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Dietary administration of *Zooshikella* sp. enhance the innate immune response and disease resistance of *Paralichthys olivaceus* against *Sreptococcus iniae*

Ju-Sang Kim [a,1], Ramasamy Harikrishnan [a,1], Man-Chul Kim [a], Chellam Balasundaram [b], Moon-Soo Heo [a,*]

[a] Marine Applied Microbes and Aquatic Organism Disease Control Lab, Department of Aquatic Biomedical Sciences, School of Marine Biomedical Sciences, College of Ocean Sciences & Marine and Environmental Research Institute, Jeju National University, Jeju 690-756, Republic of Korea
[b] Department of Animal Science, Bharathidasan University, Tiruchirapalli-620024, India

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ABSTRACT

We report the growth, innate immune response, and disease resistance in olive flounder (*Paralichthys olivaceus*) challenged with *Streptococcus iniae* after feeding with diet enriched with *Zooshikella* sp. strain JE-34 three different concentration i.e. Low (3.4×10^4, $n = 50$), medium (3.5×10^6, $n = 50$), and high (3.4×10^8, $n = 50$) cfu ml^{-1} supplemented diets, the changes were monitored on weeks 1, 2, 4, 8, 12, and 16. With all diets the innate immune parameters, such as superoxide anion production, phagocytic and lysozyme activity were not enhanced on week 1 and 4. On the other hand, all tested immune parameters in the treated groups significantly enhanced after 8th week; the weight gain significantly increased after 4th week in fish fed with enriched diets. The mortality in olive flounder after administration with high concentration diet showed 25%. With low and medium enriched diets the mortality was 40% and 35%, respectively. In the infected untreated group mortality was 85% while there was no mortality in the control group. The results suggested that *Zooshikella* sp. strain JE-34 enriched diets could be used to enhance the innate immune response and disease resistance of *P. olivaceus* against *S. iniae*.

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1. Introduction

Mariculture recently has become an important intensive economic activity in many countries. Olive flounder (*Paralichthys olivaceus*) is one of the most important marine-cultured fish in Korea, China, and Japan. In Korea, this species is produced abundantly in more than 300 farms in Jeju Island. However, in large-scale production, the aquatic animals are exposed to stressful conditions due to increased rearing density [1]. For the past 10 years, problems related to diseases have resulted in serious economic loss due to bacterial, viral, and parasitic pathogens. *Streptococcus iniae* is one of the important pathogens in fishes, an etiological cause of streptococcosis in wild and farmed fish worldwide [1]. This pathogen has been responsible for a number of disease outbreaks in different commercially cultivated fresh-water and sea-water fish species, such as channel catfish, gilthead sea bream, rainbow trout, sea bass, tilapia, and olive flounder [2–5]. The bacteria induce chronic infections, resulting in more than 50% mortality over a period of 3–7 days [6]. Recently *S. iniae* is known as a major pathogen inducing by streptococcosis in olive flounder. In olive flounder farms of South Korea also streptococcosis is an important problem causing significant economic loss [7].

To control the disease anti-microbial agents are applied in crude form. The extensive use of anti-microbials has resulted in anti-microbial resistance among pathogenic bacteria and accumulation in fish tissues. Alternatively, the use of probiotics is one of the potential tools to prevent diseases and reduce mortalities in aquaculture [8–12]. Most of the probiotic studies have been related to use lactobacillus and bacillus isolated from land, however, it has not been well known at what stage bacteria produce the metabolites and whether or how well they compete for attachment [9]. Therefore new potential candidate probiotic bacteria for aquaculture should be isolated from marine origin, such as marine sediment, seaweed, fish farm, and sea-water to ensure effective treatment.

We recently isolated *Zooshikella* sp. strain JE-34 from marine sediment in East China Sea that produce anti-microbial red-pigment prodigiosin [13]. Other strains of *Zooshikella* sp. are also known to produce prodigiosin red-pigment [13–15]. Prodigiosin red-pigments possess many bioactivities, such as antibiotics, immunomodulators, anti-diabetic, and anti-cancer activity [16]. Hence, in the present study *Zooshikella* sp. strain JE-34 was chosen as a promising candidate probiotic species and administered in

* Corresponding author. Tel.: +82 64 754 3473; fax: +82 64 756 3493.
E-mail addresses: rhari123@yahoo.com (R. Harikrishnan), msheo@jejunu.ac.kr (M.-S. Heo).
[1] The authors contributed equally to this work.

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olive flounder against *S. iniae* through supplementation diets as a feed additive to assess the innate immune response and disease resistance of olive flounder for the first time.

