Effects of *Lactococcus lactis* subsp. *lactis* I2 with β -Glucooligosaccharides on Growth, Innate Immunity and Streptococcosis Resistance in Olive Flounder (*Paralichthys olivaceus*)

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To identify and quantify the effects of a combination of dietary 1×10^8 CFU/g *Lactococcus lactis* subsp. *lactis* I2 (LI₂) and 0.1% β -glucooligosaccharides (BGO) on the growth and immunity of olive flounder (*Paralichthys olivaceus*), a feeding experiment was conducted. Flounder (14 ± 0.5 g) were divided into two groups and fed control and synbiotic feeds for 8 weeks. Investigations were carried out on growth and feed utilization, innate immunity, serum biochemical parameters, intestinal lactic acid bacterial (LAB) viability, microvillus length, and changes in the expression levels of genes encoding pro-inflammatory cytokines (tumor necrosis factor [TNF]- α , interleukin [IL]-1 β , and IL-6). Results demonstrated the synbiotic diet had significantly better ($p < 0.05$) responses in terms of weight gain and specific growth rate, three innate immune parameters (respiratory burst, serum lysozyme, and superoxide dismutase), intestinal LAB viability, and the relative TNF- α expression level ($p < 0.05$). Moreover, after challenge with *Streptococcus iniae* (1×10^8 CFU/ml), the synbiotically fed group exhibited significantly higher ($p < 0.05$) protection against streptococcosis, validating the observed changes in immune parameters and induction of the cytokine-encoding gene. Therefore, according to the results of the present study, synbiotic feed (LI₂ + BGO) increased growth, modulated innate immune parameters and protected olive flounder against streptococcosis.

Keywords: Synbiotic, growth performance, innate immunity, gene expression, olive flounder

Introduction

Olive flounder (*Paralichthys olivaceus*) is one of the most popular and commercially important fish species in far-east Asian countries including China, Japan, and Korea. Moreover, it is the foremost aquaculture species, with annual production reaching 41,636 tons and accounting for 51.1% of all aquaculture production in Korea [1] as the industry continues to grow day by day. However, large-scale production increases the risk of infectious disease outbreaks, which can cause catastrophic production and economic losses. For example, in the past 5 years (2009–

2014), cumulative annual production in Korea has fallen by about 17,647 tons, because of such outbreaks [2], 32% of which were caused by pathogenic bacteria with 19% by *Streptococcus* spp. [3].

In efforts to at least partially control bacterial infections, antibiotics like tetracycline, oxytetracycline, cephalosporin, erythromycin, and amoxicillin; chemotherapeutics like formalin, NaCl baths, and hydrogen peroxide; and various vaccines are very commonly used in aquaculture farms. However, repeated antibiotic use is associated with *tet*, *erm*, and *mef* activations in *Streptococcus* spp., creating tetracycline- and erythromycin-resistance by changing the

target ribosomal subunit by methylation or mutation, modifying the antibiotic activity via enzyme-mediated antibiotic efflux [4]. Furthermore, antibiotics nonselectively destroy both harmful and beneficial microbes in aquatic environments, and their residues in aquaculture products pose worldwide food and health biosafety problems and compromise human health [5]. The negative effects of vaccine administration on olive flounder [6]; the development of antibiotic-resistant *Streptococcus* spp. in both the olive flounder [7] and humans [8], render it essential to develop biologically-safe alternative approaches (prebiotics, probiotics, and synbiotics) to improve fish immunity against pathogens of aquaculture facilities [9, 10]. Prebiotics are low-molecular weight, non-digestible carbohydrates (usually oligosaccharides) that stimulate the growth and metabolism of beneficial intestinal bacteria [11]. Also, the administration of adequate concentrations of live bacteria to provide beneficial effects on growth and immunology of consumer is termed as probiotics [12]. Lactic acid bacteria (LAB) produce antimicrobial compounds such as nisin and pediocin [13] and directly inhibit the growth of *Streptococcus iniae* and other Gram-positive pathogenic bacteria [3, 14, 15]. Both the US Food and Drug Administration and the World Health Organization have approved such bacteria for use as probiotics for olive flounder. Moreover, intestinal LAB probiotics metabolize the usually indigestible prebiotics for growth stimulation and survival, and exert positive effects on the growth and immune status of the host [16]. Combinations of probiotics and prebiotics may exhibit additive, synergistic, or potentiation patterns termed as synbiotic [17], improving probiotic survival because the probiotic bacteria metabolize the prebiotic to a greater extent than do other intestinal bacteria. To the best of our knowledge, only one report on synbiotic supplementation of the diet of olive flounder (using *Bacillus clausii* with commercial prebiotics) has appeared [9]; no report had yet evaluated an LAB/prebiotic combination.

