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Short communication

Heat-killed *Bacillus* sp. SJ-10 probiotic acts as a growth and humoral innate immunity response enhancer in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Investigations were carried out to evaluate and quantify the effects of dietary supplementation with heat-killed (HK) *Bacillus* sp. SJ-10 (BSJ-10) probiotic (1×10^8 CFU g^{-1}) on the growth and immunity of olive flounder (*Paralichthys olivaceus*). Flounder (averagely 9.64 g) were divided into two groups, and fed control and HK BSJ-10 (HKBSJ-10)-inoculated diets for 8 weeks. Investigations were carried out on growth and feed utilizations, innate immunity, serum biochemical parameters, microvilli length, and pro- and anti-inflammatory cytokine gene (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, and IL-10) transcriptions. Compared to control, HKBSJ-10 diet significantly ($P < 0.05$) enhanced weight gain and protein efficiency ratio, 1.17 and 1.11 folds respectively. Humoral innate immune parameters, lysozyme and superoxide dismutase in treatment group were also elevated by 1.34 and 1.16 folds. Similarly, an increased ($P < 0.05$) relative expressions of TNF-α, IL-1β, IL-6 were recorded in liver (2.71, 3.38, and 4.12 folds respectively), and gill (2.08, 1.98, and 1.81 folds respectively) than that of controls. Moreover, after challenge with *Streptococcus iniae* (1×10^8 CFU mL^{-1}), the HKBSJ-10-fed group exhibited significantly higher protection ($P < 0.05$) against streptococcosis compared to controls, validating the observed changes in immune parameters and induction on the cytokine-encoding genes. Therefore, HKBSJ-10 increases growth, modulates innate immune parameters, and protects olive flounders against streptococcosis.

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is among the most common commercially farmed fish species in Northeast Asian countries, including China, Japan, and Korea. In the Republic of Korea, flounder culture contributes about 51.1% of all farmed fish, accounting for about 59.1% economy of the total fish market [1]. However, consistent farming of this species has resulted in degradation of the aquatic environment and outbreaks of bacterial pathogens, leading to production losses of about 38% from 2009 to 2017 [2]. Antibiotics and chemical drugs are common treatments used to eradicate such diseases causing pathogens. The illegal and unscientific use of chemotherapeutants (drugs/antibiotics) in aquaculture can destroy beneficial bacteria, create antibiotic-resistant pathogenic bacterial strains [3], and leave residual effects in fish and humans [4]. To overcome the detrimental effects of antibiotic use, the scientific community has explored alternative fish-feeding strategies, employing environmentally friendly natural ingredients [5,6] such as immunostimulants, prebiotics, probiotics, synbiotics and other forms of microbial management to improve the resistance of cultured fishes to pathogenic bacteria. During the advancement of the Atlantic salmon (*Salmo salar*) industry antibiotics have been nearly completely eliminated, and the maturation of the olive flounder industry in Korea could follow a similar pattern.

Probiotics are certain amount of living beneficial microorganisms that have advantageous effects on host growth and immunity when consumed [7]. Previous studies have applied the inoculation of single and combined live lactic acid bacteria (LAB) and *Bacillus* spp. probiotic to identify and quantify their effects on olive flounder growth, digestive

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enzymes, innate immunity, and bacterial disease resistance [5,8–10]. Conventional live probiotics normally experience a loss of viability during storage [11] and at low intestinal pH [12], overdoses can cause detrimental effects [13]. Moreover, probiotics that do not form spores must be mixed with feed for a certain period of time [14] requiring intensive labor practices in the field. Components and metabolites of dead probiotic cells can also act as immunostimulants for the improvement of digestive enzyme activities and can boost host immunity in a manner similar to probiotics [15–17]. Peptidoglycan, lipopolysaccharides, flagella, and microbial nucleic acids bind with pathogen pattern recognition receptors or toll-like receptors on dendritic cells (DCs), which activate and stimulate macrophages and T-cells to promote immunomodulation and pro-inflammatory cytokine transcription [18].

Live *Gordonia bronchialis* can improve growth performance, color, and skeleton formation in koi carp (*Cyprinus carpio*) [19], and heat-killed (HK) *G. bronchialis* can improve growth and innate immunity in rainbow trout (*Oncorhynchus mykiss*) [20]. Similarly, live and dead commercial probiotics increase innate immunity and survival against pathogenic bacteria in tilapia (*Oreochromis niloticus*) [15]. HK probiotics have demonstrated positive effects on growth and/or immunity in amberjack (*Seriola dumerili*) [21], red sea bream (*Pagrus major*) [16,17], and gilthead seabream (*Sparus aurata*) [22]. However, no research has been conducted to determine the effects of HK probiotic supplementation in olive flounder. Our laboratory identified *Bacillus* sp. SJ-10 (BSJ-10) [23], the full genome sequence [24] and *in vitro* characterization demonstrated its probiotic potentials [12]. Moreover, live form of BSJ-10 at 1×10^8 colony-forming units (CFU) g^{-1} of diet as a dietary supplement for 8 weeks, enumerated as a probiotic for olive flounder [25].