2. Materials and methods

2.1. Fish and experimental conditions

P. olivaceus was obtained from a commercial flounder farm in Jeju island, South Korea. Fish was transported to the laboratory alive in plastic bags containing sea-water enriched with oxygen. The fishes were acclimated for 2 week to be provided with a commercial diet *ad libitum*. Continuous aeration was also provided to maintain dissolved oxygen levels at 7.01–8.01 mg L^{-1}. The photoperiod of 10:14 h (light/dark) was provided by fluorescent light. During the experimental period water temperature ranged from 20.4 to 20.7 °C, salinity from 33.16 to 33.59 ‰, and pH from 8.38 to 8.54. After acclimation period, the fish (initial body weight 27.5 ± 1.5 g) were randomly chosen and introduced into five 150 L polyvinyl conical tanks (triplicate groups per dietary treatment) at a density of 50 fish/tank.

2.2. S. iniae culture

The strain *S. iniae* ATCC 29178 was grown in 10 ml with 1.5% sodium chloride (NaCl) in Brain Heart Infusion broth (BHIB; Difco) in a rotary shaker overnight at 200 rpm for 37 °C. The subculture was prepared from the seed. A volume of 1 ml of the seed solution was taken into a flask with 100 ml of NaCl in BHIB and was incubated at 37 °C for 24 h. The sub-cultures were grown twice under the same conditions for the experiment. Growth was measured by optical density at 700 nm and then through plate counting in BHI–NaCl.

2.3. Zooshikella strain JE-34 culture

Zooshikella sp. JE-34 strain isolated from sediment samples in the East China Sea (30°57′6.22″N, 122°32′9.30″E) was identified, cultured, and maintained by the method of Kim [17]. The strains were kept at −80 °C in marine broth (MB, Difco; 2216) containing glycerol (40 g L^{-1}) until used for experiment.

2.4. Preparation of experimental feeds

The formulated fish feed was prepared in the laboratory using soybean and fish meal as the protein sources (Table 1). To prepare the probiotics enriched diet with 3.4×10^4 (low), 3.5×10^6 (medium), and 3.4×10^8 (high) colony forming units (cfu) ml^{-1} of *Zooshikella* sp. JE-34 strain were sprayed into the feed slowly, and mixed thoroughly by part in a drum mixer, made pellets and air-dried under sterile conditions for 12 h. Further the pellets were dried in an oven at 30 °C for 18 h, packed and stored in a freezer at −20 °C until used. The amount of *Zooshikella* sp. JE-34 in each feed was determined by plate counting on marine agar (MA, Difco) by homogenate of 1 g of feed in 9 ml sterile PBS at pH 7.4 at 30 °C. The final concentration of live *Zooshikella* sp. JE-34 in the probiotic feed pellets before the feeding trials were 3.4×10^4, 3.5×10^6, and 3.4×10^8 cfu mL^{-1}. Feeding was started after challenging each fish with live *S. iniae* on Day 7.

2.5. Experimental design

All groups ($n = 50$ each) except control were inoculated intramuscularly with 50 µl of live *S. iniae* (2.3×10^5 cfu mL^{-1}) using a 1 ml tuberculin syringe with a 24-G needle on Day 1. Control fish received 50 µl of PBS alone. The control (C) and infected untreated (I, $n = 50$) groups fed with standard diet without *Zooshikella* sp. JE-34. The infected fish were fed with low, medium, and high enriched diets on day 7. The feeds were offered for 16 weeks twice a day at 09:00 and 15:00 h at a rate of 2% of their body weight. For each group a triplicate was maintained. Earlier the challenge dose was standardized to give 85% mortality in the untreated group. The cumulative mortality of fish fed with enriched diets was recorded daily for 15 days. The cause of death was confirmed by re-isolating the organism from liver and kidney of 15% of dead fish using MA medium (Sigma). Relative percent survival (RPS) was calculated by the following formula of Amend [18],

$$\text{RPS} = 1 - \frac{(\%\ \text{mortality in treated group})}{\%\ \text{mortality in control group}} \times 100$$

Table 1
Formulation and proximate composition of the experimental diets for olive flounder.

Ingredients	Concentration (g/100 g)
Fish meal	51.0
Soybean meal	7.0
Wheat flour	12.0
Defated rice bran	10.0
α-potato starch	5.0
α-cellulose[a]	0.5
Squid liver oil[b]	3.0
Blood meal	2.0
Dextrin	2.0
Casein[a]	2.0
EPA + DHA[b]	0.5
Vitamin premix[c] and minerals premix[d]	2.0
Candidate probiont or skim milk	2.0
Approximate composition (% wet weight)	
Crude protein	52.5
Crude fat	12.8
Crude starch	17.2
Crude ash	12.5