We earlier showed that nisin-Z-(bacteriocin) producing *Lactococcus lactis* subsp. *lactis* I2 (LI₂) isolated from the intestine of olive flounder, and a β -glucan oligosaccharides (BGO) from barley β -glucan, exhibited probiotic (1×10^8 CFU/g) [3] and prebiotic (0.1%) [11] effects, respectively, in olive flounder. Moreover, in vitro, LI₂ showed stimulated growth and survival associated with a 20% elevation in nisin-Z production by fermenting 0.1% BGO, as opposed to when glucose served as the growth substrate (our unpublished data). However, the potential of this synbiotic has not yet been evaluated in either a terrestrial or aquatic species.

Given the current problems in flounder aquaculture, and the potential of synbiotics, our objective was to develop an LAB-based synbiotic using a combination of LI₂ and BGO at their previously reported conditions. We explored the effects of this synbiotic on flounder growth and innate immunity, and in terms of protection against streptococcosis.

Materials and Methods

All experimental activities in this research were performed following the guidelines of the Animal Ethics Committee Regulations, No. 554, issued by Pukyong National University, Busan, Republic of Korea.

Feed Formulation

Feed formulation and storage were performed as described by Bai and Kim [18]. Fishmeal, fish oil, and wheat flour were the protein, lipid, and carbohydrate sources, respectively (Table 1). After measuring and mixing all solid feed ingredients, fish oil and 30% (v/w) water were added. Pelletizing was performed using a 2-mm-diameter die by a pelletizing machine (Baokyoung Commercial Co., Korea). The feed was dried for 48–72 h at room temperature and stored at -4°C . To prepare a prebiotic-containing feed, the cellulose (0.1%) of the control feed was replaced by 0.1% BGO.

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

Ingredients	Percent (%)
Fish meal (herring) ^a	62.0
Fish meal (tuna) ^a	10.5
Wheat flour ^b	20.0
Fish oil (menhaden) ^b	3.4
Vitamin premix ^c	1.8
Mineral premix ^d	1.8
Cellulose ^e	0.5
Feed proximate composition analysis (% DM)	
Moisture	9.19
Crude protein	56.5
Crude lipid	9.73
Crude ash	12.8

^aSuhyup Feed Co., Uiryeong, Korea

^bThe Feed Co., Goyang, Korea

^cContains (as mg/kg in diets): Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine·HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl- α -Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06.

^dContains (as mg/kg in diets): NaCl, 437.4; MgSO₄·7H₂O, 1379.8; ZnSO₄·7H₂O, 226.4; Fe-Citrate, 299; MnSO₄, 0.016; FeSO₄, 0.0378; CuSO₄, 0.00033; Ca(IO)₃, 0.0006; MgO, 0.00135; NaSeO₃, 0.00025.

^eSigma-Aldrich Korea, Yongin, Korea

The culture and addition of LI_2 to the prebiotic feed was performed as described by Heo *et al.* [3]. Adjusted concentration of LI_2 in PBS (pH 7.0, 0.1 M) was sprayed on the prebiotic feed every 3 days (LI_2 does not form spores), to ensure the presence of 1×10^8 CFU/g of LI_2 in that prebiotic diet, totally formulated as a synbiotic feed. Feed and fish body proximate compositional analyses were performed by reference to the AOAC [19] recommended methods. Isonitrogenous and isoenergetic experimental diet formulations were prepared containing 56.5% crude protein, 9.73% lipid, 9.19% moisture, and 12.8% ash (Table 1).

