Thesis for the Degree of Master of Fisheries Science

Effects of Dietary Non-Viable *Bacillus* sp. SJ-10, *Lactobacillus plantarum* and Their Combination on Growth, Humoral and Cellular Immunity, and Streptococcosis Appraisal in Olive Flounder (*Paralichthys olivaceus*)

by

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Graduate School of Global Fisheries

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February 2020

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넙치의 성장, 체액성 면역, 세포성 면역, 연쇄상 구균 감염 평가에서 *Bacillus* sp. SJ-10, *Lactobacillus plantarum*, 이들의 혼합 조합의 효과

Advisor: Prof. In-Soo Kong

by

TANKENG TERENCE NGUAFACK

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ABSTRACT

Single strain of heat killed (HK) *Bacillus sp*. SJ-10 (B), HK *Lactobacillus plantarum* (P) and their combinations were dietary administered to olive flounder (*Paralichthys olivaceus*) to quantify effects on growth, innate immunity, and disease resistance. Among four categories of test diets, control feed was free from any kind HK probiotics, and treatments were incorporated with 1×10^{8} CFU g^{-1} of each HK BSJ-10 (HKB) and HK LP

(HKP) as well as equal proportion of (0.5 HKB + 0.5 HKP) $\times 10^8$ CFU g^{-1} ($HKB_{0.5}$ $HKP_{0.5}$). After 8 weeks feeding, synergy of single probiotics ($HKB_{0.5}$ $HKP_{0.5}$) significantly ($P < 0.05$) improved growth (FBW, WG, SGR) and feed utilizations (FCR and PER) parameters compared to control group. For nonspecific immunity, $HKB_{0.5}HKP_{0.5}$ evolved alteration of respiratory burst and superoxide dismutase activities relative to control, HKB, and HKP. Serum lysozyme and *myeloperoxidase* activity was higher in both HKB and $HKB_{0.5}HKP_{0.5}$, compared to control. Serum biochemical parameters ALT and AST indicated no changes in any test diets but total cholesterol and glucose were higher in $HKB_{0.5}HKP_{0.5}$ versus control. Pro-inflammatory cytokine TNF-α in the kidney was significantly amplified with mix HK probiotic compared to control and other individual treatments. IL-6 in the liver as well as IL-1β in the liver, kidney and spleen was also improved in HKB groups. No differences were identified in the microvilli length among the groups but $HKB_{0.5}HKP_{0.5}$ demonstrated numerically elevated length. Fish when subjected to disease challenge with 1 $\times 10^8$ CFU mL^{-1} *Streptococcus iniae,* HKB and $HKB_{0.5}HKP_{0.5}$ fed fishes showed higher survival rate than control and HKP group. Therefore, dietary HK probiotics combination administration elevates growth, cellular and humoral immunity, and streptococcosis resistance in olive flounder.

Keywords: Bacillus sp. SJ-10; *Lactobacillus plantarum*; Innate immunity; Gene transcription; Olive flounder.

Abbreviation

ALT : Alanine aminotransferase

AST : Aspartate aminotransferase

BHI : Brain Heart Infusion

CD-4 : Cluster of differentiation 4

CF : Condition factor

CFU : Colony forming unit

DNA : Deoxyribonucleic acid

FBW : Final body weight

FCR : Feed conversion ratio

H_2O_2 : Hydrogen per oxide

H_2SO_4 : Sulfuric acid

HBSS : Hanks Balanced Salt Solution

HIS : Hepatosomatic Index

HKBSJ-10 : Heat killed *Bacillus sp*. SJ-10

HKLP : Heat killed *Lactobacillus plantarum*

IBM SPSS :Statistical Package for the Social Sciences

IBW : Initial body weight

IL-10 : Anti-inflammatory

IL-1β : Interleukin-1β

IL-6 : Interleukin-6

KCCM : Korean Culture Center of Microorganisms

LSZ : Serum lysozyme activity

MPO : Myeloperoxidase activity

MVL : microvillus length

NBT : Nitro blue tetrazolium

NO : Nitric oxide,

O_2^- : Reactive oxygen

OD : Optical density

OH^- : Hydroxyl radicals

$ONOO^-$: Peroxynitrite

PER : Protein efficiency ratio

*q*RT-PCR : Quantitative Reverse Transcriptase Polymerase Chain Reaction

RB : Respiratory burst

RNA : Ribonucleic acid

RPS : Percentage of survival

SD : Standard deviation

SGR : Specific growth rate

SOD : Superoxide dismutase

TC : Total cholesterol

TG : Total glucose

TMB : Tetramethylbenzidine

TNF-α : Tumor necrosis factor

TP : Total protein

VSI : Viscerosomatic Index

WG : Weight gain

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is one of the valued finfish in the world and a major alternative fish protein source overcoming food insecurity for the last 10 years in Korea [1]. This fish species is important due to its high growth rate, feed efficiency, wide range of temperature tolerance, resistance to diseases, seed availability as well as good taste and palatability. Intensive aquaculture has subjected farmed fish to stress due to overcrowding, temperature elevation, environmental degradation, and water quality deterioration [2]. Stress weakens the fish immune system leading to invasion of pathogens causing infectious diseases [3]. In flounder culture, infectious diseases cause 32% of the farm fish production loss of which 19% are caused by *Streptococcus spp* [4].

Disease outbreak commonly manifest in summer, but massive mortality in fish farm has been usually observed in winter and spring as a result of wide range of temperature fluctuation. Due to disease outbreak, national flounder production in Korea drop from 54,574 tons in 2009 to 36,921 tons in 2014 [5]. Antibiotics and chemical drugs used for disease control,

produces antibiotic resistant pathogenic strain, mass killing of beneficial bacteria, and residual effects in human [6]. The negative consequences of antibiotics and chemical drugs in olive flounder culture urgently raised an alarm for the need of pathogen control strategies by implementing an alternative feeding methods which will elevate immune activity and disease resistance [7, 8].

Probiotic act to subdue bacterial pathogens, producing antibacterial compounds such as antibiotics, bacteriocin, lysozymes, thus preventing the pathogens from colonizing the gastrointestinal tract [9, 10]. They equally guarantee the detoxification of the metabolites engendered by intestinal pathogens [11]. In novel aquaculture, probiotics are considered as biological substitute to chemical-based antibiotics and the use of that beneficial microbes have significantly reduced the use of antibiotics as therapeutic treatment in aquaculture. However, normally dietary administered live probiotics show poor viability and performance in the intestine due to lower pH and sustained microbial community [12]. Losses of live cells number during storage, negative effects by overdoses [13], and incorporation of none spore forming probiotic at different time interval need extensive labor [14]. In fish, heat-killed (HK) probiotics act as biological response modifiers that are similar to immunostimulants [15] and improved fish cellular and humoral immunity, growth performances, disease resistance, and stress overcoming [16,17].

