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Research Article

In vivo study on probiotic *Lactobacillus brevis* in *Sander lucioperca* and some of their nonspecific immune parameters, intestinal morphology and survival against *Aeromonas hydrophila*

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Abstract

Lactobacillus brevis was isolated from the intestinal tract of *Sander lucioperca* and its immunological parameters as well as intestinal morphology were studied. Thereafter, the fish were challenged with 4.5×10^8 CFU/g *Aeromonas hydrophila*. The fish were monitored daily and the rate of survival was recorded over a period of 7 days post-challenge. The results showed that feeding with supplemented *L. brevis* (10^8 to 10^{10} CFU/g) had significant effect ($p<0.05$) on the survival rate. The serum lysozyme, alternative complement activity (ACH50) and IgM was significantly enhanced by dietary probiotics during the feeding period and post challenge compared to the control group. The serum IgM levels of dietary supplementation of *L. brevis* were significantly higher than that of the control after 8 weeks ($p<0.05$). After 60 days total bacteria and probiotics in the intestinal tract in groups fed with doses of 10^8 to 10^{10} CFU/g *L. brevis* had increased ratio in compare with control sampels. The survival rates after dietary administration of *probiotics* were significantly higher than that in control in post challenge. The probiotic (10^{10} CFU g^{-1}) can be used as an effective bio-control agent and as an immune stimulant in aquaculture.

Keywords: *Lactobacillus brevis*, *Sander lucioperca*, Intestinal morphology, Immune parameters, *Aeromonas hydrophila*

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Introduction

The aquaculture industry is one of the fastest growing and economically important sector of food production worldwide (FAO, 2000; Mohapatra *et al*., 2012; FAO, 2014).

On the other hand, integrated management of aquaculture for the diseas control emphasizes on healthy and safe fish production. The indiscriminate use of antibiotics in aquaculture not only could increase the risk of bacterial resistance in fish, but also in fish consumers. Probiotics have an inhibitory effect on the growth of pathogens,as well as improving the health and survival rate of the fish (Eissa and Abou-ElGheit, 2014; Pérez-Sánchez *et al*., 2014; El-Saadony *et al*., 2021; Zorriehzahra *et al*., 2016). The main probiotics obtained from species like *Bacillus* spp., *Lactobacillus* spp., *Saccharomyces* spp., *Pseudomonas* spp., *Micrococcus* spp. (El-Saadony *et al*., 2021 ; Zorriehzahra *et al*., 2016).

Lactococcus spp. are used in aquaculture and their effects against *Aeromonas hydrophila* are presented in reports of *in vivo* and *in vitro* studies (Giri *et al*., 2011; Geng *et al*., 2012). *Lactobacillus* genus is an important lactic acid bacteria that prevent adhesion and colonization of specific fish pathogens in the gastrointestinal tract (Holzapfel and Wood, 2012). Lactic acid bacteria have different application such as an alternative method for the prevention and control of disease incidence, growth promoting factors, enhancement of immune response, improve antioxidant conditions, digestion of enzymatic contribution, anti-carcinogenic and anti-mutagenic performances (Pérez-Sánchez *et al*., 2014; Shaheen *et al*., 2014; Butt *et al*., 2021; Low *et al*., 2021). One of the most important pathogens of fish is *A. hydrophila* (Giri *et al*., 2013). According to FAO reports, *Sander lucioperca* is susceptible to disease induced by *A. hydrophila* (FAO, 2018). Pike perch (*Sander lucioperca*) is one of the main fish species of economical relevance in the Caspian Sea. It lives in Anzali wetland and Aras dam. Only few studies have been conducted on this species (Gharibkhani *et al*., 2014). The aims of the present study were to examine the effect of the probiotic *L. brevis* on growth, intestinal morphology, and survival rate against *A. hydrophila* disease and on selected immunological parameters.

