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Original Article

Effect of dietary *Lactococcus lactis* and *Bacillus subtilis* on the innate immunity, intestinal microbiota, histometrical indices, and resistance against *Aeromonas hydrophila* in Oscar, *Astronotus ocellatus* Agassiz, 1831

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Abstract: This work aimed to investigate the effect of dietary *Lactococcus lactis* and *Bacillus subtilis* on the immune responses, intestinal microbiota, and resistance to pathogens of Oscar, *Astronotus ocellatus*. During 70 days trial, 300 juveniles (8.96±0.033 g) were fed diets enriched with *L. lactis* and *B. subtilis*. The treatments included 150, 300, 450 mg kg^{-1} of dietary *L. lactis* (LL_{150}, LL_{300}, LL_{450}); 150, 300, 450 mg kg^{-1} of dietary *B. subtilis* (BS_{150}, BS_{300}, BS_{450}); 150, 300, 450 mg kg^{-1} of diet an equal mixture of *L. lactis* and *B. subtilis* (MIX_{150}, MIX_{300}, MIX_{450}); and a non-supplemented control group. At the end of the rearing period, histological, immunological, and intestinal microbiota indices in treatments were investigated. To evaluate disease resistance, 15 fish in each treatment were infected in each treatment by *Aeromonas hydrophila*. The results showed that adding *B. subtilis* and *L. lactis*, particularly in MIX_{300}, reduced the anaerobic heterotrophic bacterial microbiota and increased lactic acid bacteria (LAB) in fish. The highest white blood cell (WBC) level was recorded in the LL_{150} group. The lymphocytes in fish fed LL_{150}, LL_{300}, MIX_{150}, and MIX_{300} diets were changed and neutrophils of LL_{150}, LL_{300}, LL_{450}, MIX_{300}, and MIX_{450} were significantly increased. Monocytes in fish fed MIX_{300} and MIX_{450} diets raised significantly. The IgM, ACH_{50}, and lysozyme levels in fish-fed diets enriched by bacteria, especially in LL_{450}, were significantly higher than the control treatment. The intestinal villi in LL_{450}, BS_{150}, and MIX_{450} were significantly higher, showing lower damages than the other treatments. The survival rates of the infected fishes were higher in MIX_{150} and MIX_{300} groups.

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Introduction

Oscars (*Astronotus ocellatus*) are ornamental fish of distinct quality and value. They are known as ever-hungry fish interested in feeding even at satiation with a unique appearance, intelligence, and behavior, making them enjoyable to aquarium enthusiasts. These, combined with the fact that Oscar can live just as long as a dog, make it more like a pet than most other fishes (Yilmaz and Arsalan, 2013). Health and nutrition are two vital aspects of ornamental fish farming, as its annual international exports are around US$ 200 million, or less than 3% of the total world fish trade (Ghosh et al., 2008; Gobi et al., 2018).

The gastrointestinal tract of fish is a key area of interaction with pathogens in fish farms. It is important to enhance the cultured fish immune system by enriching the normal gut microbiota, as it affects a wide range of biological processes, including the development of gut-associated lymphoid tissue (GALT) and the ability to combat infections (Nayak et al., 2007). Microbial population diversity will be altered by manipulating microbial populations and changing environmental conditions caused by the proliferation of selected bacteria (Sayes et al., 2018). The competitive elimination of

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pathogenic bacteria by probiotics can effectively reduce or eliminate the prophylactic use of antibiotics in intensive systems (Saputra et al., 2016). It has been well-established that the GI performance of aquatic organisms is modified via microbial modification (Doan et al., 2021). Providing probiotics via diet can improve the microbial balance of the host (Nayak, 2010) and increase resistance against disease-causing bacteria in aquatics (Al-Dohail et al., 2011).

Lactic acid bacteria (LAB) of *Lactococcus lactis* are known to produce a wide range of antimicrobial compounds that can inhibit growth or kill a broad range of bacteria (Loh et al., 2017). *Bacillus subtilis* identified in the GI tract of several finfish species are spore-forming bacteria resistant to adverse environmental conditions, with various species showing unusual physiological features enabling them to survive in different environments (Díaz-Rosales et al., 2006). *Bacillus* can affect nutrition, adherence, and colonization of pathogens, affecting fish's immune system with probiotic potentials (Soltani et al., 2019).

The adhesion of Gram-positive probiotics into fish feed can improve the immuno-physiological functions of hosts and enhance their disease resistance. Probiotics multiply after settling in the intestine and use sugar to grow and produce unsaturated fatty acids (Blottiere et al., 2003). The probiotic dosage is a missing factor in earlier studies (Shenavar Masouleh et al., 2016). In addition, insufficient data are available to demonstrate the behavioral growth of Gram-positive probiotics, such as synergistic or antagonistic effects (Doan et al., 2021).