In recent times, *Bacillus* SJ-10 (B) full genome sequence possess similarity with probiotics [18] and lack of cytotoxic and haemolytic gene [12] and dietary supplementations confirmed as olive flounder probiotic [19]. *Lactobacillus plantarum* (P) is well renowned fish probiotic [20] and also shows probiotic potentials in olive flounder [21]. Previously heat inactivated or HK probiotic supplemented in tilapia (*Oreochromis niloticus*) [22], red sea bream (*Pagrus major*) [3, 15], amberjack (*Seriola dumerili*) [17], and gilthead seabream (*Sparus aurata*) [16] resulted to significant growth or immunomodulation and infectious disease resistance. Only Hasan et al. [23] reported HKB (1×10^8 CFU g^{-1}) effects in olive flounder. However, no research has been conducted by HKP (1×10^8 CFU g^{-1}) and equal proportion (1:1) mixture of HKB and HKP [$HKB_{0.5}HKP_{0.5}$; $(0.5 + 0.5) \times 10^8$ CFU g^{-1}] in olive flounder.

The objective of this study is to inquire the effects of HKB, HKP, and $HKB_{0.5}HKP_{0.5}$ on growth, cellular and humoral immunity, cytokine genes transcription, and streptococcosis challenge in olive flounder. In addition feed utilization, serum biochemistry, and intestinal microvilli length will also be quantified to confirm HK probiotics effects as an antibiotic replacer.

2. Materials and methods

2.1. HKBSJ-10 and HKLP preparation

P (KCCM 11322) [21] was purchased from the Korean Culture Center of Microorganisms (KCCM) and B was isolated from a traditional Korean fermented fish [12]. B and P was cultured and incubated (37°C for 24 h) in lysogeny broth (LB) and MRS broth respectively, then centrifuged and washed two times with distilled water. Through serial dilution, CFUs were calculated and adjusted at 3.34×10^8 mL^{-1} in 300 mL water. After that suspension was autoclaved at 121°C for 15 minutes to kill these mentioned probiotics. To ensure 1×10^8 CFU g^{-1} HKB and HKP in diet, that 300 mL of each autoclaved probiotic suspension was added with 1000g of dry mixed ingredients. To prepare $HKB_{0.5}HKP_{0.5}$ and control diet 150 mL (3.34×10^8 CFU mL^{-1}) of each HKB and HKP suspension and only distilled water respectively, was incorporated.

Fig. 1. Prepared HKBSJ-10 and HKLP

2.2 Diets formulations

Basal diet compositions were same as shown in table 1. Diet formulation and storage procedures were carried out following by Bai and kim [25]. Feed ingredients like wheat flour and fish oil were used as carbohydrate and lipid source respectively. Moreover, fish meal; soybean, poultry by-product, and tankage meal; corn and wheat gluten; and soy protein concentrate were the protein sources. At the beginning all ingredients was weighed in required amount and mixed thoroughly. After that fish oil and 300 mL kg^{-1} of water was used

to make control diet. Instead of water, autoclaved probiotic suspension was added to make HKP, HKL, and $HKB_{0.5}HKP_{0.5}$ according to the described strategy in section 2.1.

After mixing, pelleting was performed by a pelleting machine fitted with 2-mm diameter die to produce 30% moisture containing pellets. The formulated diets were air dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 °C for 48–72 h and stored at −4°C in a sealed bag. Feed and fish body proximate composition analyses were performed according to standard methods of AOAC [26]. Isonitrogenous and isoenergetic (16.88 KJg^{-1}) experimental diet prepared contain 52.25% crude protein, 7.95% lipid, 8.50% moisture, and 9.40% ash.

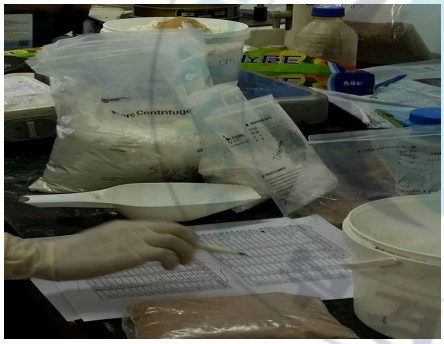

Fig. 2. Fish feed ingredients compose and weight

Fig. 3.Homogenous mixture of fish feed ingredients

Fig. 4. Fish feed pelleting

Fig. 5. Pelleted fish feed air dry

Table 1. Composition of the basal experimental diet for olive flounder [% of dry matter (DM) basis]

Ingredients	Percent (%)
Sardine fish meal[1]	22.75
Anchovy fish meal[2]	22.75
Soybean meal[3]	12
Wheat flour[4]	13
Wheat gluten[5]	4.9
Soy protein concentrate[6]	5.25
Tankage meal[7]	6.25
Poultry by-product meal[7]	4
Fish oil[8]	4.3
Lecithin[9]	0.5
Betain[7]	1
Tourine[7]	0.5
Monocalcium phosphate[10]	0.5
Mineral Mix[11]	1
Vitamin Mix[11]	1
Choline[10]	0.3

Feed proximate composition analysis (% DM)	
Moisture	7.27
Crude Protein	53.13
Crude Lipid	9.97
Crude Ash	10.75
Gross Energy (kJ g^{-1})[12]	15.87

[1]The feed Co. Orizon, Korea.
[2]Supplied by CJ CheilJedang Corporation, Seoul, Korea.
[3]The feed Co. Gangneung, Korea.
[4]The feed Co. Samhwa, Korea.
[5]The feed Co. Khawonenm, Korea.
[6]Milae ML Co. Icheon, Korea.
[7]Jejusuhyup feed Co., Jeju, Korea.
[8]Jeil feed Co. Hamman, Korea.
[9]The feed Co. Goyang, Korea.
[10]Sigma-Aldrich Korea Yongin, Korea.
[11]Contains (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.
[11]Contains (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.
[12]Estimated energy calculated: 16.8 kJ g^{-1} for protein and carbohydrate, and 37.8 kJ g^{-1} for lipid.

2.3 Experimental fish and feeding pattern

Two hundred and fifty-five (255) juvenile olive flounder free from any vaccination or antibiotics was purchased from a commercial hatchery (Won-Hong Susan, South Korea). Then distributed 15 fish $tank^{-1}$ in 17 indoor semi-recirculating seawater tanks (40 L) and acclimatized for 14 days with control diet. During the feeding trial physical condition, movement of fish, body, fin conditions, and feedback to feed were observed to grade the health status. At the starting of the trail, all stocked fish (starved for 24 h) were sampled, and then 15 physically sound flounders (13.33 ± 0.18 g) per tank were distributed randomly into 12 previously used acclimatize tanks (Fig 6). Three tanks each were randomly assigned to the control, HKP, HKB and $HKB_{0.5}HKP_{0.5}$ group. Each group were hand fed twice a day at time 900 and 1700 with the above mentioned diets, up to apparent satiation (2%–2.5% of body weight) for 8 weeks [21, 24]. The feed particles left over were syphoned 3 h later after feeding then dried and weighed to ensure the feed utilization parameters and a good physiochemical property of the milieu. Aquatic environmental parameters were maintained throughout the trail: water temperature (17.0 °C ± 0.5 °C), water flow (1.3 L min^{-1}), dissolved oxygen (6.9 mg L^{-1}), salinity (32 ± 1 ppt), photoperiod (12L:12D), and pH (7.3 ± 0.3). The experiments were carryout at the Feeds and Foods Nutrition Research Laboratory, Pukyong National University, Busan, Republic of Korea.