[a] United States Biochemical (Cleveland, OH) 44122.
[b] E-Wha oil, Pusan, Korea.
[c] Premix (g/100 g) contains DL-calcium pantothenate, 0.5; choline bitartrate, 10; inositol, 0.5; menadione, 0.02; niacin, 0.5; pyridoxine–HCl, 0.1; riboflavin, 0.1; thiamine mononitrate, 0.1; DL-α-tocopheryl acetate, 0.2; retinyl acetate, 0.02; biotin, 0.01; folic acid, 0.02; B12, 0.0002; Cholecalciferol, 0.008; α-cellulose, 85.0.
[d] Premix (g/100 g) contains Al, 0.12; Ca, 500; Cl, 10; Cu, 0.5; Co, 0.9; Na, 0.13; Mg, 50; P, 5000; K, 425; Zn, 0.3; Fe, 4; I, 0.5, Se, 0.02; Mn, 0.9.

2.6. Effect of supplemented feed on growth

The percent weight gains (PWG) were calculated as: PWG = 100 × final body weight − initial body weight/initial body weight^{-1}.

2.7. Blood sampling

Blood samples (approximately 0.5 ml) of six fish/group were collected randomly from caudal vein using a vacutainer fitted 1-ml 27-G needle on weeks 1, 4, 8, 12, and 16 after feeding. Individual fish were anaesthetised with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min. Individual fish were sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. To evaluate the blood physiological parameters and immunological assay feeding was ceased for 24 h prior to sampling. One half of each blood sample was immediately used for hematological examination, while the other half was mixed with heparin anticoagulant and kept frozen at 4 °C. The serum tubes were placed at room temperature and allowed to clot for 2 h.

Sera were separated by centrifugation at 1500 g for 20 min and sera from the same groups were pooled before being stored at −70 °C for biochemical and immunological analyses.

2.8. Blood biochemistry

Serum biochemical parameters, such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, low density cholesterol (LDL), triglycerides (TG), phosphorus, hemoglobin (Hb), triglycerides (TG), concentration of total protein (TP), and glucose (GLU) were determined in ch100 plus blood chemistry autoanalyzer (SEAC, Italy) by using analysis kits (STANBIO, Taxas, USA).

2.9. Separation of leukocytes

Leukocytes were separated from each blood sample by density gradient centrifugation. One milliliter of histopaque 1.119 (Sigma) containing 100 µl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One milliliter of a mixture of 1.077 density histopaque and hemagglutination buffer and 1-ml of blood was carefully layered on the top. The samples were centrifuged at 700 g for 15 min at 4 °C. Then the plasma was collected and stored at −80 °C for future analysis. Separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to 1×10^7 viable cells ml^{-1}.

2.10. Nitroblue tetrazolium reduction analysis (NBT)

The leukocytes (2×10^6 ml^{-1}) suspended in L-15 containing 0.1% FCS was then transferred into 96-well plates and incubated at 18 °C for 2 h. The non-adherent cells were removed by washing and the cell monolayers were maintained in L-15 containing 5% FCS. The intracellular production of the superoxide anion was estimated, based on the formation of formazan crystals in the cells. A volume of 100 µl leukocyte solution (2×10^6 ml^{-1}) was mixed with 100 µl of NBT (0.2% in PBS) containing zymosan (Sigma). Then the plates were further incubated at room temperature for 60 min with regular mixing. The plates were centrifuged at 500 g for 3 min, and the supernatants were discarded. The cells were washed twice with HBSS and fixed in 70% methanol. The formazan crystals formed were dissolved by adding a 0.12-ml potassium hydroxide (KOH) and 0.14-ml dimethyl sulfoxide (DMSO). After the turquoise-blue solutions were obtained, the absorbance was measured at 620 nm using a multiscan spectrophotometer using KOH/DMSO as the blank.

2.11. Phagocytosis assay

Phagocytic activity of blood leukocytes was determined by microscopic measurement of the number of congo red-stained yeast cells phagocytized per macrophage by the method of Seeley et al. [19]. After aseptic removal of the head kidney was placed into Petri dishes containing 4-ml of phosphate buffered saline (PBS) at pH 7.4. The single cell suspensions were made by gently drawing kidney through a 2-ml syringe and filtered over a nylon mesh funnel into 10-ml centrifuge tubes. The suspension was washed three times in 8-ml of PBS. Then the supernatant was removed and the pellet made up to 4-ml with PBS. Congo red-stained yeast cells, *Saccromyces cerevisciae* (200-ml of a 108 cells ml^{-1} suspension) were added to tubes and mixed by hand. Tubes were then centrifuged to 600 rpm for 30 min at 15 °C. Supernatant was removed and 4-ml ice-cold PBS was added. The suspension was carefully layered on 3-ml Ficoll–Paque (Sigma). Tubes were then centrifuged at 1500 rpm for 20 min at 4 °C. After centrifugation the interface layer was removed and washed in PBS, with the pellet being made up to 1-ml with PBS. Phagocytosis was evaluated by counting the number of yeast cells per phagocyte from smears prepared for each sample. One-hundred phagocytes were counted per slide (400× magnification). Data for both assays were analyzed using nested analysis of variance.