The aim of this research was to investigate the dietary supplementation effects of 1×10^8 CFU g^{-1} HK BSJ-10 (HKBSJ-10) on growth and nonspecific immune responses in olive flounder in an 8-week feeding trial for the first time. Serum biochemistry, microvillus length, cytokine gene transcription, and streptococcosis resistance in both HKBSJ-10 and control groups were also monitored.

2. Materials and methods

The feeding trials and all other experimental activities conducted in this study strictly followed the protocol of the Animal Ethics Committee (regulation no. 554), issued by Pukyong National University, Busan, Republic of Korea.

2.1. Experimental diet preparation

The ingredients and proportions described by Hasan et al. [25] were used for diet preparation and basal diet compositions are shown in Table 1. Brown fish meal, soy protein concentrate, soybean meal, corn gluten meal, and wheat gluten were provided as protein sources, and fish oil and wheat flour were the lipid and carbohydrate sources, respectively. The isonitrogenous, isoenergetic (15.75 kJ g^{-1}) experimental fish feed contained an average of 8.40% moisture, 52.50% crude protein, 8.90% crude lipid, and 8.98% crude ash.

The BSJ-10 culture was incubated in lysogeny broth (LB) at 37 °C for 24 h, centrifuged at 11,000 × *g* for 10 min, and washed twice. CFU levels were calculated using the serial dilution method following total aerobic plate counts [26] and adjusted to 3.34×10^8 mL $^{-1}$ in 300 mL water; this suspension was autoclaved at 121 °C for 15 min instead of 75 °C for 60 min as followed by Panigrahi et al. [27] to kill BSJ-10, confirmed by the absence of bacterial colonies on LB agar plate after spreading.

Feed formulation and storage procedures were carried out following the protocol of Park et al. [28] with modifications. After measuring the required amount of dry feed ingredients, fish oil and distilled water (300 mL kg^{-1}) were added and mixed thoroughly. To formulate the HKBSJ-10 or control diet, 300 mL water with or without HKBSJ-10, respectively, was added to each kilogram of mixture. A 2 mm diameter die in a pelleting machine was used to produce pellets containing 30% moisture. These pellets were air-dried in a convection oven at 50 °C for 48–72 h and stored at −4 °C. Feed and fish body composition analyses were performed according to standard protocols of the Association of Official Analytical Chemists (AOAC) [29].

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

Ingredients	Percent (%)		
Brown FM[a]	45.5		
Soybean meal[a]	12.8		
Corn gluten meal[a]	5		
Wheat flour[b]	14.7		
Wheat gluten[a]	5.85		
Soy protein concentrate [b]	5.85		
Fish oil[c]	4.7		
Lysine[d]	0.5		
Threonine[d]	0.3		
Methaonine[d]	0.3		
Lecithin[a]	0.5	Feed proximate composition analysis (% DM)	
Monocalcium phosphate [d]	1.4	Moisture	8.40
Mineral Mix[e]	1	Crude Protein	52.50
Vitamin Mix[f]	1	Crude Lipid	8.90
Choline[d]	0.5	Crude Ash	8.98
Cellulose[d]	0.1	Gross Energy (KJ g^{-1})[g]	15.75

[a] The feed Co. Goyang, Korea.
[b] Milae ML Co. Icheon, Korea.
[c] Jeil feed Co. Hamman, Korea.
[d] Sigma-Aldrich Korea Yongin, Korea.
[e] 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.
[f] 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.
[g] Estimated energy calculated: 16.8 kJ g^{-1} for protein and carbohydrate, and 37.8 kJ g^{-1} for lipid.

2.2. Fish husbandry and feeding trials

At first, 150 juvenile olive flounders were purchased from a commercial flounder hatchery; these were acclimatized to the aquatic environment at equal stocking densities in 10 indoor semi-recirculating tanks (40 L) and fed a control diet for 14 days. During this period, fish movement, body and fin condition, and feed response were very carefully observed. At the start of the experiment, all fish were fasted for 1 day and healthy olive flounder (body weight ~ 9.64 g and total length ~ 8.95 cm) were selected and distributed at the previous stocking density (15 fish tank $^{-1}$) into six previously acclimatized tanks. Three tanks were randomly assigned to control and HKBSJ-10 groups (treatments). Fish in both groups were fed 2–2.5% of their body weight to apparent satiation at 9:00 and 17:00 for 8 weeks [5,6,9,14,25]. Throughout the experimental period water flow and photoperiod were maintained at 1.2 L min $^{-1}$ and 12L:12D, respectively. Moreover, during that time dissolved oxygen, salinity, water temperature, and pH were estimated at 6.5 ± 1 mg L $^{-1}$, 32 ± 1 ppt, 18 ± 0.5 °C, and 7.2 ± 0.5 respectively by an automatic water quality parameters analyzer (Orion VersaStar Pro; Thermo scientific, USA).