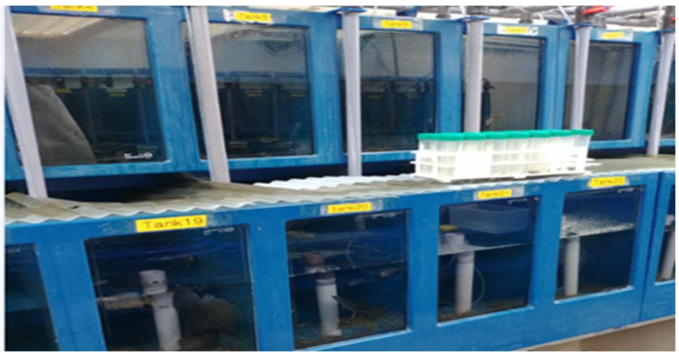

Fig. 6. Experimental fish and distribution

2.4 Sample collection and analysis

No fish casualty and death was recorded along the 8 weeks of feeding trail. After completing the trail fish was starved for 1 day and the following day all fish in the tank was caught and weighed to estimate final body weight (FBW) Fig. 7, weight gain (WG), specific growth rate (SGR), feed utilization parameters (FCR and PER). Three (03) fish $tank^{-1}$ (9 fish $group^{-1}$) were randomly selected and anesthetized with 500 µl L^{-1} 2-phenoxyethanol (Sigma-Aldrich, USA). Fish body weight and length was measured to evaluate the condition factor (CF) Fig 8. Blood sample was collected by puncturing of caudal vein with non-heparinized and heparinized syringes (NBT test). The collected blood was immediately centrifuged (5,000 × g for 10 min), serum as the supernatant was rapidly stored at −79°C. Hepatosomatic index (HSI)

and viscerosomatic index (VSI) was calculated using the weight of liver and intestine after opening the fish abdomen Fig 9. Carcass with liver and intestine was sent for body proximate composition analysis.

Fig. 7. Fish body weight measure

Fig. 8. Fish body length measure

Fig. 9. Fish liver and intestine collected

2.5. Innate immunity and serum biochemical parameters analysis

Nitro blue tetrazolium (NBT) assay was used to quantify the respiratory burst (RB) activity generated in blood during phagocytosis, according to the described methods by Anderson & Siwicki [27] with some modifications. At room temperature equal volumes (1:1) of 0.2% NBT reagent (Sigma-Aldrich, USA) and blood sample was mixed and incubated for 30-mins. Then, 50 µl of that mixture was mixed with 1ml of N-N-dimethylformamide and centrifuged at 2,000 × g for 5 min. Supernatant optical density (OD) was measured at 540 nm using a spectrophotometer (Mecasys, Optizen, Republic of Korea). Dimethylformamide was used as a blank.

Serum lysozyme (LSZ) activities was estimated by turbidometric essay according to Hultmark et al. [28] with little modification. Lyophilized *Micrococcus lysodeikticus* (0.2 mg mL^{-1}) was dissolved in phosphate buffer saline (PBS, pH 5.52) was added (180 µl) to a 96-well plate followed by serum (20 µl). Absorbance was read at 450 nm by a microplate reader after incubation at room temperature for 30 minutes. Reduction in absorbance 0.001 min^{-1} is considered as one unit of LSZ activity.

Evaluating serum myeloperoxidase (MPO) activity, slight variation of Quade and Roth [29] method was used. At the start, 80 µl of Hanks Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} was taken in a 96-well plate and 20 µl serum was diluted in the that HBSS,

afterward, 35 µl of each 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, 20 mM) and H_2O_2 (5 mM) were added. To end the color change reaction, after incubation at 35°C for 2 min, 35 µl of 4 M H_2SO_4 was added and at 450 nm absorbance reading was recorded using a microplate reader.

SOD Assay Kit (K335-100, BioVision, USA) was proceed to examine superoxide dismutase (SOD) according to kit established protocol and antiprotease activity of serum was quantified according to our laboratory established protocol [8].

Serum biochemical parameters such as total glucose, alanine aminotransferase (ALT), total cholesterol, aspartate aminotransferase (AST) and total protein were asses using an automatic chemical reader (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

Fig. 10. Blood sample collection

Fig. 11. Blood serum

2.6 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) and intestinal histopathology

The investigations on pro- [tumor necrosis factor (TNF)-α; interleukin (IL)-1β; IL-6] and anti-inflammatory (IL-10) cytokines expression by HK probiotic supplementation was workout through qRT- PCR. Flounder (9 fish group^{-1}) were chosen and anesthetized with 2-phenoxylethanol. After that ~60 mg tissue samples were collected from the lymphoid organs (liver, kidney, gill and spleen) in 1ml Trizol and total RNA collected by the manufactures instructions in a RNA isolation kit (RiboEx, GeneAll, South Korea). Collected total RNA was treated with DNAse to remove genomic DNA contamination following a DNAse-I kit protocol (Riboclear Plus, GeneAll, South Korea). RNA quantity (ng/µl) and purity (OD 260:280) were measured by a Nano Drop (Thremo Fisher Scientific, USA). cDNA was synthesized from 1µg RNA according to the manufacturers' instructions of PrimeScript cDNA Synthesis Kit (Takara, Japan).

qRT-PCR were workout according to SYBR green methods in a Thermal Cycler Dice ^{TM} (Real time machine, Takara, Japan) and gene specific primers were exactly same as our previous report [25]. PCR reaction mixture and thermal set up was done according to Abid et al. [30] with some changes. Briefly, a reaction mixture of 25 µl was prepared with 12.5 µl

SYBR green, 9.5 µl, purified sterile water, 2 µl cDNA, 0.5 µl of each forward primer and reverse primer (10 µM). Two step shuttle PCR were carry out, the initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s. β-actin was used as control gene, no primer-dimer formation was observed, and relative quantification (ΔΔCt) of each gene in different organs against β-actin was automatically estimated by V5.0x software installed in the mentioned machine.