Fish Husbandry and the Feeding Trial

The feeding trial was performed in the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan, Republic of Korea. Flounder were acclimated to the experimental environment, consuming control feed, for 14 days, and 10 flounders (body weight 14 ± 0.5 g and total length 12 ± 0.5 cm) were then placed in each of six 40-L semi-recirculated seawater tanks and the two feeds (control and synbiotic) provided to each of three replicate tanks. The fish were given approximately 2.5–3% of their body weight of feed twice daily (at 9:00 and 17:00) for 8 weeks. During the entire trial, the water temperature, water flow, dissolved oxygen level, salinity, photoperiod, and pH were $17.5 \pm 0.5^\circ\text{C}$, 1.2 l/min, 7.0 mg/l, 32 ± 1 ppt, 12-h light:12-h dark, and 7.4 ± 0.5 , respectively.

Sample Collection

At the end of the trial, all fish were weighed and weight gains (WG) and feed utilization parameters (feed efficiency ratio and protein efficiency ratio) calculated. Three fish randomly selected from each tank (9 fish/group) were anesthetized in 500 μl /l 2-phenoxyethanol (Sigma-Aldrich, USA), and their lengths and weights were measured for condition factor (CF) calculations. Then, blood was collected from the caudal vein using heparinized and non-heparinized syringes. Serum was prepared from clotted blood by centrifugation at $5,000 \times g$ for 10 min and stored at -79°C . After that, the livers and intestines of the fish were weighed to allow calculation of the viscerosomatic and hepatosomatic indices (VSI and HSI); these tissues, along with the fish carcasses, underwent proximate composition analyses.

Estimation of Feed LI_2 and Intestinal LAB Viabilities

We used a slight modification of the method of Nikoskelainen *et al.* [20] to measure LAB viability in feed and fish intestines. One-gram amounts of feed were mixed with 9 ml (10-fold dilution) of PBS (pH 7.0, 0.1 M) and held at room temperature for 10 min. After simple vortexing, the suspension was held at room temperature for a further 7–8 min to allow the feed to settle. Then, 1-ml amounts of supernatant were serially diluted and spread on lactobacilli selective MRS (de Man, Rogosa & Sharpe Merck, Germany) agar plates, incubated in 37°C for 24 h and colonies were counted. To determine intestinal LAB viability, one fish per tank (3 fish/group) was starved for 24 h, anesthetized, and 1 g of

intestinal tissue was collected, cut into small pieces, ground well, and then 0.5 g of ground tissue was diluted with 4.5 ml of PBS, and processed as described above for the feed samples.

Innate Immune Parameters and Serum Biochemical Parameters

The turbidometric assay used to determine serum lysozyme activity was that of Ellis [21], with slight variations. Briefly, 190- μl of a 0.2 mg/ml lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) suspension in PBS (pH 6.2, 0.05 M) were added to wells of a 96-well plate and 10- μl of serum were then added. Absorbances were read at 530 nm after incubation at room temperature for 0.5 and 4.5 min by a microplate reader (Infinite M200 nanoquant, Tecan, Zurich, Switzerland). One unit of lysozyme activity corresponded to a reduction in absorbance of 0.001/min.

Superoxide dismutase (SOD) activities in 20- μl of serum were measured using an SOD assay kit (K335-100, BioVision, USA) according to the manufacturer's instructions. SOD inhibited the action of xanthine oxidase on a water-soluble tetrazolium dye. Absorbances at 450 nm were read after 20 min reaction at 37°C and percentage inhibitions calculated using absorbance reading through a kit optimized equation.