The effects of probiotics on the growth indices and nutrition of some fish species were reviewed in previous studies e.g. Oscar and the Nile tilapia (*Oreochromis niloticus*) (Safari and Atash, 2013; Won et al., 2020). The *B. subtilis* as a probiotic has recently been introduced to Iranian fish farmers; however, its effect on Oscar has not been studied. Based on the above-mentioned background, the present study aimed to investigate the effects of dietary *L. lactis* and *B. subtilis* on immune responses, intestinal microbiota, and resistance to pathogens in Oscar fish.

Materials and methods

Experimental conditions: During 70 days of the experimental period, 300 Oscar juveniles were stocked in 50-liter aquaria, ten fish per aquarium (0.16 g L^{-1}). There were 10 experimental treatments, each with three replicates. The mean weight and length of juveniles were 8.96±0.03 g and 8.23±0.02 cm, respectively. Adaptation to the lab condition and feed was made for ten days. *Bacillus subtilis* (Persian type culture collection: PTCC No.:1204) and *L. lactis* (registered at NCBI under No. JF831150) were added to the basic diet (Coppens, Germany) in different concentrations, including 150 mg kg^{-1} (1.5×10^6 CFU g^{-1}), 300 mg kg^{-1}, (3×10^6 CFU g^{-1}) and 450 mg kg^{-1} (4.5×10^6 CFU g^{-1}), as an equal mixture of *B. subtilis* and *L. lactis* (Table 1). The predicted powder-containing bacteria was dissolved in 50 mL of ringer solution and added per kg of diet. The control group received the same basal diets without added bacteria.

The pelleted diet from Coppens Company (TROCO CRUMBLE HE) with size of 0.8-1.2 mm was used as basic diet, containing 56% crude protein, 15% crude fat, 0.5% fibre, 8.4% ash, 2.3% calcium, 0.7% sodium, and 1.4% total phosphorus. The juveniles were fed at 3-5% of their body weight based on the water temperature and biomass of each aquarium (according to initial and mid-term biometric measurements) 3 times a day (8 a.m., 12 a.m., and 2 p.m.) for 70 days. The growing juveniles were transferred to 200 L aquaria to maintain their biomass. The physico-chemical parameters of water, including temperature, dissolved oxygen, pH, hardness (DH), and total ammonia before and after feeding, are shown in Table 2.

Blood sampling and serum preparation: Feeding was stopped for 24 hours, allowing gut evacuation, with three fish randomly selected from each aquarium. Using a syringe, 2 mL of blood was taken from their caudal vein. Then, 1.5 ml of blood was taken and

Table 1. Experimental treatments based on added bacteria to basic diet.

Treatment	*L. lactis* added to basic feed (mL kg^{-1})	*B. subtilis* added to basic feed (mL kg^{-1})
Control	0	0
LL_{150}	150	-
LL_{300}	300	-
LL_{450}	450	-
BS_{150}	-	150
BS_{300}	-	300
BS_{450}	-	450
MIX_{150}	75	75
MIX_{300}	150	150
MIX_{450}	225	225

Table 2. Physico-chemical parameters of water during the 70-days of rearing

Nitrite (mg l^{-1})	Ammonia (mg l^{-1})	Hardness	pH	Dissolved Oxygen (ppm)	Temperature (ºC)
<0.04	0.07-0.10	172.4±0.4	7.36±0.21	7.6±0.44	27.84±0.32

saved in non-heparinized tubes for measuring immunological parameters (Abarike et al., 2018). The blood serums of non-heparinized samples were separated by centrifuging at 1409 g force (3000 rpm) for 10 min (Labofuge, manufactured by Heraeus Sepatech, Germany), then stored at -80°C until analysis. The remaining blood samples (0.5mL) were used for blood cell counts. The blood sample was diluted with a Natt-Herrick solution to calculate white blood cells (WBC) by a Neubauer hemocytometer slide. The blood smears on glass microscope slides were stained with Giemsa for the differential leukocyte count, and the percentage of different leukocytes was determined (Mohammadian et al., 2020).

Measurement of immunological parameters: The immunoglobulin M (IgM) of the serum was measured by an immunoturbidimetric assay in a spectrophotometer (2100-VIS, Unico, the US) at a wavelength of 340 nm with distilled water as the control (Teige et al., 2019). To determine lysozyme activity through the gradual lysis of a Gram-positive bacterium (*Micrococcus lysodeikticus*, Sigma, USA), 50 µL of serum was added to 950 µL of the *M. lysodeikticus* solution (200 mg mL^{-1} of the bacterium in 5% sodium phosphate at a pH of 6.2). The solution turbidity observation was at 530 nm and 22°C after 0.5 to 4.5 minutes by a turbidimetric assay in an Elisa reader (Awareness, USA). To record the data (mg mL^{-1}), egg lyophilized albumen lysozyme (Sigma) was applied based on Sharifuzzaman and Austin (2009) and Merrifield et al. (2010). The Alternative complement pathway (ACH_{50}) was measured based on the photometric method at 414 nm using a spectrophotometer (Awareness, USA) through hemolysis of rabbit red blood cells (RaRBC; TCS Biosciences Botolph clydon, UK) (Tukmechi et al., 2011).