2.12. Lysozyme activity

The serum lysozyme activity was measured spectrophotometrically according to method of Ellis [20]. A suspension of *Micrococcus lysodeikticus* (0.02%, w/v) made up in 0.05 M PBS at pH 6.2 was used as substrate. Lyophilised hen egg white lysozyme was used as a standard. A standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25 °C. The results were expressed as µg ml^{-1} equivalent of hen egg white lysozyme activity.

2.13. Microbiological analysis

The microfloral analyses were performed following Nikoskelainen et al. [21] except that the fish were starved for 36 h before sampling. The probiotic strain counts in the posterior intestines of fish were determined by plate counting on MA. Six fish for each treatment and time were used for these studies.

2.14. Statistics

The data (mean ± SE, standard error) were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test to compare the means between individual treatments with SPSS at $P < 0.05$ levels.

3. Results

3.1. Growth

Percent weight gain (PWG) was not significantly changed in infected olive flounders fed with low, medium, and high enriched diets on the first week. On the 4th week, the PWG did not vary fish fed with low enriched diet. On the other hand, in infected flounder fed on medium and high supplementation diets registered a significant weight gain on 4th week. After 8th week with all the diets there were significant weight gain when compared to control group (Fig. 1).

3.2. Serum biochemical constituents

The aspartate aminotransferase, low density cholesterol, triglyceride, and hemoglobin concentration significantly increased

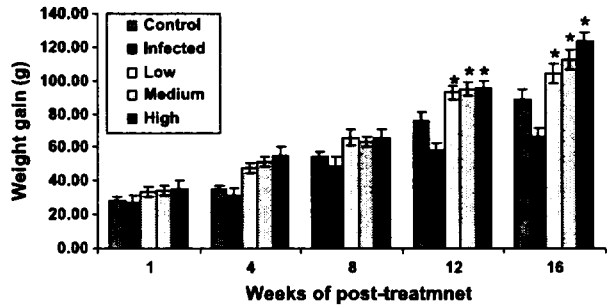

Fig. 1. Percent weight gain of olive flounder (mean ± SE, $n = 6$) control and infected after feeding with low (3.4×10^4), medium (3.5×10^6), and high (3.4×10^8) cfu ml^{-1} of *Zooshikella* supplemented diets at chosen weeks. Statistical differences ($P < 0.05$) from the control group are indicated by asterisks.

when fed with all three diets on 8, 12, and 16 week. However, alanine aminotransferase, glucose and phosphorus concentration did not significant change (Table 2).

3.3. Superoxide anion production

The superoxide anion production of respiratory burst activity of the anterior kidney leukocytes are shown in Fig. 2. All the diets did not significantly increase the superoxide anion production on 1st week when compared to control group. Interestingly, after fourth week of all the enriched diets significantly increased when compared to the control.

3.4. Phagocytosis activity

The phagocytosis activity of head-kidney leucocytes did not significantly increase in all the diets groups on 1st and 4th week.

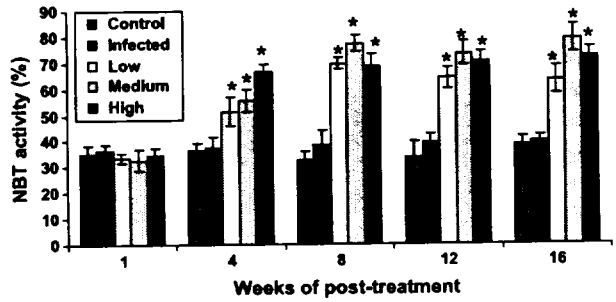

Fig. 2. Superoxide anion production in olive flounder (mean ± SE, $n = 6$) control and infected after feeding with low (3.4×10^4), medium (3.5×10^6), and high (3.4×10^8) cfu ml^{-1} of *Zooshikella* supplemented diets at chosen weeks. Statistical differences ($P < 0.05$) from the control group are indicated by asterisks.

Table 2
Serum biochemical parameters of olive flounder (mean ± SE, $n = 6$) control, infected untreated, and treated (low, medium, and high supplemented diets) on week 1, 4, 8, 12, and 16.