Serum myeloperoxidase (MPO) activity was estimated using the method of Quade and Roth [22], with slight modifications. Serum samples (20 μl) were diluted with 80- μl of Hanks' balanced salt solution (without Ca^{2+} or Mg^{2+}) in wells of a 96-well plate and 35 μl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich, USA) and 35 μl of 5 mM H_2O_2 were added to each well. After 2 min of incubation, 35 μl of 4 M H_2SO_4 was added to stop the color change and the absorbance read at 450 nm.

The generation of oxidative radicals by neutrophils engaging in phagocytic activity during a respiratory burst was measured with the aid of the nitroblue-tetrazolium (NBT) assay, as described by Hasan *et al.* [11], and serum antiprotease levels were estimated using the method of Heo *et al.* [3].

Total cholesterol, serum alanine aminotransferase (ALT), serum total glucose, serum protein and aspartate aminotransferase (AST) levels were determined with the aid of an autoanalyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Japan).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Middle Intestinal Histopathology

To quantify the effects of synbiotic feeding on the levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 in four distinct organs (liver, kidney, gill and spleen) RT-PCR was performed. Organ samples were collected from 1 fish per tank (3 fish/group) and we performed RT-PCR as described by Hasan *et al.* [11]. Briefly, 55–60-mg of organ tissues were homogenized in 1-ml of Ambion Trizol (Thermo Fisher Scientific, USA) under sterile conditions, and RNA was extracted according to the instructions of the manufacturer. The RNAs were treated with DNase-I to remove genomic DNA, following the protocol of a DNase kit (Riboclear plus, GeneAll, Korea). RNA purity (OD

Table 2. Gene specific primers, annealing temperature, gene bank accession number^a and cycle number^b of olive flounder β -actin and cytokines genes used in this study.

Name of gene	Sense	Oligonucleotide Sequence (5' to 3')	Base pair (bp)	Annealing temperature (°C)
β -actin	F	TTTCCTCCATTGTTGGTCG	200	58
	R	GCGACTCTCAGCTCGTTGT		
TNF- α	F	CAGCTGCAGCTCAGCAGGCACCTGGAGAC	168	60
	R	GGACGACTTCTTCTCCACCAGAACC		
IL-1 β	F	ATGGAATCCAAGATGGAATGC	250	57
	R	GAGACGAGCTTCTCTCACAC		
IL-6	F	CGAATACGAGCCACCGACAGTCC	460	58
	R	GTGGAAAGTGCTGGGGTTGTGG		

^aGene bank accession number: β -actin (HQ386788.1), TNF- α (AB040448.1), IL-1 β (AB070835.1), IL-6 (DQ267937.1).

^bCycle numbers: 30

260:280) and concentration (ng/ μ l) were measured via NanoDrop (Thermo Fisher Scientific, USA) spectrometry and 1- μ g of RNA were used for cDNA synthesis employing a kit protocol (PrimeScript, Takara, Japan) as instructed by the manufacturer. PCR was performed on an equivalent of cDNA using gene-specific primers (Table 2) and the products were electrophoresed on a 1.5% (w/v) agarose gel. The photostimulated luminescence values of PCR bands were measured with the aid of ImageJ software (version 1.38; National Institutes of Health, USA). The relative levels of RNAs encoding various cytokines were determined and normalized to those encoding endogenous β -actin.

Fishes used for RT-PCR (3 fish/group) were also used for middle intestine histopathology evaluation as described by Abid et al. [23]. Photographs were taken by a light microscope (Olympus BX50, Japan) and microvillus length was determined with the aid of Image-Pro Plus software (Version 5.1, Germany).