Flounder (9 fish group^{-1}) used for qRT-PCR tissue collection were used to evaluate the posterior intestinal histopathology following the methods of Cerezuela et al. [31]. Sectioned posterior part of intestine was condition in 10% formalin and dehydrated with graded levels of alcohols. Xylene and paraffin was used for clearing and embedding, respectively. Paraffin embedded block was sectioned at 4 µm diameter by a microtome apparatus, then haematoxylin and eosin staining was effectively workout. With the help of light microscope, sectioned intestine picture was taken, and microvillus length (MVL) was read by Image-Pro plus software (Version 5.1 Germany).

Fig. 12. Polymerase Chain Reaction (PCR), Nanodrop, Real-time polymerase chain reaction (RT-PCR)

Table 2. Gene specific primers and gene bank accession number

Name of gene	Sense	Oligonucleotide Sequence (5´ to 3´)	Base Pair	Gene bank accession number
β-actin	F	CATCAGGGAGTGATGGTGGGTA	107	HQ386788.1
	R	ATACCGTGCTCGATGGGGTACT		
TNF-α	F	CAGCAGCGTCACTGCAGAGTTA	120	AB040448.1
	R	GTTACCACCTCACCCCACCATT		
IL-1β	F	CATCACCACTGTCTGCTGGAAA	122	KF025662.1
	R	GCTACTCAACAACGCCACCTTG		
IL-6	F	CAGTGCCAACTTCAGCAAGGAG	130	DQ267937.1
	R	GTGATATCTGGCGTGCAAGAGG		
IL-10	F	AGCGAACGATGACCTAGACACG	114	KF025662.1
	R	ACCGTGCTCAGGTAGAAGTCCA		
CD4-1	F	AGGTGCCAGTGAGGTGGTTTAT	112	AB716323.1
	R	GCCGTCCTGTTTACCAAAACTC		
CD4-2	F	CTCTGTTTCATGCCAAGGTGTC	109	AB716324.1
	R	CTTGCAGGTAAACATCCCACTG		

2.7 Challenge test

At the end of feeding trail, eight fish tank^{-1} (24 flounders group^{-1}) were anasthatized and intra-peritoneally injected with *Streptococcus iniae* (1×10^8 CFU / ml) [19, 21] and kept in quarantine tank. Along the pathogenic challenge, flounders were subjected to similar diet stress (starve) with no water exchange. Fish physical and morphological properties were monitor every 6h day^{-1} and mortality data was taken upto 11 days. Swabs from death fish skin, gill, and liver was spread on BHI agar plate. Presence of *S. iniae* colony authenticated

the streptococcosis outbreak. The formula of Amend [32] was used to calculate the relative percentage of survival (RPS). RPS= 100 – [(test mortality / control mortality) × 100.

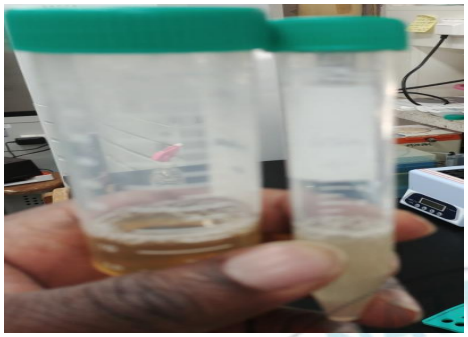

Fig. 13. *Streptococcus iniae* ($1x10^8$CFU/ml)

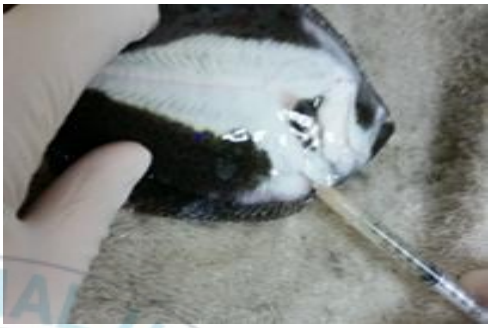

Fig. 14. Intra-peritoneal injection with *Streptococcus iniae*

2.8 Statistical analysis

Obtained data set normality and variance homogeneity was effectuated by Shaprio-Wilk and Levene tests. Then all data were analyzed following one-way ANOVA using IBM SPPS software (SPSS Inc., version 17.0). Analyzed results are depicted as mean ± SD and a *P* value less than 0.05 ($P < 0.05$) was considered as a level of significance.

3. Results

3.1 HK probiotic effects on growth performances, feed utilizations, body indices, and final body proximate composition

The formulated diets were well balanced to meet the nutritional requirement of olive flounder and only HK probiotics (1×10^8 CFU g^{-1}) were incorporated with the diets to make treatments (HKB, HKP, and $HKB_{0.5}HKP_{0.5}$). After 8 weeks of feeding, FBW was significantly ($P < 0.05$) higher in all treatments groups compared to control. WG, SGR, FCR, and PER in HKB and HKP were similar ($P > 0.05$) to the control group. However, these parameters in $HKB_{0.5}HKP_{0.5}$ were significantly higher relative to control, but statistically similar with HKB and HKP (Table 3). Olive flounder HSI, VSI, and CF remained unchanged after 8 weeks feeding and showed no difference among the feeding groups. Moreover, similar to these body somatic indices, no significant difference ($P > 0.05$) were also observed in moisture, crude lipid, crude protein, and crude ash content among the investigated and control groups after final body proximate compositions analysis, depicted in Table 4.

Table 3. Growth performance, feed utilization, and organosomatic indices of olive flounder supplemented with the experimental feed additives for 8 weeks[1].

Experimental groups	Growth performance, feed utilization, and organosomatic parameters								
	[2]IBW	[3]FBW (g)	[4]WG (%)	[5]SGR(%day^{-1})	[6]FCR	[7]PER	[8]CF (%)	[9]VSI (%)	[10]HSI (%)
Control	13.36 ± 0.21	38.42 ± 1.90^{a}	167.85 ± 12.50^{a}	1.68 ± 0.77^{a}	0.82 ± 0.04^{b}	1.84 ± 0.98^{a}	0.79 ± 0.09	1.49 ± 0.29	0.99 ± 0.12
HKB	13.33 ± 0.20	41.11 ± 1.52^{b}	186.31. ± 8.78^{ab}	1.79 ± 0.05^{ab}	0.76 ± 0.02^{ab}	1.98 ± 0.06^{ab}	0.86 ± 0.09	1.46 ± 0.22	0.96 ± 0.14
HKP	13.31 ± 0.30	41.02 ± 0.93^{b}	186.25 ± 11.85^{ab}	1.79 ± 0.06^{ab}	0.79 ± 0.03^{ab}	1.89 ± 0.08^{ab}	0.87± 0.08	1.48 ± 0.10	1.01 ± 0.16
$HKB_{0.5}HKP_{0.5}$	13.32 ± 0.18	42.56 ± 0.33^{b}	197.30 ± 5.53^{b}	1.86 ± 0.03^{b}	0.73 ± 0.02^{a}	2.01 ± 0.04^{b}	0.84 ± 0.07	1.45 ± 0.23	0.95 ± 0.33
P value	0.978	0.029	0.041	0.040	0.048	0.059	0.251	0.977	0.910

[1]Values are mean ± SD of three replicates (9 fish group^{-1}). Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences ($P > 0.05$).