Parameters	Groups	Weeks				
		1	4	8	12	16
AST (U/L)	Control	23.3 ± 4.6	23.5 ± 5.4	23.6 ± 5.2	23.8 ± 4.8	24.0 ± 5.6
	Infected	20.7 ± 3.4	22.1 ± 3.6	23.4 ± 4.2	24.2 ± 3.8	24.7 ± 3.5
	Low	23.7 ± 6.4	24.2 ± 6.2	25.2 ± 5.8	25.5 ± 6.6	25.8 ± 7.4
	Medium	23.8 ± 3.8	24.8 ± 5.4	26.2 ± 5.0	27.1 ± 5.2*	27.7 ± 4.8*
	High	24.0 ± 4.8	24.4 ± 5.8	27.6 ± 5.2*	28.4 ± 5.6*	28.7 ± 6.0*
ALT (U/L)	Control	6.1 ± 0.8	6.2 ± 0.6	6.2 ± 0.6	6.4 ± 0.8	6.4 ± 0.8
	Infected	3.8 ± 0.6	3.8 ± 0.6	4.0 ± 0.4	4.2 ± 0.6	4.9 ± 0.6
	Low	4.5 ± 0.4*	4.5 ± 0.6*	5.2 ± 0.2*	5.4 ± 0.4*	5.7 ± 0.2
	Medium	4.1 ± 0.4*	4.4 ± 0.4*	5.1 ± 0.6*	5.5 ± 0.4	5.8 ± 0.3
	High	4.3 ± 0.6*	4.5 ± 0.4*	5.6 ± 0.6*	5.7 ± 0.4	5.9 ± 0.5
LDL-C (mg dl^{-1})	Control	18.6 ± 6.2	18.8 ± 6.4	19.0 ± 6.2	19.1 ± 5.8	19.2 ± 6.5
	Infected	20.0 ± 6.0	20.2 ± 5.8	20.3 ± 6.2	20.5 ± 6.6	21.3 ± 7.2
	Low	19.4 ± 5.4	20.2 ± 5.0	22.4 ± 4.6	23.6 ± 4.8*	24.7 ± 5.1*
	Medium	21.6 ± 4.6	22.4 ± 3.8	25.2 ± 4.6	26.4 ± 5.2*	27.7 ± 4.8*
	High	22.2 ± 5.4	22.5 ± 5.6	26.6 ± 5.6	27.8 ± 5.8*	28.7 ± 6.0*
TP (g dl^{-1})	Control	6.0 ± 0.6	6.1 ± 0.8	6.2 ± 0.6	6.2 ± 0.6*	6.4 ± 0.8*
	Infected	6.2 ± 0.6	5.8 ± 0.6	5.2 ± 0.8	5.0 ± 0.6	4.9 ± 0.6*
	Low	4.5 ± 0.8*	4.6 ± 1.1*	4.8 ± 1.4*	5.3 ± 1.0	5.5 ± 1.2
	Medium	4.2 ± 0.4*	4.4 ± 0.3*	5.1 ± 0.6	5.5 ± 0.4	5.7 ± 0.2
	High	4.5 ± 0.6*	4.9 ± 0.4*	5.3 ± 0.6	5.8 ± 0.5	5.9 ± 0.5
GLU (mg dl^{-1})	Control	30.3 ± 0.6	30.5 ± 0.8	30.7 ± 0.6	31.1 ± 0.8	31.3 ± 0.6
	Infected	27.4 ± 0.8*	24.7 ± 0.7*	21.6 ± 1.2*	16.4 ± 0.9*	14.0 ± 1.0*
	Low	29.6 ± 1.4	26.5 ± 0.8*	23.6 ± 1.4*	21.7 ± 1.2*	30.3 ± 4.9*
	Medium	30.1 ± 1.2	30.6 ± 2.4	30.4 ± 3.6	30.2 ± 4.2	25.3 ± 1.5
	High	30.0 ± 1.2	28.6 ± 1.6	27.5 ± 1.2	26.4 ± 1.4*	25.3 ± 1.5*
PHO (mg dl^{-1})	Control	9.0 ± 1.1	9.2 ± 1.4	9.2 ± 1.2	9.3 ± 1.4	9.4 ± 1.3
	Infected	8.6 ± 1.8	7.4 ± 1.5*	6.5 ± 1.4*	6.1 ± 2.2*	5.7 ± 2.4*
	Low	8.8 ± 2.4	8.2 ± 2.8	7.8 ± 2.2	7.4 ± 2.6	7.2 ± 3.0
	Medium	9.2 ± 1.2	9.4 ± 1.2	9.9 ± 1.4	10.3 ± 0.8	10.5 ± 1.4
	High	9.2 ± 1.1	9.3 ± 1.4	9.5 ± 1.6	9.6 ± 1.4	9.8 ± 1.2
TRI (mg dl^{-1})	Control	1.5 ± 0.8	1.6 ± 0.5	1.6 ± 0.5	1.7 ± 0.4	1.7 ± 0.6
	Infected	1.4 ± 0.5	1.4 ± 0.5	1.3 ± 0.6	1.4 ± 0.4	2.3 ± 0.6
	Low	1.8 ± 0.4	2.1 ± 0.4	2.2 ± 0.4	2.2 ± 0.4	2.3 ± 0.6
	Medium	2.0 ± 2.2	2.6 ± 2.4*	3.1 ± 2.2*	4.2 ± 1.8*	4.7 ± 2.9*
	High	2.1 ± 0.8	2.5 ± 0.6*	2.8 ± 0.6*	3.0 ± 0.4*	3.3 ± 0.6*
Hg (g dl^{-1})	Control	17.6 ± 5.2	17.7 ± 5.6	17.7 ± 5.4	17.8 ± 5.2	18.0 ± 5.5
	Infected	16.8 ± 5.4	15.8 ± 5.8	15.3 ± 6.2	15.1 ± 6.4	14.8 ± 6.5
	Low	17.8 ± 6.7	18.1 ± 8.8	18.2 ± 7.4	18.2 ± 8.8	18.4 ± 10.7
	Medium	19.6 ± 6.4	22.4 ± 7.6*	24.8 ± 8.4*	26.3 ± 5.9*	29.0 ± 9.5*
	High	17.4 ± 4.8	17.8 ± 5.2	18.6 ± 4.6	18.9 ± 4.9	19.2 ± 5.6