2.3. Sample collection and analyses

During the feeding period, no fish mortality or body disorders were recorded in any of the tanks. At the end of the 56-day feeding trial, all fish in the tanks were caught and body weight was measured to determine the final body weight (FBW), weight gain (WG), specific growth rate (SGR), and feed conversion and protein efficiency ratios (FCR and PER). Then randomly selected three fish per tank (9 fish group^{-1}) were used for anesthetization using 2-phenoxyethanol (500 μL L^{-1}, Sigma-Aldrich, USA).

The body weight and total length of these fish were determined to calculate the condition factor (CF); then caudal vein was punctured with non-heparinized (serum preparation) and heparinized (NBT test) syringes to collect blood samples. The abdomen was opened and the liver and intestine were weighed to calculate the hepatosomatic and viscerosomatic indices (HSI and VSI), respectively. The full bodies with liver and intestine of these fish were sent for proximate composition analyses. For serum sample collection, clotted blood was centrifuged at 5000 × *g* for 10 min and the collected supernatant was immediately stored at −79 °C. Parameters were calculated according to the following equations:

Initial body weight = (Initial weight of total fish in tank) × (Fish number) $^{-1}$; Final body weight = (Final weight of total fish in tank) × (Fish number) $^{-1}$; Weight gain (%) = [(Final weight − Initial weight) × (Initial weight)$^{-1}$] × 100; Specific growth rate (%) = {(ln final weight − ln initial weight) × days^{-1}} × 100; Feed conversion ratio = (Dry feed intake) × (Wet body weight gain) $^{-1}$; Protein efficiency ratio = (Wet weight gain) × (Protein fed) $^{-1}$; Condition factor (%) = {Body weight (g)} × [{Total body length (cm)}^{3}] $^{-1}$ × 100; Viscerosomatic index (%) = (Visceral weight) × (Body weight) $^{-1}$ × 100; Hepatosomatic index (%) = (Liver weight) × (Body weight) $^{-1}$ × 100.

2.4. Innate immunity and serum biochemical parameters analyses

To quantify the respiratory burst (RB) generated during phagocytosis, blood samples were analyzed using a nitro blue tetrazolium (NBT) assay performed according to the method of Anderson and Siwicki [30] with slight modifications. At first, equal volumes (1:1) of 0.2% NBT reagent (Sigma-Aldrich, USA) and blood sample were mixed and incubated for 30 min at room temperature. Then 50 μL of that mixture was mixed with 1 mL N—N-dimethylformamide and centrifuged it at 2000 × *g* for 5 min. The supernatant optical density (OD) was measured at 540 nm using a spectrophotometer (Mecasys, Optizen, Republic of Korea), where dimethylformamide was used as a blank.

A turbidometric assay was performed to estimate serum lysozyme (LSZ) activity following the method of Hultmark et al. [31] with slight variation. At the beginning, 180 μL of 0.2 mg mL^{-1} dissolved lyophilized *Micrococcus lysodeikticus* in phosphate buffer saline (PBS, pH 5.52) was added to a 96-well plate and then 20 μL serum was added. Following incubation at room temperature for 0 and 30 min, absorbance was read at 450 nm using a microplate reader (Infinite M200 nanoquant, Tecan, Zurich, Switzerland); a 0.001-min^{-1} reduction in absorbance was considered 1 unit of LSZ activity.

Determination of serum myeloperoxidase (MPO) activity was done following the method of Quade and Roth [32] with modifications. Initially, 20 μL serum was diluted in 80 μL Hanks Balanced Salt Solution (HBSS) into a 96-well plate. Then 35 μL each of 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, 20 mM) and H_2O_2 (5 mM) were added in that well. After incubation at 35 °C for 2 min, 35 μL 4 M H_2SO_4 was mixed with that previous mixture to stop the color change reaction and absorbance was read at 450 nm using a microplate reader.

The superoxide dismutase (SOD) activity of the serum was quantified using a SOD Assay Kit (K335-100, BioVision, USA) according to the manufacturer's instructions. Antiprotease activity was quantified using methods previously optimized by Heo et al. [8] in our laboratory.

By using an automatic chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan) serum biochemical parameters including total glucose, alanine aminotransferase (ALT), total cholesterol, aspartate aminotransferase (AST), and total protein were quantified.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) and intestinal histopathology

To estimate the effects of the HKBSJ-10 on the expression of pro- and anti-inflammatory cytokines, qRT-PCR analyses was carried out. Total RNA collection and cDNA preparation were done following the method of Hasan et al. [6,14] with slight modifications. After anesthetization of fish (9 fish group^{-1}), tissue samples (55–60 mg) were collected from the liver, kidney, gill, and spleen, and homogenized in 1 mL RiboEx (Trizol, GeneAll, South Korea). Total RNA was collected using an RNA extraction kit (Hybrid-R, GeneAll, South Korea) according to the manufacturer's instructions. To remove genomic DNA contamination, collected RNA was treated with DNase-I using a DNase kit (Riboclear Plus, GeneAll, South Korea), following the manufacturer's protocol. The quantity (ng μL^{-1}) and purity (OD 260:280) of RNA were measured using a NanoDrop (Thremo Fisher Scientific, USA). cDNA was synthesized from RNA (1 μg) using a PrimeScript cDNA Synthesis Kit (Takara, Japan), according to the manufacturers' instructions.