[2]IBW (g): Initial body weight = Initial weight of total fish in tank / Fish number

[3]FBW (g): Final body weight = Final weight of total fish in tank / Fish number

[4]WG: Weight gain (%) = [(Final weight − Initial weight) / Initial weight]] × 100.

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

[6]FCR: Feed conversion ratio = Dry feed intake / Wet body weight gain

[7]PER: Protein efficiency ratio =Wet weight gain / Protein fed.

[8]CF: Condition factor (%) = [Body weight (g) / {Total body length (cm)} ^{3}] × 100.

[9]VSI: Viscerosomatic Index (%) = (Visceral weight / Body weight) × 100.

[10]HSI: Hepatosomatic Index (%) = (Liver weight / Body weight) × 100.

Table 4. Final whole-body proximate compositions (% of wet weight) of olive flounder supplemented with the experimental feed additives for 8 weeks[1].

Experimental groups	Proximate compositions (% of wet weight)			
	Moisture	Crude protein	Crude lipid	Crude ash
Control	76.35 ± 1.19	17.9 ± 1.07	1.70 ± 0.08	3.78 ± 0.20
HKB	75.8 ± 1.28	18.0 ± 0.40	2.36 ± 0.53	3.64 ± 0.36
HKP	75.2 ± 1.53	18.6 ± 0.75	2.63 ± 0.55	3.85 ± 0.07
$HKB_{0.5}HKP_{0.5}$	76.1 ± 1.26	17.9 ± 0.56	2.39 ± 0.58	3.53 ± 0.24
P value	0.73	0.67	0.18	0.39

[1]Values are mean ± SD of three replicates (9 fish group^{-1}). All values within the same column in the table are not significantly different ($P > 0.05$).

3.2 HK probiotic effects on nonspecific immunity and serum biochemical parameters

Among five nonspecific immunity parameters, except antiprotease, others (RB, SOD, LSZ, and MPO) were modulated at different extent. Compared to the control group, flounder fed with both HKB and HKP elevated LSZ up to the significant ($P < 0.05$) levels and no effects on RB and SOD. However, only HKB increased MPO activity but HKP showed no effect ($P > 0.05$). $HKB_{0.5}HKP_{0.5}$ produced higher effect on all these 4 modulated parameters verses control. RB and SOD were higher not only compared to control but also both HKB and HKP (synergistic effects) (Table 5). However, LSZ in $HKB_{0.5}HKP_{0.5}$ was similar to other two treatments, and MPO was significantly increased compared to HKP, but same as HKB. No improvement in antiproteases was revealed among the test and control group.

Analyzed serum biochemical parameters demonstrated no statistical differences ($P > 0.05$) among the diet groups in terms of ALT and AST. TP in HKB was significantly higher compared to $HKB_{0.5}HKP_{0.5}$ group. Similar statistical phenomenon of observed in TG but opposite diet groups (Table 6). $HKB_{0.5}HKP_{0.5}$ group's TC levels was higher ($P < 0.05$) compared to control and other two treatments, although both of HKB and HKP feed fed group TC was higher compared to control.

Table 5. Influence of the experimental feed additives on non-specific immune parameters of olive flounder supplemented for 8 weeks[1].

Experimental groups	Innate immune parameters				
	RB[2]	SOD[3]	LSZ[4]	MPO[5]	Antiprotease[6]
Control	0.45 ± 0.02[a]	38.28 ± 3.09[a]	0.47 ± 0.03[a]	1.12 ± 0.08[a]	58.29 ± 4.09
HKB	0.47 ± 0.04[a]	41.41 ± 3.76[a]	0.64 ± 0.11[b]	1.35 ± 0.09[b]	60.47 ± 6.77
HKP	0.53 ± 0.10[a]	39.43 ± 4.96[a]	0.61 ± 0.06[b]	1.12 ± 0.07[a]	63.59 ± 2.21
$HKB_{0.5}HKP_{0.5}$	0.69 ± 0.03[b]	49.43 ± 2.83[b]	0.75 ± 0.06[b]	1.39 ± 0.146[b]	59.74 ± 2.87
P value	0.005	0.025	0.011	0.021	0.532

[1]Values are mean ± SD of three replicates (9 fish group^{-1}). Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). Same or lack of superscript letter indicates no significant differences ($P > 0.05$).

[2]RB: Respiratory burst (absorbance at 540nm)

[3]SOD: Superoxide dismutase (% superoxide inhibition)

[4]LSZ: Serum lysozyme activity (Units mL^{-1})

[5]MPO: Myeloperoxidase activity (absorbance at 450nm)

[6]Antiprotease: % of trypsin inhibition

Table 6. Biochemical parameters of serum in olive flounder supplemented with experimental feed additives for 8 weeks.

Experimental groups	Serum biochemical parameters				
	ALT[2] (U L^{-1})	AST[3] (U L^{-1})	Total glucose (mg dL^{-1})	Total cholesterol (mg dL^{-1})	Total protein (mg mL^{-1})
Control	5.67 ± 0.51	18.78 ± 4.9	25.04 ± 2.37^{ab}	94.56 ± 2.87^{a}	41.94 ± 3.02^{ab}
HKB	6.56 ± 0.51	17.58 ± 5.81	22.66 ± 2.73^{a}	118.36 ± 8.31^{b}	52.34 ± 6.83^{b}
HKP	6.56 ± 0.51	15.80 ± 0.51	33.98 ± 7.32^{bc}	130.88 ± 8.51^{b}	41.08 ± 8.03^{ab}
$HKB_{0.5}HKP_{0.5}$	6.56 ± 1.03	17.29 ± 2.87	35.47± 6.77^{c}	150.86 ± 9.48^{c}	37.76 ± 5.80^{a}
P value	0.344	0.846	0.43	0.000141	0.089

[1]Values are mean ± SD of three replicates (9 fish $group^{-1}$). All values within the same column in the table are not significantly different ($P > 0.05$)

[2]ALT: Alanine aminotransferase

[3]AST: Aspartate aminotransferase

3.3 HK probiotic effects on immune related genes and intestinal MVL

Inflammatory cytokine [tumor necrosis factor (TNF)-α; interleukin (IL)-1β; IL-6], anti-inflammatory (IL-10), cluster of differentiation 4 (CD4)-1, and CD4-2 were quantified in the various fish organs (liver, gills, kidney and spleen) after 8 weeks (Fig 15).

In liver (Fig 15.A) TNF-α and IL-10 were significantly ($P < 0.05$) upregulated in HKP as well as both HKB and HKP group respectively, compared to $HKB_{0.5}HKP_{0.5.}$ In this same organ, relative to control IL-6 and IL-1β were expressed in HKB and HKP respectively. CD4-1 showed downregulated ($P < 0.05$) pattern in treatment groups and no difference was observed among control, HKB, and HKP in CD4-1.