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDL-C: Low density cholesterol, TP: Total protein, GLU: Glucose, PHO: Phosphorus, TRI: Triglycerides, Hg: Hemoglobin, *: $P < 0.05$.

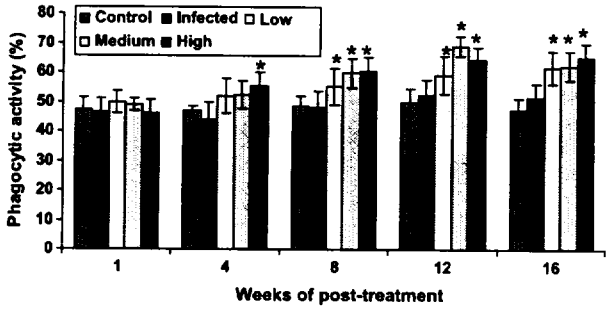

Fig. 3. Phagocytic activity of isolated phagocytic cells from olive flounder (mean ± SE, $n = 6$) blood control and infected after feeding with low (3.4×10^4), medium (3.5×10^6), and high (3.4×10^8) cfu ml^{-1} of *Zooshikella* supplemented diets at chosen weeks. Statistical differences ($P < 0.05$) from the control group are indicated by asterisks.

On the other hand, all the diets significantly enhanced the phagocytosis activity after 8th week (Fig. 3).

3.5. Lysozyme assay

The serum lysozyme activity did not significantly enhance in all the diet treated groups on first week. The serum lysozyme activity did not significantly enhance in groups given with low concentration of *Zooshikella* sp. JE-34 supplementation diet on 4th week. On further in groups treated with medium and high concentration of *Zooshikella* sp. JE-34 supplementation diets the serum lysozyme activity significantly increased, but not in group fed with low enriched diet. In all treated groups, the serum lysozyme activity significantly increased after 8th week when compared to control (Fig. 4).

3.6. Microbiological analyses

The *Zooshikella* sp. JE-34 was not detected in the whole intestines of control group. Infected untreated group also did not have *Zooshikella* from 1st to 16th week. In low and medium diet fed groups were detected at 3.4×10^1 and 7.1×10^1 on the 1st week. On 16th week of these groups the concentration increased to 3.1×10^4 and 1.4×10^4. On the other hand in the high supplementation diet group was concentration of *Zooshikella* detected at 3.1×10^2; on the 16th week, it reach the high level of 9.6×10^6 (Table 3).

3.7. Cumulative mortality

There is no mortality in control group. However, the infected untreated group suffered a cumulative mortality of 80% on 15 days. The cumulative mortality of infected fish fed with low and medium

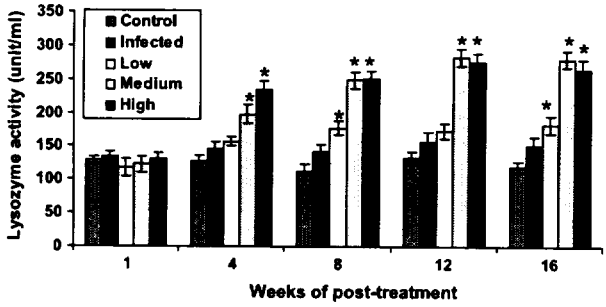

Fig. 4. Lysozyme activity of olive flounder (mean ± SE, $n = 6$) control and infected after feeding with low (3.4×10^4), medium (3.5×10^6), and high (3.4×10^8) cfu ml^{-1} of *Zooshikella* supplemented diets at chosen weeks. Statistical differences ($P < 0.05$) from the control group are indicated by asterisks.