Intestinal bacteria count: Three samples from each treatment were randomly selected and euthanized with 1500 ml L^{-1} of clove oil (CPCSEA, 2021). Then intestine was removed under sterile conditions (Nayak et al., 2007). The ringer solution was applied, making 10^1 to 10^8 dilutions of the intestinal fecal contents. After sampling, dilutions of 10^1 through 10^7 of intestinal extracts were prepared using a sterile Ringer solution. Then 0.1 mL of each dilution was inoculated on the Tryptic Soy Agar (TSA) medium and De Man, Rogosa, and Sharpe agar (MRS) media (LAB specific medium) for the intestinal bacterial count. TSA incubation plates were in aerobic condition at 25°C and MRS plates

Table 3. Total and differential WBC of *Lactococcus lactis* and *Bacillus subtilis* added diets for Oscar after 70 days.

Treatments	WBC (mm3)	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)	Eosinophils (%)
Control	3316.67±72.64^{c}	82.67±0.88^{a}	12.33±0.33^{b}	4.67±0.33^{bc}	0.33±0.33
LL_{150}	5000.00±493.28^{a}	77.33±0.33^{c}	16.00±0.57^{a}	6.00±0.57^{abc}	0.67±0.33
LL_{300}	3633.87±0.007^{bc}	77.33±1.20^{c}	16.00±1.52^{a}	6.00±0.57^{abc}	0.67±0.33
LL_{450}	4033.87±284.80^{bc}	78.00± 0.53^{bc}	15.67±0.06^{a}	5.67±0.33^{abc}	0.67±0.33
BS_{150}	3816.87±120.19^{bc}	81.00±1.53^{ab}	14.00±1.20^{ab}	4.67±0.67^{bc}	0.33±0.33
BS_{300}	3500.00±208.17^{c}	79.00±1.00^{bc}	14.67±1.15^{ab}	5.33±0.33^{abc}	1.00±0.58
BS_{450}	3450.00±125.83^{c}	78.33±0.88^{bc}	14.33±0.88^{ab}	6.33±0.33^{ab}	1.00±0.58
MIX_{150}	4033.33±272.84^{bc}	79.67±0.88^{bc}	15.67±0.66^{ab}	4.33±0.33^{c}	1.00±0.58
MIX_{300}	4433.33±437.16^{ab}	77.00±1.15^{c}	15.67±0.33^{a}	6.67±0.88^{a}	0.67±0.33
MIX_{450}	4133.33±176.38^{bc}	77.00±1.00^{c}	14.93±0.30^{a}	6.67±0.33^{a}	0.67±0.33

Values expressed as mean±SD. Different letters in each column indicate the significance of the difference (P<0.05).

were incubated at 30°C and under anaerobic conditions. The bacterial count (CFU) took place after the incubation.

Bacterial challenge test (BCT): Fish were exposed to pathogens based on Al-Dohail et al. (2011) and Sharifuzzaman and Austin (2009). After final sampling, *Aeromonas hydrophila* (ATCC:15309, obtained from Pasture Institute, Iran) was injected into fishes to evaluate their resistance to pathogens. Five specimens, 30-35 g., were randomly selected from each replicate in all treatments. Upon anesthetizing, 0.1 ml of *A. hydrophila* (a dosage of 1×10^{8} cells/fish) was injected intraperitoneal (McFarland Standard Kit No. 1, Dalynn biologicals). During the 14-day BCT period, the water temperature of the tanks was 28°C. Individual infections, mortalities, and disorders were monitored and recorded daily. At the end of this period, fins, skin, internal organs, liver, and swim bladder were sampled and examined to identify probable lesions.

Intestinal histology: After opening the abdomen, the anterior, middle, and posterior parts of the intestines were removed and fixed into Buen's solution. Following 48 hours, the fixed intestinal tissues were processed, and histological slides were prepared based on Eagderi et al. (2013) and stained with hematoxylin-eosin. Images were taken using a light microscope (BEL, BIO2, Italy) equipped with a EUREKAM 10.0 camera. ImageJ 1.46r software was used for the histomorphometry of tissue expansions. Mucous folds and other histometric parameters were measured in 6 random fields. Measurements were made on the mucosal fold's height, the thickness of the mucosal epithelium, the parine layer, submucosa, and the muscle layer. The tissue indices were compared between treatments based on the average size of the three parts of the intestine (Suvarna et al., 2012).

Data analysis: The data normality of all measured parameters was determined using the Shapiro-Wilk test. The treatments were compared using the one-way analysis of variance (ANOVA). After ensuring the homogeneity of variances, Duncan Multiple Range Test (DMRT) was used to compare treatments. Statistical data analyses were done using SPSS-23, and the graph was plotted in Excel-2016.