qRT-PCR was conducted using the SYBR green method in a Thermal Cycler Dice™ (Real time machine, Japan) system with gene-specific primers. Primers (Table 2) and qRT-PCR conditions were identical to those of our previous study [25]. A 25 μL reaction mixture was prepared with 12.5 μL SYBR green, 9.5 μL sterile purified water, 2 μL cDNA, 0.5 μL each of the forward and reverse primer (10 μM). The two-step shuttle PCR protocol parameters were as follows: initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s. β-actin (mean Ct = 22.12 ± 0.27) was used as control, no primer–dimer formation was observed, and relative quantification (ΔΔCt) was automatically estimated using the V5.0x software preinstalled in the Thermal Cycler Dice™.

Table 2
Gene specific primers and gene bank accession number of olive flounder β-actin and immune related genes used in this study.

Name of gene	Sense	Oligonucleotide Sequence (5′ to 3′)	Base Pair (bp)	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		

Fish used for qRT-PCR tissue collection (9 fish group^{-1}) were subjected to posterior intestinal histopathology according to the method of Cerezuela et al. [33] with some modifications. The posterior portion of the intestine was sectioned, fixed, and re-fixed in 10% formalin. Tissues were dehydrated using graded alcohol treatments, cleared with xylene, and embedded in paraffin wax. Hematoxylin and eosin staining was performed after embedded tissue blocks were sectioned (4 μm diameter) using a microtome machine (Leica RM 2125 RTS, Germany). Light microscopic photograph was used to measure the microvillus length using the Image-Pro plus software (Version 5.1, Germany).

2.6. Pathogenic challenge

Pathogenic *Streptococcus iniae* was purchased from Korean Collection for Type Cultures (KCTC3657, South Korea). At first, single colony of that bacteria was inoculated in 3 mL brain heart infusion (BHI; Becton, Dickinson and Company, USA) broth and incubated at 37 °C for 24 h in a shaking incubator. Next day from that culture 1% was inoculated again in BHI broth and after incubation when growth reached at mid-log phage (previously optimized at OD600nm) was centrifuged and washed two times with PBS (pH 7.0, 0.1 M). Contamination identification and CFU mL^{-1} estimation were performed on BHI agar plate by serial dilution method. After that final concentration was adjusted at 1×10^8 CFU mL^{-1} in PBS solution.

The remaining 9 fish in each tank (27 fish group^{-1}) were intraperitoneally (i.p.) injected with 100 μL of *S. iniae* at 1×10^8 CFU mL^{-1} [6,8,14,25] and kept in challenge test tanks. During pathogenic challenge, fish were starved without water exchange. The mortality in each tank was recorded every 6 h on a daily basis to 13 days; because after 11 days, no mortality was observed in HKBSJ-10. To confirm the systemic infection of streptococcosis, swabs from kidney of dead fish were spread on to BHI agar plates to observe growth of *S. iniae*. The survival (%) of fish was calculated following an equation [6], as follows.

Survival (%) = [(initial fish – dead fish) × initial fish^{-1}] × 100

2.7. Statistical analyses

Normality and homogeneity of variance were assessed for all data using the Shaprio–Wilk and Levene tests, respectively. All data were analyzed using the IBM SPSS software (SPSS Inc., version 17.0, Chicago, IL, USA) following student t-test. Statistical significance was determined at a level of $P < 0.05$. Data are presented as means ± standard deviations (SD).

3. Results

3.1. Effects on growth, feed utilization, and organosomatic indices

Following the 56-day feeding trial, growth (FBW, WG, and SGR) (Fig. 1) and feed utilization (FCR and PER) parameters of the HKBSJ-10 group were positively upregulated ($P < 0.05$) compared to the control group (Table 3). WG (%) values were 232.10 ± 4.78 and 271.57 ± 2.86, and FCR values were 1.16 ± 0.04 and 1.05 ± 0.04 in the control and HKBSJ-10 groups, respectively. These results clearly demonstrate positive growth and feed utilization modulation following HKBSJ-10 supplementation. However, there were no significant differences ($P > 0.05$) in flounder organosomatic indices (CF, VSI, and HSI) (Table 3) or initial and final body proximate composition between groups (Table 4).

3.2. Effects on nonspecific immunity and serum biochemical parameters

Among the five-important innate immune parameters, two (LSZ and SOD) were significantly ($P < 0.05$) elevated by the dietary supplement inoculated with HKBSJ-10 (Table 5). RB, MPO, and antiprotease activity values statistically similar ($P > 0.05$) between the control and treatment groups. However, LSZ and SOD activity values for the HKBSJ-10 supplement group were 0.67 ± 0.03 and 62.06 ± 2.47, respectively, significantly higher than 0.50 ± 0.04 and 53.25 ± 4.60, respectively, in the control group. These results demonstrate the humoral innate immunity modulation properties of HKBSJ-10.