Challenge Test

At the end of the feeding trial, five fish from each (15 fish/group) tank were anesthetized, intraperitoneally injected with 100- μ l of an *S. iniae* (KCTC 3657) suspension at a concentration of 1×10^8 CFU/ml [3, 11], and kept in quarantine tanks without water exchange or feeding. Fish condition and mortality were checked every 6 h daily up to 14 days. Streptococcosis was confirmed by the growth of *S. iniae* on brain heart infusion (BHI) agar plates after spreading of swabs from the skins, livers, and gills of dead fish. We calculated survival as:

Survival (%) = [(Initial fish number – Dead fish number)/Initial fish number] $\times$ 100

Statistical Analysis

The normality and variance homogeneity of the datasets were checked by Shapiro–Wilk and Levene tests, respectively. Before analysis, non-normally distributed data were log-transformed through Kruskal–Wallis test. All data were analyzed with the aid of IBM SPSS software (SPSS Inc., version 17.0, USA). One-way

ANOVA (Analysis of variance) was used to determine if parameters of the control and synbiotic groups differed significantly. A *p*-value < 0.05 was considered to reflect significance and values are presented as means $\pm$ standard deviations (SD).

Results

Effect of the Synbiotic on Growth, Feed Utilization, and Body Indices

The WG and specific growth rate (SGR) values of the

Table 3. Growth, feed utilization and body indices of olive flounder fed with experimental diets for 8 weeks

Growth Parameters	Diets		Level of significance (<i>p</i> -value) ^a
	Control	Synbiotic	
WG ^b	222.90 $\pm$ 6.68	252.74 $\pm$ 5.61	S
SGR ^c	2.09 $\pm$ 0.08	2.24 $\pm$ 0.04	S
FER ^d	117.53 $\pm$ 6.46	125.62 $\pm$ 3.17	NS
PER ^e	2.07 $\pm$ 0.11	2.23 $\pm$ 0.05	NS
CF ^f	0.84 $\pm$ 0.02	0.87 $\pm$ 0.05	NS
VSI ^g	3.22 $\pm$ 0.26	3.31 $\pm$ 0.14	NS
HSI ^h	1.37 $\pm$ 0.25	1.50 $\pm$ 0.13	NS

^aValues are mean $\pm$ SD of three replicates (3 fish/replicate). “S” or “NS” indicates values within the same row in the table are significantly (*p* < 0.05) or not significantly (*p* > 0.05) different, respectively.

^bWG: Weight gain (%) = [(Final weight – Initial weight) / Initial weight] $\times$ 100.

^cSGR: Specific growth rate (%/day) = [(ln final weight – ln initial weight)/days] $\times$ 100.

^dFER: Feed efficiency ratio (%) = (Wet weight gain/Dry feed intake) $\times$ 100.

^ePER: Protein efficiency ratio = Wet weight gain/Protein fed.

^fCF: Condition factor (%) = [Body weight (g)/[Total body length (cm)]³] $\times$ 100.

^gVSI: Viscerosomatic Index (%) = (Visceral weight/Body weight) $\times$ 100.

^hHSI: Hepatosomatic Index (%) = (Liver weight/Body weight) $\times$ 100.

Table 4. Olive flounder initial and final body proximate composition.

Proximate analysis (% of DM basis)				
Nutrient composition	Initial	Final		Level of significance (<i>p</i> -value) ^a
		Control	Synbiotic	
Moisture	76.76 ± 1.43	74.47 ± 1.64	74.81 ± 1.35	NS
Crude protein	16.67 ± 0.63	18.51 ± 1.15	18.51 ± 1.15	NS
Crude lipid	3.25 ± 0.78	3.26 ± 0.15	3.26 ± 0.15	NS
Crude ash	3.99 ± 0.20	3.53 ± 0.28	3.53 ± 0.28	NS

^aValues are mean ± SD of three replicates (3 fish/replicate). "NS" indicates values within the same row in the table are not significantly different (*p* > 0.05).

synbiotic group were significantly higher (*p* < 0.05) than the control group (Table 3). However, fish body indices (CF, HSI, and VSI) or feed utilization parameters were unchanged compared with control after 56 days of feeding trial. Moreover, the synbiotic feed did not affect (*p* > 0.05) flounder body proximate composition (Table 4).