Table 3
Zooshikella sp. JE-34 counts on 16 week.

Weeks	Groups				
	Control	Infected	Low	Medium	High
1	ND	ND	3.4×10^1	7.1×0^1	3.1×10^2
4	ND	ND	3.4×10^2	5.1×10^2	3.1×10^3
8	ND	ND	3.8×10^3	1.4×10^3	2.5×10^4
12	ND	ND	4.6×10^3	9.4×10^3	6.8×10^5
16	ND	ND	3.1×10^4	1.4×10^4	9.6×10^6

enriched diets was 40% and 35%, respectively. On the other hand in the cumulative mortality was low as 25% in infected fish fed with high supplementation diet (Fig. 5).

4. Discussion

Probiotics are defined as live microbial or cultured product, which beneficially affect the host by producing inhibitory compounds that compete for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance [11,22,23]. They are aquaculture to control disease, enhance the immune response, supplement or even in some cases to replace the use of anti-microbial compounds and for providing nutrients, enzymatic contributions, and improving water quality [24]. In the present study, olive flounder fed with all the three enriched diets did not significantly increase PGW on first week. After fourth week all the enriched diets significantly increased the PGW after certain period. We evaluated the benign anti-microbial and anti-oxidant activity of *Zooshikella* solvent extracts against a number of fish pathogens *in vitro* [17]. Before using any organism as a probiotics it is essential to evaluate its potential, origin of strain, acid and bile tolerance, adhesion to the intestinal mucus, production of anti-microbial substances, and antibiotic resistance or sensitivity [25]. Further the importance of selecting the probiotic strains from the homologous species is uncertain as the adhesive activity of candidate probiotic to mucus in different hosts is species-specific [26,27].

In the present study *Zooshikella* strain JE-34 bacteria to analysis any improvement in immune parameters against *S. iniae* by supplementation fish feeding low (3.4×10^4), medium (3.5×10^4), and high (3.4×10^8). In the present study the number of viable *Zooshikella* sp. JE-34 in the posterior intestines of control and infected untreated group did not vary at any time. Infected groups fed with low and medium enriched diets had lower number of bacteria (3.1×10^4 and 1.4×10^4) in the posterior intestines on week 16, but the concentration was higher (9.6×10^6) in group fed with high enriched diet. Gill, skin, and gastrointestinal tract have been demonstrated to be the portal of entry for many microbial pathogens [28].

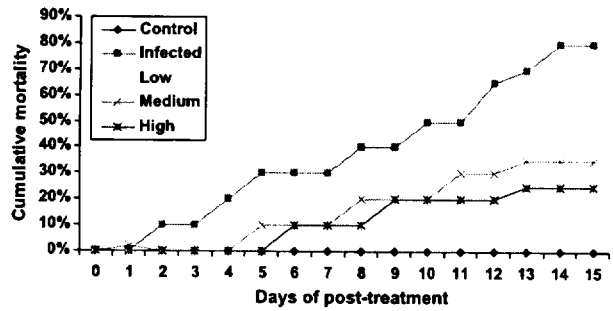

Fig. 5. Cumulative mortality of control and infected after feeding with low (3.4×10^4), medium (3.5×10^6), and high (3.4×10^8) cfu ml^{-1} of *Zooshikella* supplemented diets for 15 days.

The probiotic bacteria might be able to protect the host from pathogens by blocking the integumental attachment sites (adhesion receptors). The *Zooshikella* colonizing epidermal mucus may probably originate from the intestinal tract via faeces. A good adhesion ability to mucosal surfaces is a prerequisite for bacterial colonization of *Lactobacillus rhamnosus* (ATCC 53103) to fish mucus [29] and human mucus [21,30,31]. To our knowledge, this is the first report where a dose-dependent colonization of *Zooshikella* is shown over such a wide range (10^1–10^6 cfu mL^{-1}). This result is in agreement with the observations by Joborn et al. [32] a full washout of probiotic bacteria was observed in 3 days. The above studies clearly show that the probiotics containing feed must be given to fish continuously to retain facilitate the retention of the probiotic bacteria in the gut, skin, and tank water and that the adhesion properties of probiotic bacteria may influence the speed of washout. The relative proportion of *Zooshikella* spp. in the intestinal flora of olive flounder fed diets containing probiotics increased with probiotics supplement in treated groups. A high proportion of probiotics is probably related to an increase in suitable attachment sites affording improved internal environmental conditions for bacterial growth [33].