Results

WBC and differential count: The lowest WBC was recorded in the control group, while the highest value was observed in the LL_{150} group (P<0.05; Table 3). The lymphocyte of all treatments that were fortified with probiotics was lower than the control group (P<0.05) (Table 3). Neutrophils of all treatments receiving *B. subitilis* and *L. lactis* were higher than the control one (P<0.05; Table 3). The highest monocyte percentage was recorded in MIX_{300} and MIX_{450} (P<0.05). There was no significant difference in eosinophile between treatments (P>0.05; Table 3).

Serum immunological parameters: The serum lysozyme activity in the LL_{450} and MIX_{450} was

Table 4. The IgM, Lysozyme and ACH50 value of *A. ocellatus* fed with diets containing *Lactococcus lactis* and *Bacillus subtilis* after 70 days.

Treatments	IgM (mL dL^{-1})	Lysozyme (μg mL^{-1})	ACH_{50} (U%)
Control	32.50±0.50^{c}	28.00±1.00^{c}	128.0±1.0^{d}
LL_{150}	33.00±1.00^{c}	29.00±1.00^{c}	129.0±1.0^{cd}
LL_{300}	38.00±1.00^{abc}	30.00±1.00^{bc}	135.0±2.0^{abc}
LL_{450}	42.00±1.00^{a}	36.50±2.50^{a}	137.0±1.0^{a}
BS_{150}	34.00±1.00^{c}	28.00±2.00^{c}	129.0±2.0^{cd}
BS_{300}	40.50±3.50^{a}	34.50±2.50^{abc}	136.5±4.5^{a}
BS_{450}	38.50±0.50^{ab}	32.50±1.50^{abc}	134.0±1.0^{abcd}
MIX_{150}	32.50±0.50^{c}	30.00±1.00^{bc}	130.0±0.0^{bcd}
MIX_{300}	37.00±1.00^{abc}	31.00±1.00^{abc}	135.0±1.0^{abc}
MIX_{450}	38.00±3.00^{abc}	37.50±3.50^{a}	137.0±2.0^{a}

Values expressed as mean±SD (n=81). Different superscript lowercase letters within each column represent significant differences (P<0.05).

Table 5. The number of aerobic heterotrophic bacteria of intestinal mucus on TSA and MRS (log CFU g^{-1}±SD).

Treatments	TSA (CFU g^{-1})	MRS (CFU g^{-1})
Control	8.29±0.006^{a}	3.54±0.06^{e}
LL_{150}	5.71±0.05^{g}	4.50±0.01^{ab}
LL_{300}	6.33±0.02^{e}	4.59±0.01^{b}
LL_{450}	7.25±0.01^{d}	4.42±0.01^{c}
BS_{150}	6.11±0.04^{f}	4.03±0.01^{d}
BS_{300}	8.09±0.04^{b}	4.02±0.04^{d}
BS_{450}	8.13±0.02^{b}	4.004±0.01^{d}
MIX_{150}	7.75±0.07^{c}	4.81±0.02^{a}
MIX_{300}	7.78±0.026^{c}	4.88±0.01^{a}
MIX_{450}	8.07±0.03^{b}	4.62±0.01^{b}

Values were expressed as mean±SD (n=3): different superscript lowercase letters within each column represent significant differences (P<0.05).

significantly higher than the control treatment (P<0.05; Table 4). The ACH_{50} was higher in all treatments than in the control one (P<0.05; Table 4). Treatments of the LL_{450}, BS_{300}, and MIX_{450} were the highest from others (P<0.05; Table 4). Based on the results, the IgM, lysozyme, and ACH_{50} were influenced by increasing bacterial dosage in all treatments (Table 4). The IgM level of LL_{450} and BS_{300} was significantly increased in treatments (P<0.05; Table 4).

Intestinal microbiota counts: The results revealed that aerobic heterotrophic bacteria of intestinal mucus and LAB were well-colonized, and their number in the intestine was significantly elevated by increasing bacterial dosage (P<0.05). Considering the total intestinal microbiota on TSA and MRS media, there was a significant difference between all treatments and the control (P<0.05). The lowest and the highest bacterial counts were observed in the LL_{150} and the control, respectively. The total LAB count in the intestine of all treatments significantly decreased compared with control and *B. subitilis* treatments. However, MIX_{150} and MIX_{300} exhibited a significant difference from the control (P<0.05). There was an increasing value in the number of intestinal mucus bacteria related to bacterial dosage of diets (Table 5).

Intestinal histology: In the histometeric indices (Table 6), based on the average size in the anterior, middle, and posterior portions of the intestine, the villius average height in MIX_{450}, villi epithelium average diameter, villi average diameter, the

Table 6. Mean intestinal histomteric indices (µm) of Oscar fed by *Lactococcus lactis* and *Bacillus subtilis* and mixed probiotic diets.