LAB Viability in Feed and the Intestine

After spray, the LI₂ concentration in the synbiotic feed was more or less constant for three days, averaging 9.73×10^7 CFU/g ($\sim 1 \times 10^8$ CFU/g). In control fish, the intestinal LAB count was too few to count (TFTC, < 30 CFU/ml), but that in fish fed the synbiotic averaged $\sim 3.91 \times 10^4$ CFU/g (*p* < 0.05).

Effects of the Synbiotic on Non-Specific Immune and Serum Biochemical Parameters

Among five innate immune parameters three were

Table 5. Non-specific immune responses of olive flounder fed the experimental diets for 8 weeks.

Innate Immunity Parameters	Diets		Level of significance (<i>p</i> -value) ^a
	Control	Synbiotic	
RB ^b	0.38 ± 0.02	0.48 ± 0.03	S
SOD ^c	60.03 ± 3.29	76.79 ± 1.94	S
LSZ ^d	307.33 ± 14.75	456.66 ± 11.27	S
MPO ^e	1.57 ± 0.23	1.84 ± 0.17	NS
Antiprotease ^f	73.28 ± 2.39	77.19 ± 1.98	NS

^aValues are mean ± SD of three replicates (3 fish/replicate). "S" or "NS" indicates values within the same row in the table are significantly (*p* < 0.05) or not significantly (*p* > 0.05) different, respectively.

^bRB: Respiratory burst (Absorbance at 540nm)

^cSOD: Superoxide dismutase (% Superoxide inhibition)

^dLSZ: Serum lysozyme activity (Units/ml)

^eMPO: Myeloperoxidase activity (Absorbance at 450nm)

^fAntiprotease: Percent (%) of Trypsin inhibition

upregulated by synbiotic inoculated feed. Table 5 clearly illustrated, serum lysozyme activity, respiratory burst, and SOD inhibition percentage in the synbiotic group were significantly higher (*p* < 0.05) than the control group. The synbiotic had no effect on serum antiprotease and MPO activity. Also, all serum biochemical parameters including the ALT and AST levels were very similar (*p* > 0.05) in both groups depicted in Table 6.

Effects of the Synbiotic on Cytokine Gene Expression Levels and Microvillus Length

Fig. 1A shows that, in the synbiotic group, only the levels of mRNA encoding TNF-α in liver and spleen were higher (by about 1.70-fold) than the control levels (Fig. 1B) (*p* < 0.05). However, the TNF-α expression levels in gills and kidneys, and the IL-1β (Fig. 1C) and IL-6 (Fig. 1D) expression levels in all organs, were not affected (*p* > 0.05) by the synbiotic. In addition, the microvillus lengths were 1.66 ± 0.14 and 1.72 ± 0.20 μm in the control (Fig. 2A) and synbiotic (Fig. 2B) groups respectively, thus mid-intestinal microvillus length and structure were not affected by synbiotic feeding for 8 weeks.

Table 6. Serum biochemical parameters of olive flounder fed the experimental diets for 8 weeks.

Biochemical Parameters	Diets		Level of significance (<i>p</i> -value) ^a
	Control	Synbiotic	
ALT ^b (U/l)	28.00 ± 2.64	29.04 ± 4.00	NS
AST ^c (U/l)	11.39 ± 3.05	11.00 ± 2.64	NS
Total Glucose (mg/dl)	71.00 ± 5.56	72.66 ± 2.51	NS
Total Cholesterol (mg/dl)	228.33 ± 4.16	232.33 ± 3.71	NS
Serum Protein (mg/ml)	56.71 ± 3.80	58.23 ± 2.26	NS

^aValues are mean ± SD of three replicates (3 fish/replicate). "NS" indicates values within the same row in the table are not significantly different (*p* > 0.05).