In the present study aspartate aminotransferase, cholesterol, triglyceride, and hemoglobin concentration significantly increased in groups fed with all supplemented diets on week 16. The blood biochemical and physiological parameters are important for general health, toxicology, and bio-monitoring [34–36]. However, in this study, alanine aminotransferase, glucose, and phosphorus concentration were not significantly changed when compared to the control group indicating that the fish may be protected from the infection when diet supplemented with *Zooshikella* sp. JE-34. The probiotic bacteria *L. rhamnosus* reduced mortality of rainbow trout when challenged with a virulent strain of *Aeromonas salmonicida* [37].

The immune system comprises into innate and acquired components. The innate response generally precedes the adaptive response, activates and determines the nature of the adaptive response and cooperates in the maintenance of homeostasis [38]. Further, the innate immune system's recognition of non-self is mediated by germline-encoded pattern recognition proteins or receptors that identify molecular patterns [39]. It is particularly the phagocytic, lysozyme, and spontaneous haemolytic activity, and in some cases pentraxins, have been used as indicators of the effects of inherent or external factors on the immune system and the disease resistance of fish [40].

The superoxide anion production of respiratory burst activity in the present study in all the treated groups did not significantly increase on first week. However, when compared to control group in all supplemented diet fed groups it significantly enhanced after fourth week. Neutrophils are an important component of host defence against a variety of bacterial, viral, and fungal infections, and the evaluation of neutrophil function is valuable in assessments of the health status of individuals and animal [41]. In response to inflammatory stimuli, neutrophils migrate from the circulating blood into infected tissues, where they efficiently bind, engulf, and kill bacteria via the activity of proteolytic enzymes and anti-microbial proteins, coupled with the generation of reactive oxygen species [42]. The measurements of neutrophil functions have been extensively utilized for evaluations of immune response in humans, mice, and fish species.

The phagocytosis activity was not significantly enhanced 1st and 4th week, whereas after 8th week in all treated groups it significantly enhanced when compared to control group. The phagocytic cells are the most important cellular components of the innate immune system of fish [43]. It is a primitive defence mechanism [44], and an important characteristic of the innate immune system [19]. In humans certain probiotic bacteria are able to stimulate phagocytic activity [45] and complement receptor expression [46]. The respiratory activity correlates well with phagocytosis of bacteria in fish [47–49] as well as in humans [49]. In our experiments a high enriched *Zooshikella* in the diet was able to significantly enhance the phagocytic activity of leukocytes in infected olive flounder only after 4th week. *A. salmonicida* can resist both cellular [50] and humoral defence [51] of fish but activated macrophages are able to kill the pathogen. Probiotic bacteria administered at an optimal dose of 10^4 to 10^8 cfu/g of feed to fish are capable to stimulating the respiratory activity and this could be the protective mechanism as reported earlier [29]. The probiotic bacteria may also induce cell-mediated immunity by stimulating cytokine production in fish as well as in mammals [52].

In the present study, the serum lysozyme activity did not significantly enhance on first week. After 8th week, all treated groups the activity significantly increased when compared to control group. Lysozyme is an important parameter in the immune defence of both invertebrates and vertebrates. Lysozyme is bactericidal, hydrolyzing-β [38] linked glycoside bonds of bacterial cell wall peptidoglycans resulting in lysis. Although primarily associated with defence against Gram-positive bacteria, Gram-negative bacteria can also be lysed by this enzyme. Lysozyme is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species [53] and is well known to be an opsonin and activate the complement system and phagocytes [54]. This action has been shown to attack primarily Gram-positive bacteria, in addition to some Gram-negative bacteria, in conjunction with the complement [55] and increasing protection of fish against bacterial infections; this could be correlated with an increment in serum lysozyme levels, as well as the phagocytic and bactericidal activity of head-kidney leukocytes [56]. However, several other marine species like cod, haddock, Pollack, and wolffish show very little or no lysozyme activity in their tissues or body fluids [40].

In the present study, the cumulative mortality in infected untreated group was 80%, whereas in low, medium, and high supplemented diet fed groups it was 40, 35, and 25%, respectively. Similar results demonstrated that administration of *L. rhamnosus* to fish feed could reduce mortality of fish challenged with a virulent strain of *A. salmonicida* [29]. We found that *Zooshikella* supplemented diets in olive flounder starter with the proper density could be beneficial for fast growth and survival [57]. The best survival rate was observed in groups treated with medium and high concentration of *Zooshikella* supplemented diets. The enhancement of innate immune parameters by *Zooshikella* supplementation diets are possibly an important factor in reducing the percentage mortality and thereby protecting the fish against live *S. iniae*. In addition, it is suggested that adding *Zooshikella* strain JE-34 may help to control infections and improve the innate immune system in olive flounder.

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