HKBSJ-10 did not affect ($P > 0.05$) total cholesterol, ALT, total

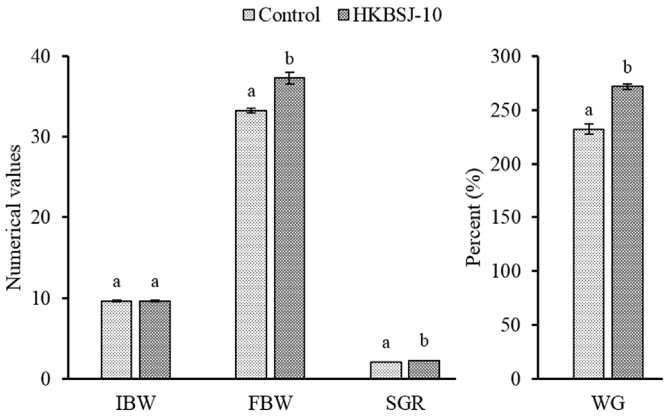

Fig. 1. Growth parameters of olive flounder fed control and HKBSJ-10 supplement diets for 56 days. IBW: Initial body weight (g); FBW: Final body weight (g); SGR: Specific growth rate (% day^{-1}); WG: Weight gain (%). Data represent mean ± standard deviation; means with the same or different letters are not significantly ($P > 0.05$) or are significantly ($P < 0.05$) different, respectively.

Table 3
Growth performance, feed utilization and organosomatic indices of olive flounder supplemented with the control and HKBSJ-10 diets for 8 weeks.

Growth, feed utilization and organosomatic parameters	Diets		*P* value
	Control	HKBSJ-10	
Initial body weight (g)	9.64 ± 0.05	9.64 ± 0.12	0.970
Final body weight (g)	33.26 ± 0.30^a	37.28 ± 0.70^b	0.001
Weight gain (%)	232.10 ± 4.78^a	271.57 ± 2.86^b	0.00025
Specific growth rate (% day^{-1})	2.08 ± 0.03^a	2.28 ± 0.03^b	0.002
Feed conversion ratio	1.16 ± 0.04^b	1.05 ± 0.04^a	0.042
Protein efficiency ratio	1.48 ± 0.05^a	1.65 ± 0.07^b	0.029
Condition factor (%)	0.68 ± 0.02	0.78 ± 0.06	0.083
Viscerosomatic Index (%)	1.33 ± 0.13	1.52 ± 0.07	0.200
Hepatosomatic index (%)	2.62 ± 0.16	2.78 ± 0.07	0.120

Values are mean ± SD of three replicates (3 fish replicate^{-1}). Values with different superscript letters within the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences ($P > 0.05$).

Table 4
Whole-body proximate compositions (% of wet weight) of olive flounder supplemented with the control and HKBSJ-10 diets for 8 weeks.

Compositions	Body proximate composition (% of wet weight)			*P* value
	Initial	Final		
		Control	HKBSJ-10	
Moisture	75.46 ± 1.21	74.50 ± 1.93	74.82 ± 2.77	0.875
Crude protein	17.89 ± 1.68	18.66 ± 1.55	18.42 ± 1.21	0.843
Crude lipid	3.29 ± 0.31	3.15 ± 0.22	3.18 ± 0.19	0.869
Crude ash	3.94 ± 0.19	3.74 ± 0.09	3.63 ± 0.10	0.217

Values are mean ± SD of three replicates (3 fish replicate^{-1}). All values that are within the same row in final body proximate composition are not significantly different ($P > 0.05$).

Table 5
Influence of the control and HKBSJ-10 diets on non-specific immune parameters of olive flounder supplemented for 8 weeks.

Innate Immunity Parameters	Diets		*P* value
	Control	HKBSJ-10	
Respiratory burst[a]	0.39 ± 0.05	0.40 ± 0.03	0.894
Superoxide dismutase[b]	53.25 ± 4.60[a]	62.06 ± 2.47[b]	0.043
Serum lysozyme activity [c]	0.50 ± 0.04[a]	0.67 ± 0.03[b]	0.009
Myeloperoxidase[d]	1.20 ± 0.05	1.28 ± 0.08	0.227
Antiprotease[e]	68.51 ± 1.89	68.61 ± 4.24	0.974

Values are mean ± SD of three replicates (3 fish replicate^{-1}). Values with different superscript letters within the same row are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences ($P > 0.05$).

[a] Respiratory burst: Absorbance at 540 nm.
[b] Superoxide dismutase: % Superoxide inhibition.
[c] Serum lysozyme activity: Units mL^{-1}.
[d] Myeloperoxidase: Absorbance at 450 nm.
[e] Antiprotease: % of Trypsin inhibition.

Table 6
Serum biochemical parameters of olive flounder fed with the control and HKBSJ-10 diets for 8 weeks.