Treatments	Villus Height (µm)	Epitelium Diameter (µm)	Villus Width (µm)	Muscular Thickness (µm)	Lamina Propria (µm)	Sub Mucosa (µm)
Control	369.77±14.85^{e}	32.42±0.90^{f}	68.57±1.99^{g}	85.01±3.37^{c}	13.42±0.38^{e}	32.66±1.04^{d}
LL_{150}	514.65±17.19^{bc}	47.65±1.16^{ab}	98.77±2.74^{b}	74.13±2.61^{d}	46.87±1.88^{b}	36.30±1.27^{d}
LL_{300}	493.64±12.78^{c}	49.07±1.67^{a}	105.58±3.76^{a}	85.22±3.44^{c}	58.55±1.89^{a}	42.69±1.93^{c}
LL_{450}	547.28±12.97^{ab}	44.73±1.26^{bc}	92.01±2.32^{cd}	86.91±2.49^{c}	60.16±1.54^{a}	50.37±1.32^{a}
BS_{150}	546.48±17.72^{ab}	41.66±1.06^{cd}	88.44±2.20^{cd}	85.33±2.15^{c}	47.83±1.41^{b}	51.63±1.73^{a}
BS_{300}	421.33±12.15^{d}	37.37±1.08^{e}	78.38±2.14^{ef}	117.20±5.35^{a}	42.17±1.47^{c}	49.46±1.60^{ab}
BS_{450}	473.31±9.78^{c}	41.28±1.10^{cd}	86.58±2.21^{cd}	114.26±3.57^{a}	42.67±0.96^{c}	47.54±1.32^{ab}
MIX_{150}	495.22±18.27^{c}	34.13±0.95^{f}	72.78±1.87^{fg}	100.37±2.72^{b}	38.16±1.15^{d}	47.91±1.46^{ab}
MIX_{300}	503.54±16.63^{bc}	41.83±1.26^{cd}	87.94±2.47^{cd}	61.91±1.84^{e}	47.06±1.63^{b}	41.77±1.12^{c}
MIX_{450}	580.03±15.05^{a}	39.03±0.98^{de}	82.23±1.91^{e}	97.49±3.75^{b}	46.31±1.33^{bc}	45.44±1.36^{bc}

Different letters in each column indicate significant differences between them (P<0.05). (Mean±SD).

thickness of parine layer in LL_{300}, the thickness of muscle layer in BS_{300}, BS_{450}, the thickness of the submucosal layer in LL_{450} and BS_{150} were significantly different compared to the control (P<0.05) (Fig. 1).

Bacterial challenge test (BCT): After two weeks of monitoring *Aeromonas*-infected Oscars, the highest mortality rate was detected in the control group (P<0.05), while the lowest mortality rate (P<0.05) was recognized in MIX_{150} and MIX_{300} (Fig. 2). The first mortality was recorded after six days of injection in the control group. The most common lesions observed during BCT, were bleeding in the anus, base of the anal, caudal fins, and around the mouth. Limited necrospy hemorrhage was detected in the liver, swim bladder, peritoneal cavity, and hyperemia in the kidney (Table 7).

Discussion

The results showed a significant increase of WBC as an immunity index in the LL_{150}, and MIX_{300}. Increased levels of WBC were observed in rainbow trout and Nile tilapia fed *B. subtilis* and *L. lactis* treated diets (Balcázar et al., 2007; Opiyo et al., 2019), indicating that an increase in probiotic supplementation may reflect improved immunity of fish. Probiotics such as *B. subtilis* (Lee et al., 2017) and *L. lactis* (Xia et al., 2018) have been proven useful in aquaculture. The immune system response of fish can be greatly influenced by adding *B.subtilis* and *L. lactis* in different fish diets (Nayak et al., 2010; Dias et al., 2020). After colonizing *Bacillus* in the mucosal epithelium of the intestine, protection signs show against pathogens by competitive exclusion or competition for available energy and the production of inhibitory compounds (Soltani et al., 2019).

The results showed a significant IgM and lysozyme improvement in Oscar, especially in treatments LL_{450}, BS_{300} and BS_{450}. These findings are similar to those reported for *Lactobasilus rhamnosus*, *Carnobacterium maltaromaticum*, and *C. divergens* in rainbow trout (Panigrahi et al., 2004; Kim and Austin, 2006), *Pediococcus acidilactici* in the Nile tilapia (Ferguson et al., 2010), and the Oscar (Safari and Atash, 2013). According to Nayak (2010), probiotics can stimulate the intestinal immune system by increasing the number of IgM and acidophilic granulocytes. Safari and Atash (2013) reported that the lysozyme level significantly increased in the blood serum of Oscar fed with diets enriched with *P. acidilactici*. The effects of *B. subtilis* and *L. lactis* on the immune responses of the Nile tilapia showed that the immune system