^bALT: Alanine aminotransferase

^cAST: Aspartate aminotransferase

Fig. 1. Relative expression levels of genes encoding various cytokines in olive flounder fed a control or synbiotic diet for 56 days. RT-PCR was used to quantify gene expression levels in various organs (A). Densitometric quantification of mRNAs encoding tumor necrosis factor (TNF)- α (B); interleukin (IL)-1 β (C); and IL-6 (D), relative to that of mRNA encoding β -actin, in livers, kidneys, gills, and spleens. Data represent means $\pm$ standard deviations, and means with different letters differed significantly ($p < 0.05$). The lack of letter indicates that the difference was not significant ($p > 0.05$).

Cumulative Mortality after *S. iniae* Challenge

The first deaths in the control and synbiotic groups occurred at 7 and 8 days, respectively, after challenge with *S. iniae*. Moreover, after about 10 days control group mortality attained 100%, but at 14 days the synbiotic group survival proportion was 20%, thus significantly better ($p < 0.05$) than that of the control group (Fig. 3), although both groups had been identically (*S. iniae*; 1×10^8 CFU/ml) challenged.

Discussion

In this present study, synbiotic-fed olive flounder group showed positive WG and SGR compared with those in the control group. Similar results were demonstrated by some previous reports on LAB synbiotics, supplemented to rockfish (*Sebastes schlegelii*) [10], basa fish (*Pangasius bocourti*)

[24], and juvenile Siberian sturgeon (*Acipenser baerii*) [25]. However, supplementation with individual LI_2 (1×10^8 CFU/g) or BGO (0.1%) depicted no improvement of WG in olive flounder [3, 11]. The fish innate immune system is a key weapon when combating foreign invaders. Several types of blood cells (neutrophils, monocytes, and macrophages) act with cytokines to engulf, kill, and eradicate pathogens. Moreover, after synbiotic feeding, microbial fermentation of prebiotic produces short-chain fatty acids that bind to G-protein receptors (GPR43), modulating immune parameters [26]. During macrophage and neutrophil phagocytosis in the course of a respiratory burst, oxygen (O_2) is converted to superoxide (O_2^-) and MPO then transforms O_2^- to hypochlorous acid (HClO). Both O_2^- and HClO are bactericidal, aiding in pathogen destruction and eradication [27]. However, the highly reactive O_2^- anion is also toxic to host cells, and is converted to the less reactive H_2O_2 , and

Fig. 2. Mid-intestinal histopathology of olive flounder fed without (A) or with synbiotic 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).

finally water, by SOD activity. Importantly, fish lysozyme cleaves not only the β -(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid of Gram-negative bacteria [28], but also destroys all types of pathogenic bacteria engulfed by macrophages or neutrophils [29]. The significant changes in lysozyme activity, the respiratory burst, and the SOD level constitute clear evidence that the synbiotic exercised immunomodulatory effects through positive alteration of different immune cells and enzymes activities in our olive flounder. Similarly, feeding of synbiotics to olive flounder [9]; cobia (*Rachycentron canadum*) [30]; Atlantic salmon (*Salmo salar*) [23] and rainbow trout [31] over various periods was accompanied by positive changes in innate immune parameters. In contrast, feeding with various graded levels of a synbiotic (*B. subtilis* + FOS) to juvenile large croaker (*Larimichthys crocea*) did not modulate their immunological status [32].

Immune system-related cytokines generally act in cascades.

Fig. 3. Cumulative survival rates of olive flounder after challenge with *Streptococcus iniae* (1×10^8 CFU/ml).

Means were compared at identical times; means with different or lack of letters indicates significant ($p < 0.05$) or non-significant differences ($p > 0.05$) respectively.