In kidney (Fig 15.B), synergistic TNF-α expression ($P < 0.05$) was observed in $HKB_{0.5}HKP_{0.5.}$ IL-10 showed no difference ($P > 0.05$) among the diet groups, and IL-6 in control was higher verses HKB. IL-1β in HKB was transcript compared to control and $HKB_{0.5}HKP_{0.5.}$ Expression of CD4-1 and CD4-2 in control group was significantly higher than HKB and HKP.

TNF-α expression in HKB and $HKB_{0.5}HKP_{0.5}$ was higher in gill (Fig 1.C) compared to both control and HKP. Compared to control, IL-10 and IL-6 positive transcription was observed in all treatments and $HKB_{0.5}HKP_{0.5}$ group, respectively. Similar, statistical phenomenon of

TNF-α was also followed by IL-1β in the same organ. CD4-1 showed no expression among the diet groups and CD4-2 transcriptions showed lower ($P < 0.05$) trend in all treatments relative to control.

No expression of TNF-α and CD4-1 was determined between control and treatment groups in spleen (Fig 1.D). IL-10 and IL-6 in both HKP and $HKB_{0.5}HKP_{0.5}$ was higher compared to control. IL-1β transcription in this organ was higher ($P < 0.05$) in treatment groups verses control and opposite statistical pattern was observed in CD4-2.

No inflammation, deformation or dilation of the intestinal microvilli was observed in any diet group (Fig. 16). The goblet cells in all diet groups intestine were uniformly distributed around the intestinal epithelial layer with its normal cup-shape structure. MVL in control group was 1.54 ± 0.08 µm (Fig. 16A), HKB was 1.55 ± 0.25 µm (Fig. 2B), HKP was 1.56 ± 0.07 µm (Fig. 2 C), and in $HKB_{0.5}HKP_{0.5}$ was 1.73 ± 0.20 µm (Fig. 1D). Although $HKB_{0.5}HKP_{0.5}$ demonstrated numerically maximum MVL among the diet groups, but statistically there was no difference ($P > 0.05$).

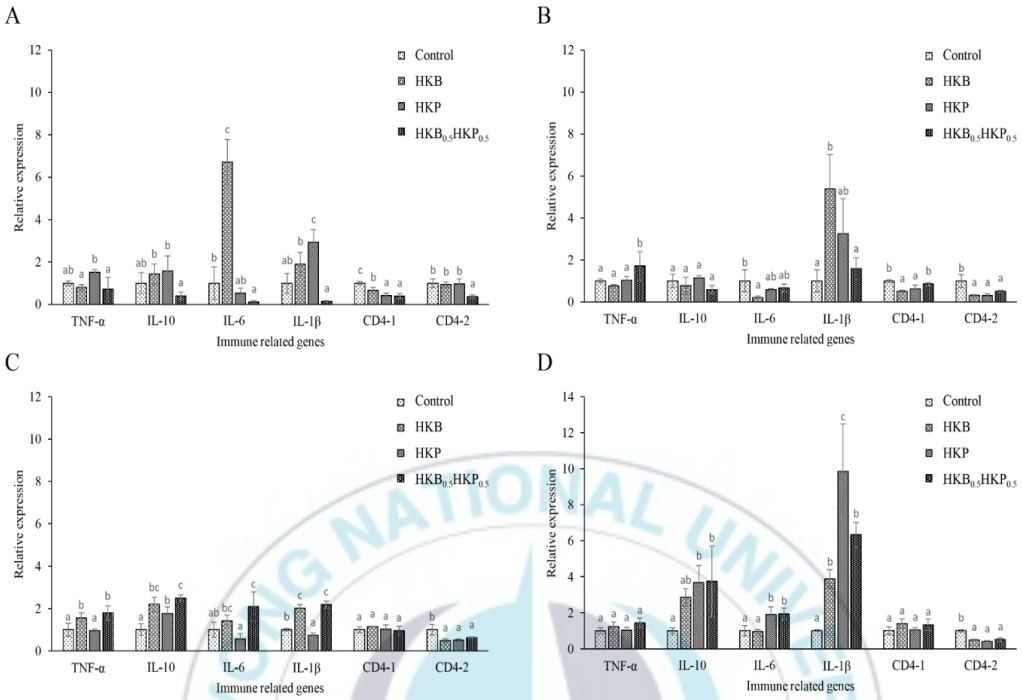

Fig. 15. Immune related gene transcription in (A) liver, (B) kidney, (C) gill, and (D) spleen in olive flounder were measured using qRT-PCR after weeks feeding with control, HKB, HKP, and $HKB_{0.5}HKP_{0.5}$. Expression of these genes was quantified relative to β-actin transcription in specific organs. Data represent mean ± standard deviation; means (9 fish group^{-1}) with the same or different letters are not significantly ($P > 0.05$) or are significantly ($P < 0.05$) different, respectively.