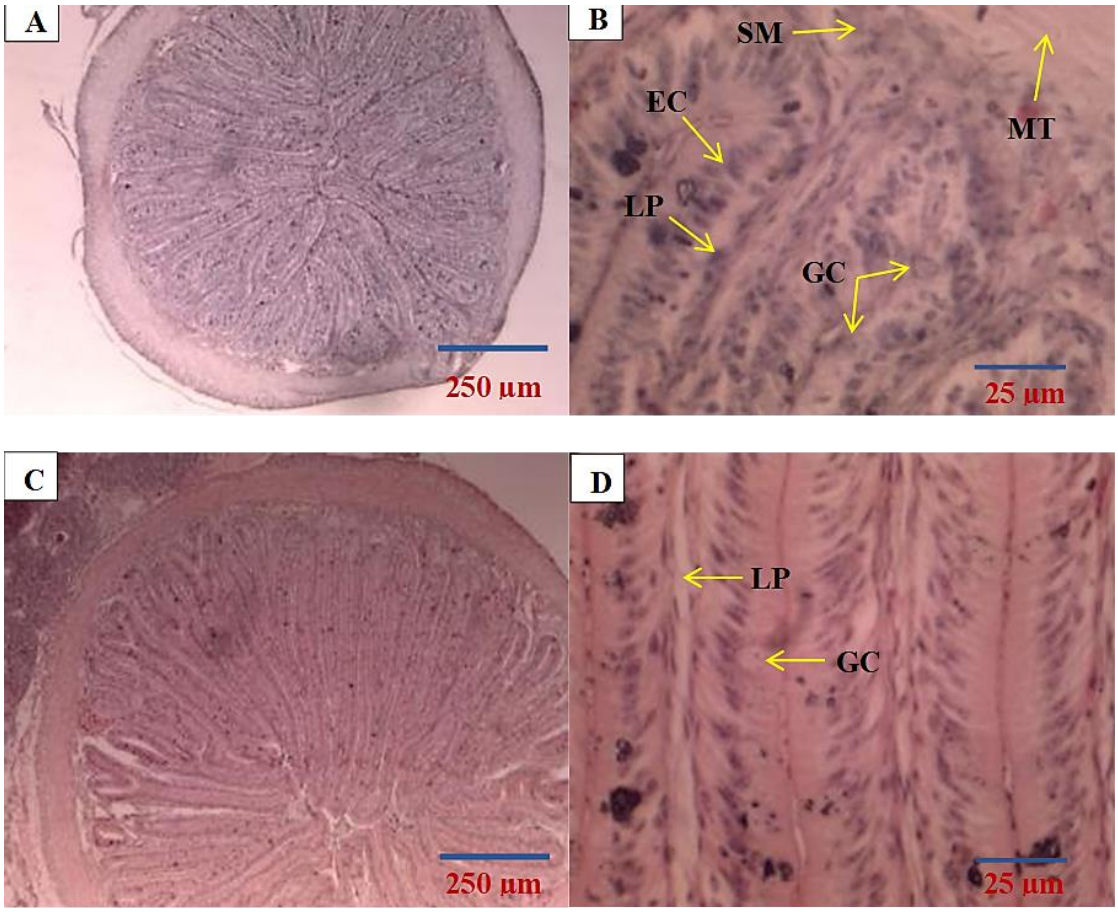

Figure 1. Histologic cross-section view of the posterior intestine of Oscar in the control group (A&B) and the treatment 9 (MIX_{450}) (C& D) (Abbreviations: GC: Goblet Cell, EC: Enterocyte, LP: Lamina Propria, Muscular thickness (MT), SM: Submucosa. Eosin-hematoxylin staining.

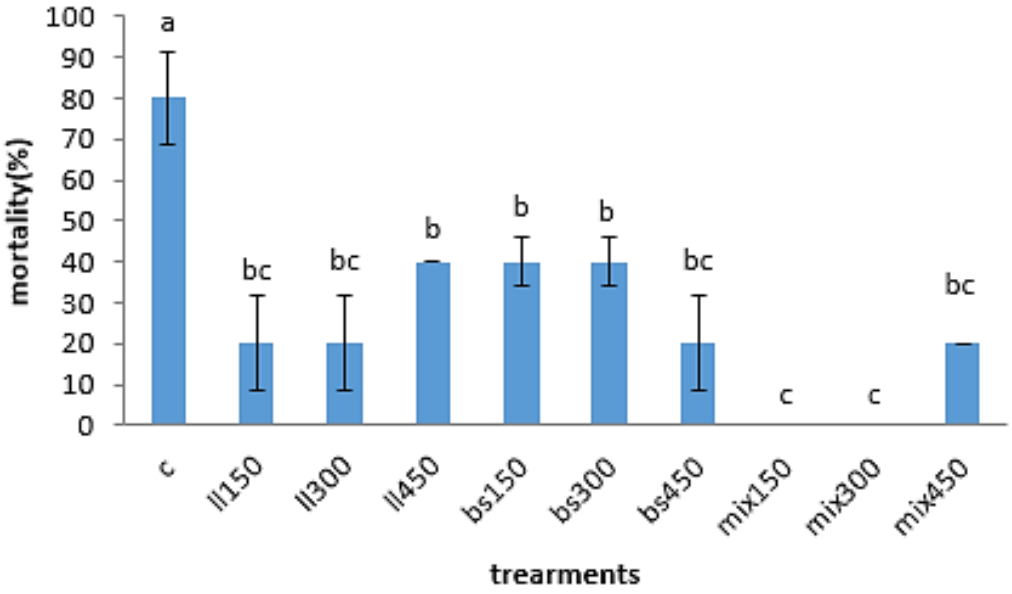

Figure 2. The mortality rate of differently treated *Aeromonas ocellatus* exposed to *A. hydrophila* after 14 days.