LAB can activate dendritic cells, specifically the T_H1 subset $CD4^+T$, which stimulates pro-inflammatory gene expression to eliminate invasive pathogens during the acute phase of the immune response [15]. Cytokine expression profiles reflect the extent of their involvement in immune defense, and such expression is sometimes associated with variations in other immune parameters. $TNF-\alpha$ is an immune system biomarker that orchestrates the host defense against pathogen invasion and colonization [33]. $TNF-\alpha$ is secreted principally by activated macrophages and stimulates neutrophil-associated immunity [34]. In this study, it is possible that the significant changes in the blood respiratory burst (NBT assay) and $TNF-\alpha$ expression levels in liver and spleen after synbiotic feeding may be interrelated because the NBT assay measures neutrophil activation and proliferation. $TNF-\alpha$ expression in olive flounder showed a positive correlation with innate immunity after feeding with LAB [15] and LAB synbiotic fed to Atlantic salmon [23] have been studied and the results were similar to present study. As previously stated by Dziarski [35], after peptidoglycan (LAB cell wall component) binding with the specific sites of macrophage and lymphocyte, they get activated and stimulated, which could cause secretion of $TNF-\alpha$. Moreover, the liver and spleen are enriched with different types of immune cells including macrophages and lymphocytes [36, 37]. Probably this is the reason why these two organs showed higher mRNA transcription levels of $TNF-\alpha$ in the present study. However, very high levels of $TNF-\alpha$ is also undesirable for cells [38]. Moreover, as in this study, an 8-week feeding trial of 0.1% β -glucan plus

10^9 CFU/g *Shewanella putrefaciens* had no effect on IL-1 β expression in the head-kidney of gilthead seabream (*Sparus aurata*) [39].

It is generally assumed that a greater microvillus length in the apical brush border may improve nutrient uptake and improved the apparent digestibility coefficient. Similar to our current finding, a 63-day synbiotic feeding trial in Atlantic salmon had no effect on microvillus length in the posterior intestine [23]. However, Zhou *et al.* [40] reported that only prebiotics increased microvillus length in the middle intestine of red drum (*Sciaenops ocellatus*). It is not clear, but lack of alteration in metabolism governing IL-6 [41] and MVL by synbiotic feeding might explain the no effect on FER and PER in this study. In the cited study, the significantly higher LAB levels in intestines of the synbiotic group reflected microbial community alterations positively affecting both growth and innate immunity [42], or innate immunity only [20], in agreement with our present findings. Serum ALT is a biomarker of liver dysfunction or damage, and AST is expressed by kidney, liver, skeletal and cardiac tissue. Cellular degradation caused by stress or toxins triggers high-level ALT and AST release into the bloodstream. The absence of changes in ALT and AST levels indicates that our synbiotic is safe for aquaculture feeding. Oral administration of 0.1% BGO had no effects on ALT and AST levels in olive flounder [11]; and a combination of *Enterobacter faecium* with 0.1% FOS did not affect the levels of triglyceride or total glucose, or the albumin:globulin ratio, in rainbow trout [43].

LAB may inhibit fish pathogens by producing a bacteriocin, lactic acid, or another antibacterial compound [44]. The feeding of synbiotics to cobia [30] and rainbow trout [31] afforded significant resistance against *Vibrio harveyi* and *S. iniae* infections, respectively. We found that fish fed the synbiotic were significantly protected against streptococcosis. It was previously shown that a single administration of 1×10^8 CFU/g LI₂ or 0.1% BGO to olive flounder that were then challenged with 1×10^8 CFU/ml *S. iniae* was associated with 100% mortality in 12 and 11 days, respectively [3, 11] whereas the combination (LI₂ + BGO) improved survival duration and percentages (20% survival at 14 days). Such protection against streptococcosis may reflect BGO fermentation by LI₂ for stimulating growth in the intestine, positively affecting the immune parameters of olive flounder. Thus the relationship between BGO and LI₂ appeared to be very effective when they were combined to create a synbiotic.

According to the findings of this research, the combination of LI₂ and BGO (LAB-based synbiotic) supplement not only

improved fish growth, innate immunological parameters, TNF- α transcription, and intestinal microbial community but also protected fishes from streptococcosis. In future, this newly identified synbiotic feeding strategy could be implemented in field level to encourage fish farmers to cease antibiotic use in aquaculture practices, in turn ensuring food and nutritional biosafety. Various mixtures of LI₂ and BGO should be tested in the future to establish an optimum dietary synbiotic for the olive flounder.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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