Biochemical Parameters	Diets		*P* value
	Control	HKBSJ-10	
Alanine aminotransferase (U L^{-1})	10.41 ± 2.50	8.52 ± 3.41	0.482
Aspartate aminotransferase (U L^{-1})	27.76 ± 2.89	30.29 ± 3.41	0.384
Total Glucose (mg dL^{-1})	17.67 ± 2.38	12.93 ± 3.32	0.116
Total Cholesterol (mg dL^{-1})	253.40 ± 7.17	239.83 ± 9.85	0.126
Serum Protein (mg mL^{-1})	18.60 ± 1.44	18.75 ± 2.15	0.926

Values are mean ± SD of three replicates (3 fish replicate^{-1}). All values within the same row in the table are not significantly different ($P > 0.05$).

glucose, AST, or total protein levels. Although ALT and total cholesterol were numerically lower in the HKBSJ-10 group, these differences were not significant ($P > 0.05$) (Table 6).

3.3. Effects on cytokine gene expression and intestinal microvillus length (MVL)

Anti-inflammatory cytokine (IL-10) expression did not change ($P > 0.05$) in the liver, kidney gill, or spleen at the end of the feeding trial (Fig. 2). Except kidney (Fig. 2B), TNF-α expression in the liver (Fig. 2A), gill (Fig. 2C), and spleen (Fig. 2D) of the HKBSJ-10 group were about 2.71, 2.08, and 2.00 times higher ($P < 0.05$), respectively, than the control. A similar pattern was observed in IL-1β expression, which was 3.38, 4.76, 1.98, and 1.94 times higher in the liver, kidney, gill, and spleen, respectively. IL-6 expression levels were 4.12, 3.30, and 1.81 times higher ($P < 0.05$) in the liver, kidney, and gill in the HKBSJ-10 group than in the control, whereas there was no significant difference ($P > 0.05$) in expression in the spleen.

Posterior intestinal images showed clear and intact epithelial barriers, goblet cell arrangements, and microvillus structures in both feeding groups. Mean MVL were 1.20 ± 0.04 and 1.21 ± 0.05 μm in the control and HKBSJ-10 groups, respectively (Fig. 3), demonstrating that there were no statistical differences ($P > 0.05$) between groups.

3.4. Streptococcosis resistance after S. iniae challenge

In the control group, the first fish death was recorded 6 days following i.p. injection with pathogenic *S. iniae*, whereas that in the HKBSJ-10 group occurred after 8 days (Fig. 4). At day-9 post-challenge, fish survival in HKBSJ-10 was 59.27 ± 5.78% which was significantly ($P < 0.05$) higher than 37.04 ± 4.34% in controls. After 11 days, no mortality was observed in the HKBSJ-10 group and a cumulative survival of 18.52 ± 2.00% continued to 13 days, possibly indicating resistance against streptococcosis. These results demonstrate a significantly higher ($P < 0.05$) death rate in the control and greater survival and protection against streptococcosis in the HKBSJ-10 supplement group.

4. Discussion

To date, there has been no study of the effects of orally administered HK probiotics in olive flounder, although live probiotic supplements have been shown to increase growth, feed utilization, cellular and humoral innate immunity, cytokine gene transcription, and infectious disease resistance in olive flounder [10,25,34]. HK probiotics offer an attractive alternative to live probiotics, because their activity is similar to that of an immunestimulant [35]. Binding with the active sites of pathogen, immunostimulants can eradicate/decrease pathogenicity and adhesion to the intestinal wall [36], and interaction with dendritic cells stimulates innate immune phagocytic cells to engulf and destroy the pathogenic antigen [36,37].

In this study, supplementation with HKBSJ-10 increased growth (WG and SGR) and feed utilization (FCR and PER) parameters compared to the control. These findings are consistent with those of a previous study involving administration of live *Bacillus* probiotics (*B. subtilis* and BSJ-10) to olive flounder for 8 weeks [5,9,25]. Importantly, our HK probiotic growth parameter results exhibited patterns identical to those reported for other commercially important farmed aquatic species including kuruma shrimp (*Marsupenaeus japonicas*) [38], rainbow trout [39], amberjack [21], and red sea bream [16,17]; however, a different trend was observed in rohu (*Labeo rohita*) [40]. The mechanism underlying the improvement of these parameters by HK probiotics in the present study remains unclear. Nevertheless, oral supplementation with different forms (HK, dead, or inactivated) of probiotics increased digestive enzymes activities, resulting in better digestion of lipids, carbohydrates, and proteins, leading to positive effects on weight and feed utilization in fish [40,41].

Fish innate immunity is considered the key weapon by which self and non-self are distinguished and foreign invaders are removed from the body. The immune system is complicated, comprising different types of immune cells, as well as pro-/anti-inflammatory and regulatory cytokine genes. Cytokine activity occurs in a cascading manner; cytokine transcription is an indicator of fish immune system modulation/alteration. Supplementation with HKBSJ-10 had no effect on RB, MPO, or antiprotease activity, but upregulated serum SOD and LSZ activity. LSZ cleaves β-(1, 4)-linked N-acetylglucosamine and N-acetylmuramic acid in the bacterial cell wall [42] and destroys all types of pathogens engulfed by macrophages and neutrophils [43], which is the hallmark of an immune responses to feed additives. Highly reactive O^{2-} produced by neutrophils, macrophages, and monocytes during RB are converted into less reactive H_2O_2 and finally to H_2O through the action of SOD in the main antioxidant pathway to maintain immunological balance [44]. The increased activity of these two important enzymes in this study demonstrated the higher immunological status and immune pathway toxicants stress-free fish of the HKBSJ-10 group than in the control.