performance, including lysozyme, became significantly efficient in the fishes fed-diets enriched with probiotic bacteria (Won et al., 2020). The probiotic bacteria could improve the lysozyme level

in different species, including rainbow trout (Balcázar et al., 2007), the Malabar grouper (*Epinephelus coioides*) (Sun et al., 2012) and Nile tilapia (Han et al., 2015; Abarike et al., 2018; Opiyo et al., 2019). The use of LAB in aquaculture can affect the IgM level as an important humoral immunity against pathogens (Panigrahi et al., 2005). It has been shown that *B. subtilis* in the diet of rainbow trout and Rohu (*Labeo rohita*) can improve the IgM level (Nikoskelainen et al., 2003; Nayak, 2010), which is in line with the findings of the present study on Oscar. Our study results indicated that the IgM level in LL_{450} and BS_{300} was significantly higher. The proof of higher IgM levels in some treatments can be attributed to stimulating antibodies produced by LAB (Yu et al., 2020).

Panigrahi et al. (2004) reported that feeding rainbow trout with diets enriched with *L. rhmanosus* increased ACH_{50} in the anterior part of the kidney. A similar increasing trend in ACH_{50} has been reported in the brown trout (*Salmo trutta*) (Balcázar et al., 2006), rainbow trout (Ramos et al., 2015) and the gilthead seabream (*Sparus aurata*) (Díaz-Rosales et al., 2006) fed diets containing *L honi*, and *L. lactis* and also in rainbow trout fed diets enriched with *L. rhamnosus* (Nikoskelainen et al., 2003). Compared to the control, the increase of ACH50 in all treatments indicated the positive effect of probiotics. The findings of the present study showed that the addition of *L. lactis* and *B. subtilis* to the basal diet of Oscar could be due to the increasing colonization rate of microbiota or a significant reduction of the intestinal bacterial count.

Based on our results, diets with 10^{10} CFU *B. subtilis,* and *L. lactis* led the intestinal bacterial count to log 5.71 CFU mL^{-1} and the intestinal LAB count ranged between log 3.33 to log 4.81 CFU mL^{-1}. The highest bacterial count was found in MIX_{300}. Feeding the Persian sturgeon and Beluga (*Huso huso*) fry with diets enriched with two types of LAB (*L.mesenteroides* and *L. curvatus)*, at a dosage of 10^9, showed the intestinal bacterial count about log 3 CFU mL^{-1} and LAB count was log 2.27 to log 3.02 CFU mL^{-1} (Askarian et al., 2011). During the Persian sturgeon feeding on diets enriched with *L. lactis* at a dosage of 10^8, the intestinal bacterial count reached log 4.05 CFU mL^{-1} and the intestinal LAB count ranged between 3.35 and log 4.19 CFU mL^{-1} (Shenavar Masouleh et al., 2016). The inhibited growth of pathogenic bacteria by beneficial bacteria could be due to the individual or combined production of antibacterial metabolites (e.g., bacteriocins, siderophores, lysozymes, proteases), competition for essential nutrients, alteration of pH by organic acid production, and competitive exclusion (Kim and Austin, 2006; Mukherjee and Ghosh, 2016). In addition, the inhibitory effect is related to the organic acid excreted by *L. lactis* (Loh et al., 2017). The differences in CFU are due to the feeding behavior of species, the initial dosage of added bacteria, its strain, and rearing environmental conditions, particularly water temperature (Martínez Cruz et al., 2012; Sayes et al., 2018).

The intestinal microbiota showed a significant difference between juveniles fed diets enriched with bacterial species and those in the control group. Both added bacteria were effective in reducing the populations of common intestinal bacteria. Moreover, the results showed that LABs were well-colonized in the intestine of Oscar, and their population in the intestine significantly increased with the increase of enrichment. The results of probiotic bacteria in the MRS medium showed that the intestinal wall in the control group lacked these bacteria. Adding probiotics to the diet caused a rise in the number of probiotic bacteria in the intestine, with the highest number observed in mixed diets i.e., a mixture of *B. subtilis* and *L. lactis* could easily survive in the gastrointestinal tract, attach to the mucosal surface of the intestine, proliferate, and act synergistically.