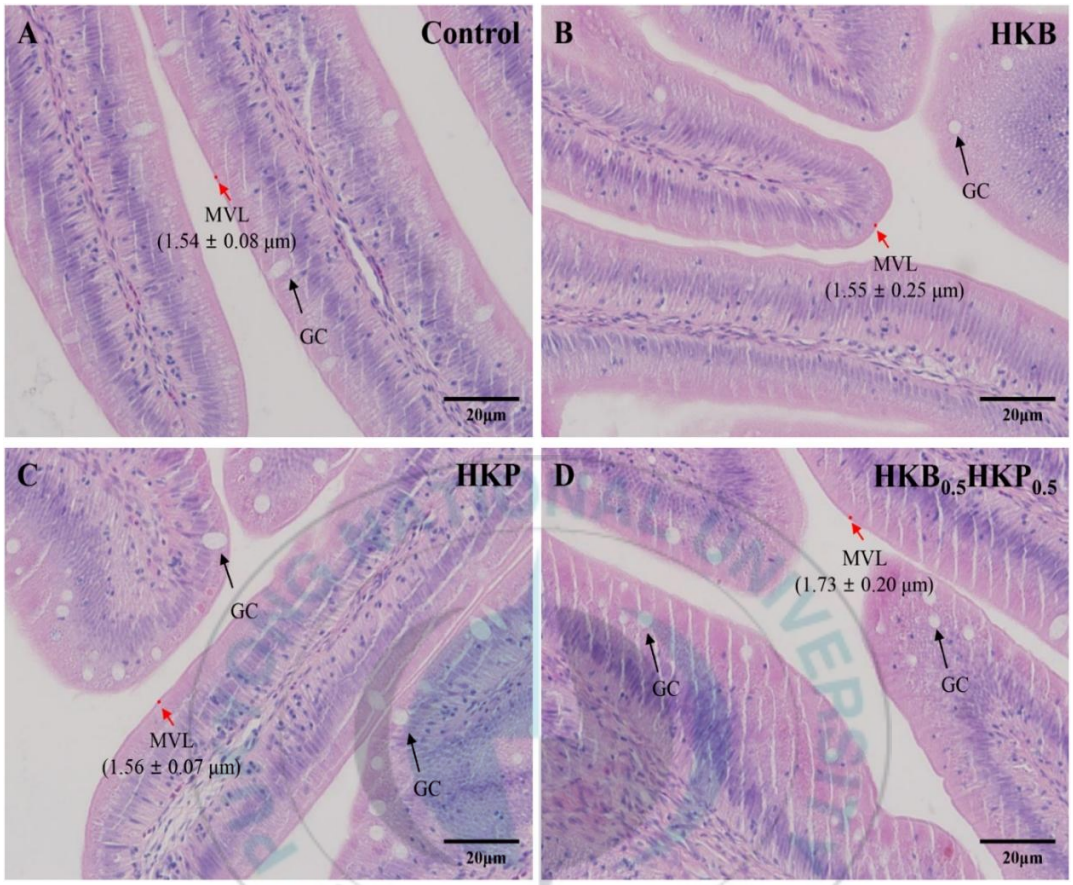

Fig. 16. Olive flounder posterior intestinal histopathology after 8 weeks HK probiotic supplementation A) Control B) HKB C) HKP, and D) HKB0.5HKP0.5. Pictures were taken with light microscope, MV: microvilli; GC: goblet cell; light microscopy staining: hematoxylin and eosin. Scale bars 20 µm (A, B, C, and D)

3.4 Olive flounder appraisal to streptococcosis challenge

After post injection, single mortality in control as well as both HKP and $HKB_{0.5}HKP_{0.5}$ group was recorded after 5 and 6 days respectively. However, first mortality in HKB was observed at day-8 (Fig. 17). Compared to control and HKP, fish fed with HKB were more persistence to *S. iniae* challenge. At 9.75 days RPS in HKP was 9.09 ± 4.09% was statistically similar ($P > 0.05$) to control mortality. However, at the same time the RPS value in HKB was 59.09 ± 6.64% far greater ($P < 0.05$) than HKP and the control. Although $HKB_{0.5}HKP_{0.5}$ first mortality was observed earlier than HKB, but after 9.75 days best RPS (72.73 ± 5.64%) was observed in that group, which was significantly ($P < 0.05$) higher compared to control, HKB, and HKP. This result indicated that mixed HK probiotic can improve flounder resistance to infectious *streptococcosis* compared to control and individual probiotics.

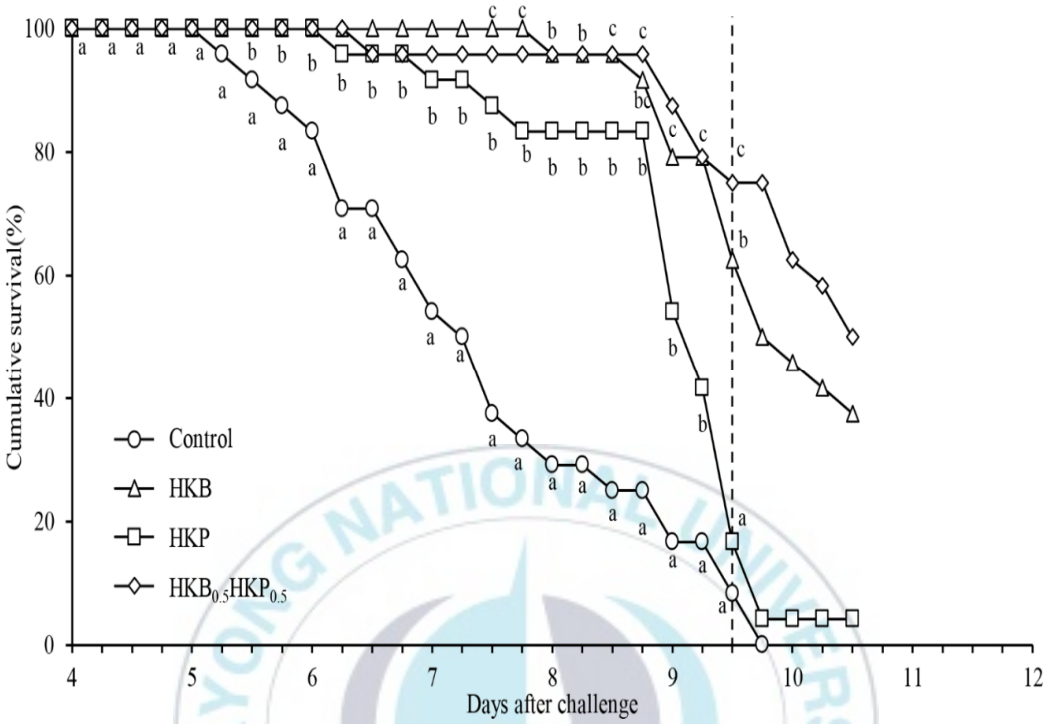

Fig. 17. Olive flounder cumulative survival rate after *S. iniae* challenge (1×10^8 CFU mL^{-1}). Means with different letters are significantly different ($P < 0.05$) and without letter do not differ significantly ($P > 0.05$).

4. Discussion

Fish blood and serum assessment gives an indication of physiological status and enzymes lysosomal activities in body fluids which play a crucial role in defending host from harmful foreign invaders. Single and multi-probiotic as well as their inactivated or HK form have been demonstrated to be excellent immunostimulants, which exerted positive effects on growth performances, innate immune parameters, feed utilizations, and intestinal floral stabilization in aquatic species [23,33,34]. HK probiotic has proven similar or slightly lower efficacy compared to live probiotics in aquatic vertebrae or invertebrates [16, 35] and a very limited number of research was conducted on HK probiotic mixtures effects quantifications in commercial fish species especially in olive flounder. However, in this regard, this research was to find out the effects of HK probiotics and it synergy effects on flounder's growth, immunomodulation, immune gene transcription, and halting ability to streptococcosis.

In this research, after 56 days feeding no alteration was observed in growth (WG and SGR) and feed utilizations parameters (FCR and PER) in the single HK probiotic supplemented groups (HKB and HKP). Similar pattern of results was also reported in rainbow trout

(*Oncorhynchus mykiss)* fry supplemented with formalin inactivated probiotic (*Aeromonas hydrophila* A3-51 and *Carnobacterium* BA211 [36]. However, Tung et al. [37, 38] reported HKP increased WG and SGR in larvae and post-larvae of Kuruma shrimp (*Marsupenaeus japonicas*) which was in contrast with the current findings. Feeding at very early life stage, about 3300 times higher (3.3×10^8 CFU g^{-1}) concentration of HKP, and species difference may be the causal factors of shrimp higher growth performance.