HKBSJ-10 had no effect on serum biochemical parameters or posterior intestinal MVL. Specifically, higher serum ALT and AST levels indicate toxin stress and liver dysfunction in fish [14]; the lack of change observed in these two stress-inducing enzymes indicates that HKBSJ-10 is safe for use as a feed additive for olive flounder, as previously *B. subtilis/licheniformis* shown in starry flounder (*Platichthys stellatus*) [28]. Microvilli increase nutrient absorption by lengthening the apical brush border area of the intestine. Single administration of HK *B. subtilis* has been shown to increase the number of folds and lymphoid cells in the posterior intestine of gilthead seabream [22];

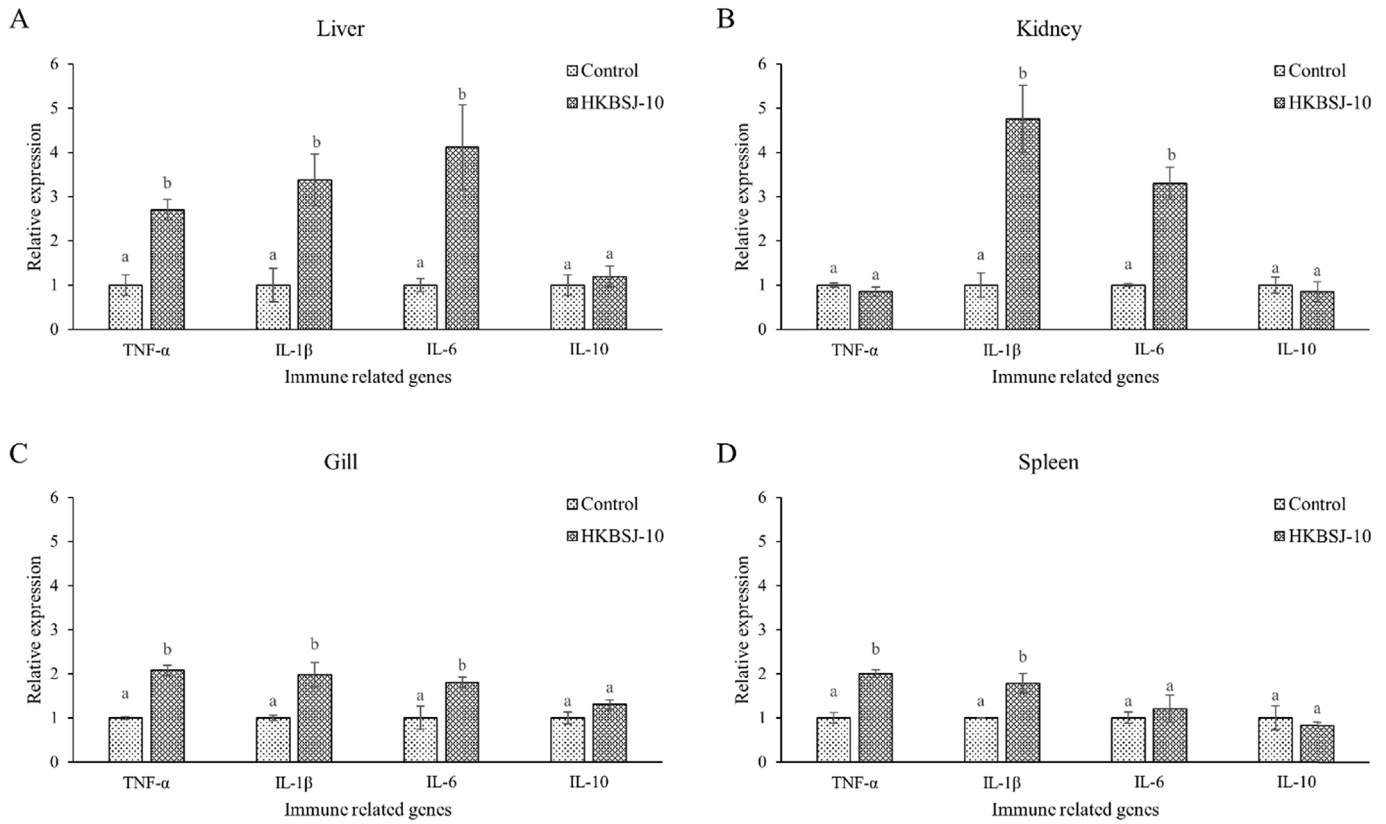

Fig. 2. Profiles of different immune-related gene expression in specific organs, (A) liver, (B) kidney, (C) gill, and (D) spleen in olive flounder were measured using qRT-PCR after 8 weeks feeding with control and HKBSJ-10. Expression of these genes was quantified relative to β-actin transcription in 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.

however, administration of the same live probiotic in the same fish species produced no effects on MVL [33]. In addition, HK *G. bronchialis* administration also showed no change in the structure and number of intestinal folds in rainbow trout [20].

