globicatella* species (*G. sanguinis*) has been isolated from patients and was reported to cause human diseases [92]. No significant difference was found regarding the relative abundance of *Bacillus* (mainly composed of *B. subtilis*) among the dietary treatments, but it was observed to be more evenly distributed in each individual gut content. In contrast, the enrichment of *Bifidobacterium* and *Streptomyces* (belonging to Actinobacteria) were remarkably reduced by the MSP administration. *Bifidobacterium* is a well-known probiotic strain for human use, but it has not been widely reported in aquaculture. The probiotic potential and limitations of using *Streptomyces* in aquaculture have been highlighted [93]. The gut is an organ with a very complex environment involving digestion, adsorption, and immune response, thereby giving selective pressure on the ingested probiotics. As for the view of taxonomic results, only the supplementary *L. brevis*, *L. plantarum*, and *B. subtilis* seems to successfully colonize in the gut of olive flounder. Indeed, the probiotic persistence in the gut is species-dependent [94], and it is also associated with the inclusion dosage and duration as well as the developmental stage of the animal [95,96].

The predictive genomic function revealed probiotic potential on enhancing metabolisms with respect to terpenoids and polyketides, nucleotides, lipids, and carbohydrates in the gut of olive flounder, which might be due to the production of diverse digestive enzymes and antimicrobial substances. Polyketides are known for antibacterial and antiviral activities [97]. The enriched purine metabolism is likely to aid commensal microbes to colonize in the intestine of host [98]. In addition, the enriched bile synthesis could improve the lipid digestive ability in the host gut [99]. The increased glycolysis and gluconeogenesis could reduce the adverse effects caused by the non-digestible carbohydrate and anti-nutritional factors present in plant protein sources [100]. Previously, an enhanced carbohydrate-related metabolism was also reported in the gut of herbivorous fish, as compared with that in carnivorous fish based on PICRUSt analysis [101]. These enhanced functional metabolisms indicate the positive impacts of probiotic supplement on physiological activities of olive flounder. In contrast, the metabolisms related to amino acid, other secondary metabolites, cofactors, and vitamins were found to be increased in the FM30-fed fish. The improved alkaloid biosynthesis might be due to the abundant alkaloid content in plants [102]. *Streptomyces* is known to produce novobiocin [101], which was increased in the FM30-fed fish. The enhanced metabolism of cofactors and vitamins in the fish fed the FM30 diet might have resulted from the increased abundance of Bacteroidetes, Proteobacteria, and Actinobacteria phyla [103]. In addition, the enriched abundance of *Peptostreptococcus* and *Clostridium* might be a reason for the enhancement of amino acid metabolism and biosynthesis in the FM30 fed-fish, as is also reported for other fish species [104,105]. Since PICRUSt analysis only provides an indication of genetic potential, future studies in relation to shotgun *meta*-transcriptomics are imperative to gain insight into the true functional activities of these gut microbial communities in olive flounder.

5. Conclusion

In conclusion, our results indicate that the inclusion of a low-FM diet, with or without MSP supplementation, in olive flounder did not affect the growth performance, feed utilization, and body and muscle proximate composition when compared to the basal FM diet (Con). Non-specific immune responses in relation to MPO, lysozyme, and GPx activities as well as specific immune responses regarding liver pro-inflammatory gene expression of IL-1β, IL-6, and TNF-α were significantly improved with the MSP administration. The probiotic dietary treatment differentially affected gut microbiota composition in the fish, and the variations in community structure among the fish fed the low-FM diet have the potential to change on several microbial metabolic pathways. The MSP supplementation (Pro) significantly influenced host carbohydrate, lipid, terpenoid, and polyketide metabolism, unlike the low-FM diet (FM30) groups, and that might be associated to the modulation of specific bacterial populations. Although some significant links among the low-FM diet, probiotic inclusion, immune parameters, and gut microbiota structure, as well as their predictive gene functions were observed, further mechanistic studies are required to fully understand the low-FM diet-driven changes in olive flounder. Moreover, the MSP used in this study is expected to be included in low-FM aquafeed as an immunostimulant for olive flounder which needs to be validated by a disease challenge test in the future.

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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.2019.07.056.

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