However, flounders fed with 1:1 HKB and HKP mixtures ($HKB_{0.5}HKP_{0.5}$) showed outstanding results in growth and feed utilizations compared to control. Previously Park et al. [39] reported multiple probiotic administration can improve growth and feed utilizations of starry flounder (*Platichthys stellatus*). Furthermore, multi-strains of *L. acidophilus* and HKP L-137 with other probiotics also elevated the growth and feed utilizations of terrestrial animal [40, 41]. Dead probiotics increased beneficial bacterial colonization in gut [42] which increased the secretion of proteolytic enzymes like tannase and phytase [3,43] to breakdown food nutrients, facilitating digestion that improve absorption and assimilation thus improving WG as observed in aquatic animals. Body somatic indices gives us an indication about the fish well-being, the saint breeding milieu, energy stock, fish liver status, and healthy fish ensure better growth [44]. In this research, HK probiotic showed no effect on flounder body somatic indices like CF, VSI, and HSI among the test and control group. Olive flounder final

body proximate compositions were not elevated after HK probiotic administrations, and this result was in line with some previous reports of commercial fish species [37-39].

Sound immunological status attributed to less stress on fish which may contribute to accelerate the growth and feed utilizations performance [63]. The nonspecific immune system has a role to protect animals from pathogenic infection [45]. Yoshitaka et al. [46] reported that oral intake of HKP L-137 augmented innate and acquired immunity in mice and human subjects. In this study, individual HKB and HKP showed no significant modulation in RB and SOD but both HK probiotics elevated LSZ, and only HKB increased MPO. HK probiotic from vibrionaceae family (Pdp11 and 51M6), were unable to increase RB and serum peroxidase content in rainbow trout (*Oncorhynchus mykiss*) [47], however *Lactococcus lactis* was able to alter innate immune parameters [48]. Very importantly, $HKB_{0.5}HKP_{0.5}$ improved one cellular and three humoral parameters compared to control, among which RB and SOD was synergistically higher. During RB neutrophils, macrophages, natural killer cells, and cytotoxic cells are engaged in phagocytosis to produce antimicrobial compounds such as reactive oxygen (O_2^-), nitric oxide (NO), peroxynitrite ($ONOO^-$), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) to get rid of pathogens from host body [49]. SOD transforms O^{2-} to H_2O_2, after which HClO and H_2O_2 are converted to H_2O via this predominant antioxidant pathway [50]. Among different serum biochemical parameters, ALT and AST gives an indication on the levels of stress, adverse environmental

condition, liver malfunction, and presence of toxicants [14]. No elevation of these two-stress indicator hormones was previously reported after HK probiotic supplementation [17, 23] and concluded that our inoculated HK probiotics are safe for flounder feeding.

Among different organs liver synthesize immune hormones and lymphocytes [51], macrophages and B-lymphocytes produced in spleen and kidney respectively, and gill is embedded with lymphoid tissues [52]. In immune signaling pathway, fish cytokines play a vital role in fish immunomodulation [53] and their transcription can be amplified through supplementation with live, HK, single, or mixed probiotics [40]. Live and HK probiotic have been recognized as biological response enhancer, upregulated innate immunity and/or pro-inflammatory and others immune genes expression [54]. In our study, HKB, HKP, and $HKB_{0.5}HKP_{0.5}$ not only triggered pro-inflammatory (TNF-α, IL-6, and IL-1β) but also anti-inflammatory (IL-10) genes in different immune organs like liver, kidney, gill, and spleen. TNF-α orchestrated defense mechanism [55], IL-1β proliferates lymphocytes and macrophages [56], and IL-6 governs metabolism and bone formation [57]. HK probiotic dietary administration elevated the pro-inflammatory (IL-1β, IL-6, IL-17A/F-3, TNF-α, and TNF-N), cell-mediated immune regulatory (IL-12p35, IL-12p40, and IL-18), antiviral (IFN-1 and IFN-γ), and other regulatory (IL-2, IL-7, IL-15, IL-21, IL-10, and TGF-β1) genes in Japanese pufferfish (*Takifugu rubripes*) [58] and *B. amyloliquefaciens* increased IL-1 and TNF-α expression in Nile tilapia [59]. Different cell fragments of HK probiotics might bind

with immune cells receptors (lymphocytes and macrophages) and increased the transcription levels of mentioned genes in different immune organs. Compared to control immune genes transcription in important organs is an indication of robust immunological status in treatment groups in this study. Moreover, in different commercial fish species HK probiotics increased immunological parameters and/or immune related genes transcription [15, 17, 23, 60] which are the supporting evidence of this study. The absorption and assimilation of available digested nutrients is possible via the gut microvilli [16]. In our investigation, the MVL shows no difference between the control and the treatment groups. Certain duration of feeding probiotics were unable to alter the MVL [23, 41] in aquatic species.

The major role of probiotics is to improve fish immunity to combat against infectious pathogens. As an immunostimulant probiotic stimulate immune cells to search and engulf foreign invaders. HK probiotic have been reported to increases the colonization of useful bacteria in the gut of animal and aquatic animal species [41, 42], these useful bacteria have that capacity to stick and colonize the intestinal lining of fish as a result exhibiting a competitive embargo to pathogenic microbes' adhesion on fish intestinal mucus thus overriding the colonization of any pathogens [61]. Previously, in tilapia HK *B. pumilus* and HKP L-137 improved fish resistance to *Aeromonas hydrophila* and *S. agalactiae* challenge, respectively [62, 63], and a mixture of three strains of HK *L. acidophilus* evaluated protection against *Salmonella typhimurium* challenge compared to single probiotic in mice [40]. HKB

and $HKB_{0.5}HKP_{0.5}$ showed better protection against streptococcosis, whereas $HKB_{0.5}HKP_{0.5}$ demonstrated synergistic resistance. *B. cereus* administrated tambaqui (*Colossoma macropomum*) depicted similar immunological profile and survival rate at juvenile stage against infectious bacterial challenge [64]. Previously, dietary supplementation with live or HK probiotics, and probiotics + prebiotics increased cellular and humoral immunity as well as *S. iniae* infections [14, 19, 23, 48, 65] in commercial fish species including olive flounder. Higher elevation in immunological parameters and positive alteration in immune related genes might cumulatively protect experimental flounders from streptococcosis.

Conclusion

Addition of live probiotics in feed and retention of their activities in field level is really labor intensive and time consuming. Findings of this research suggested that HK probiotics also act as immunological response modifier similar to live probiotics. Moreover, mixture of HK probiotics showed better performance compared to individual probiotic supplementations. Increasing growth and immunity by $HKB_{0.5}HKP_{0.5}$ will ensure production enhancement and protection against disease ultimately eradicate antibiotics use in flounder farm. In future, mixed HK probiotic should be supplemented in the field level to identify the difference with these laboratory results. Moreover, supplementations of different graded levels of HKB and HKP mixture to identify the optimum dietary levels for olive flounder should be farther specific study.

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