Unlike IL-10, TNF-α, IL-6, and IL-1β expression was significantly higher in the liver, kidney, gill, and spleen of fish in the HKBSJ-10 group. HK probiotics clearly act as a biological response modifier and induce pro-inflammatory cytokine expressions [13]. TNF-α is secreted by activated macrophages [45] and orchestrate fish immune defense mechanisms against pathogen entrance, colonization, and proliferation [46]. IL-1β is a key response mediator that eradicates invading pathogens in the body by stimulating macrophages, natural killer cells, and lymphocytes [47]. IL-6 is a bone formation and metabolism-governing gene [48]; the positive change in WG and FCR in the HKBSJ-10 group may have been correlated with significant IL-6 transcription. Disrupted fragments of HKBSJ-10 cell walls component (peptidoglycan) bind with specific receptors in DCs [10,18], stimulating and activating phagocytic cells (macrophage and lymphocytes) to transcribe these pro-inflammatory cytokines in different localized organs in olive flounder. Moderate transcription of pro-inflammatory cytokines may be beneficial for the maintenance of immunological balance and increase resistance against infection [13]. HK probiotic administration significantly increased cytokine gene transcription [49,50] and upregulated innate immunity in fish [15–17,20–22,27]; our findings are consistent with the results of these previous studies.

The increase in two humoral innate immune parameters and expression of three important cytokines in different organs may have cumulatively resulted in higher survival against streptococcosis in the

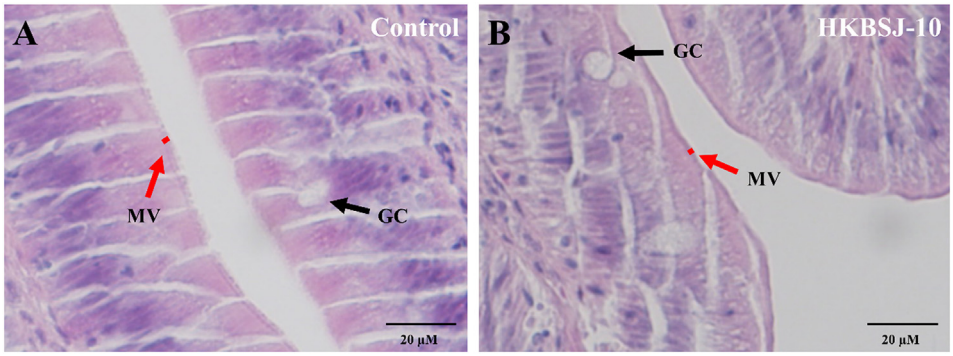

Fig. 3. Posterior-intestinal histopathology of olive flounder fed control (A) or with HKBSJ-10 supplement (B) for 56 days. Pictures were taken with a light microscope, MV: microvilli; GC: goblet cell; light microscopy staining: hematoxylin and eosin. Scale bars 20 μm (A and B).

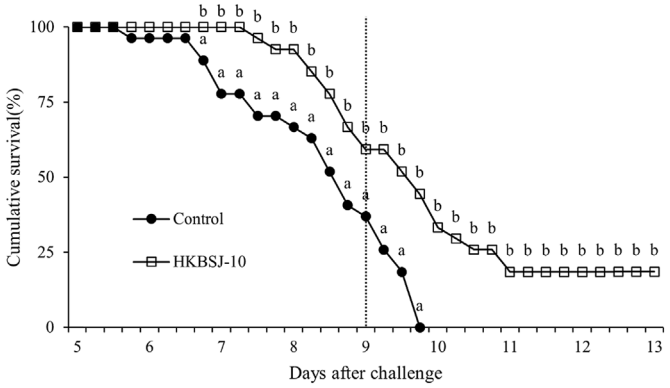

Fig. 4. Cumulative survival rates of olive flounder after challenge with *Streptococcus iniae* (1×10^8 CFU mL^{-1}). Means with different letters are significantly different ($P < 0.05$) and those without a letter do not differ significantly ($P > 0.05$).

HKBSJ-10 compered to control. Previous reports on supplementation with HK probiotics or probiotic cell components have demonstrated increased disease resistance against infectious *S. iniae* and *Yersinia ruckeri* challenges in rainbow trout [51,52]. Similar to the disease protection exhibited by HK probiotics in the current study, live *B. subtilis* and *B. licheniformis*, as well as BSJ-10 [9,25] and LAB [8,10] supplementation, have previously demonstrated improved innate immunity resulting in streptococcosis resistance.

5. Conclusion

In this first-ever report of HK probiotic supplementation in olive flounder, higher growth, feed utilization, and improved immunity alongside resistance to infectious disease were demonstrated amongst the HKBSJ-10 supplement group. The application of these findings in the field will reduce the reliance on live probiotic viability in feed, increasing production, decreasing fish mortality, and progressively reducing antibiotic use to ensure the health bio-safety of humans and aquatic species. After this initial investigation, further study should be conducted by the oral administration with different concentrations levels of HKBSJ-10 to identify its optimum dietary requirement for olive flounder.

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