Histological examinations of the intestines show a significant extension of intestinal folds in the treatments containing *L. lactis* and *B. subtilis*. It was found that *B. subtilis* probiotic could improve the immunity, and digestive system, especially the fish intestinal morphology parameters (Lee et al., 2017). Probiotics increase food absorption and enzyme

digestion process and improve the intestinal tissue of common carp (Yanbo and Zirong, 2006), sea bass using *B. mojavensis* (Hamza et al., 2016), the barb fed by mixed probiotics (Allameh et al., 2017) and Nile tilapia fed by *B. subtilis* and *L. lactis* (Liu et al., 2017; Xia et al., 2018). Also, using two probiotic strains (Pdp11 and Pdp13) from Alteromonadaceae, similar results were reported i.e. including the increased size and number of microvilli in the intestine of *Solea senegalensis* fry (Saenz et al., 2009). Probiotics have been reported to increase the thickness of the muscle layer due to the modulation of the physiological activities of intestinal mucosal cells (Lazado and Caipang, 2014). The combined use of *Lactobacillus, Entrococous, Pedicoccus* and *Bacillus* probiotics in feeding rainbow trout has increased the anterior surface of the intestine by increasing the length of the villi and the number of goblet cells in the diet containing probiotics (*Ramos et al., 2015*). Recent studies linked the improvement of intestinal histological indices in Nile tilapia fed-diet containing *B. subtilis* and *L. lactis* to better growth performance, feeding efficiency, and increased activity of intestinal tissues. Probiotics could facilitate the absorption of effective nutrients by improving the length of the intestinal villi, the muscle layer's thickness, and the trypsin's activity (Won et al., 2020).

This study concerning the increased disease resistance induced by probiotic supplements confirmed the results of previous studies (Raida et al., 2003; Panigrahi et al., 2007; Soltani et al., 2019). *Aeromonas hydrophila* is a known pathogenic bacterium that causes diseases in the Cichlids family (Saputra et al., 2016). Our findings indicated that adding probiotic bacteria to the basal diet improved Oscar resistance against *A. hydrophyla.* The mortality rate after 14 days was lower in groups fed diets enriched with *B. subtilis* and *L. lactis* and started later than in control. In the Nile tilapia, *L. lactis* improved immune responses and resistance against diseases (Xia et al., 2018). The symptoms observed in intentionally *A. hydrophila* infected fishes were similar to those reported for Persian sturgeon exposed to the same bacterium (Soltani and Kalbassi, 2001). The symptoms in both experiments were imbalance, lethargic swimming, bruising with bleeding spots on the external surfaces, and hemorrhage in internal organs.

In the rainbow trouts receiving diets supplemented with 10^9 and 10^{12} CFU of *L. rhamnosus*, the survival rate increased by 33.7 and 6.3%, respectively, after exposing fish to *A. salmonicida* (Nikoskelainen et al., 2001). Kim and Austin (2006) applied diets enriched with 10^7 CFU g^{-1} of *Clostridium maltaromatieum* (B26) and *C. divergens* (B33) for two weeks. They reported that the immunological protection improved following rainbow trout exposure to *A. salmonicida* and *Yersinia ruckeri.* Brunt et al. (2007) supplemented diets with 2×10^8 CFU g^{-1} of *Bacillus* (JB-1) and *A. sobria* (GC2) applied for 14 days on rainbow trout, showing lower mortalities than that in the control when the fishes were exposed to *Vibrio ordalii*, *V. anguillarum*, *Streptococcus iniae*, *A. salmonicida*, and *Y. ruckeri.* Won et al. (2020) listed many studies confirming improvements in *E. malabaricus* fed a diet containing *L. plantarum,* Chinese drums (*Miichthys miiuy*) with a diet added by *C. butyricum* (CB2), the Nile tilapia diet enriched by *B. subtilis* and *L. acidophilus* (mixture of both) showed a reduced mortality rate among fish exposed to *A. hydrophila*, *V. anguillarum, Pseudomonas fluorescens*, and *S. iniae* respectively. They also conducted 13 days of BCT trial with *A. hydrophila* on the Nile tilapia, revealing a significantly increased survival rate of the tilapia fed with diets enriched with *L. lactis* and *B. subtilis* than control one and the diets enriched with Oxytetracycline (OTC) (Won et al., 2020). Differences in the probiotic efficacy are related to bacteria strains and their concentration in basic diet, target organism criteria, including fish species, age, its disease-causing intensity, challenge duration, and environmental condition, including water temperature, salinity, pH, and alkalinity (Lee et al., 2017; Elsabagh et al., 2018; Won et al., 2020).

Conclusion

Based on the results of the immunological analysis, the two probiotics of *L. lactis* and *B. subtilis* improved the performance of Oscar's immune system. The Oscar juveniles fed diets enriched with probiotic bacteria had more efficient intestines and showed higher resistance against stressors and diseases. The mixture of *B. subtilis* and *L. lactis* (150 mg+150 mg) per kg of feed could modulate the intestinal microbiota, immunity, and resistance in exposure to *A. hydrophila*. The better results of mixed treatments can be attributed to the symbiotic effect of using two different probiotics simultaneously.

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