Materials and methods

Bacterial strain

In this research, *L. brevis* was isolated and identified by biochemical methods and sequencing of 16 SrRNA gene (Gen bank Accession number KR021404) from the intestinal tract of juvenile *Sander lucioperca*. In general, 50 µL of *L. brevis* suspension was inoculated into 20 mL De Man, Rogosa and Sharpe (MRS) broth and maintained at 30°C for 24 h. Then the bacteria were inoculated into 500 mL fresh MRSB and incubated for 48h at 30°C under aerobic condition. Bacterium cells were harvested and supernatant of the suspension was

discarded and the precipitate was resuspended in phosphate buffered saline (PBS). It was compared to the half-McFarland standard and adjusted in a spectrophotometer with a wavelength of 625 nm (total bacterial flora per gram). Incubation was performed for 30h at 30°C in anaerobic condition and then transferred to 10 mL of *Aeromonas hydrophila* grown in trypticase soy agar at 30°C to 8 mL of Muller Hinton agar medium at 45 to 50°C, and then poured into a plate containing *Lactobacillus brevis*.

The plates were incubated again for 24h at 30°C, and then the diameter of *Aeromonas* growth inhibition zone was measured. Then the two dilutions that had the greatest impact on *Aeromonas hydrophila* were identified (10^8 and 10^{10} CFU /mL).

Diet preparation

The basal diet was prepared by mixing special Gammarid powder with trout pellet (Faradaneh, Iran) containing 44.8% crude protein, 22.5% crude lipid, 11.2% ash (Table 1). Proximate analysis of the diet was conducted according to the AOAC (1990) method. The basal diet was given as control and the bacterial suspension with doses (10^8 and 10^{10} CFU/g) were sprayed and mixed with basic diet by the mixer. The diet was air dried at room temperature under sterile condition for 2h. and stored at 4°C until use. The diets were prepared nearly every week.

Experimental design

Sander lucioperca specimens with average weight of 14.5g were collected from a private fish farm in Guilan province, Iran. The fish had no sign of any bacterial or parasitic diseases or viral infections. Fish specimens were acclimatized in tanks to the conditions in the wet laboratory for two weeks. The treatments with 30 fish were replicated three times. Feeding was performed using 3% of the body weight of the fish. All fish were fed with basal diet during the acclimatization period, water temperature was 21±2°C and approximately 30% of the illumination were natural, during feeding period it was artificially dark. The physicochemical factors were evaluated as, pH (7.2 to 7.8), oxygen (7.5 to 9 mg O_2 per L, ammonium (NH3) and nitrite (NO^{-2}) at 0.04-0.09 mgL^{-1} and 0.03 - 0.07 mgL^{-1}ppm.

Table 1: Dietary formulation and proximate composition of the experimental basal diet (%).

Ingredients	
Fish meal	40
Gammarid powder	20
Soybean meal	7
Maize gluten	6
Wheat gluten	5
Wheat	7
Fish oil	11
Mineral premix	2
Vitamin premix	1
Antioxidant	0.5
Anti fungi	0.5
Proximate composition (% dry matter basis)	
Crude protein	44.8
Crude lipid	22.5
Ash	11.2

Measurement of immunological parameters

Serum lysozyme activity

Serum lysozyme activity was evaluated using list Gram positive bacteria (*Micrococcus lysodeikticus*) that was sensitive to lysozyme enzyme, according to Clerton's method (Clerton *et al*., 2001).

Alternative complement pathway activity assay

ACH50 was determined as described by Yano (1992) and was calculated by using the following formula:

ACH50 (unit/mL) = 1/K × r × ½

K= the rate of serum giving 50% hemolysis and ½ is the correction factor

IgM Level

The serum IgM level was measured by enzyme-linked immunosorbent assay (ELISA). This test uses antibodies and color change to identify a material. samples were read at 450 NM in a plate reader (Bio Tek), and mean absorbance of the negative controls were subtracted from the optical density at 450 NM, negative control was without biotin antibody (Nikoskelainen *et al*., 2001).

Phagocytic activity

Phagocytic activities were investigated using method described by Fujiki and Yano (1997). The first 50 µl. of leukocytes ($5x10^6$ cells) was placed on a glass slide, and allowed to adhere for 20 min at 25°C in a moisture incubation chamber. Then 50 µl. of latex beads (10^7 beads/ml., Sigma-Aldrich) was added to the leukocytes monolayer, and incubated for 30 min. at 25°C. The percentage of phagocytes ingesting beads (Phagocytic rate, PR) and the number of ingested beads per phagocyte (Phagocytic index, PI) were evaluated by enumerating 100 phagocytes under a microscope. Phagocytic activity was remarked as the phagocytic index (PI) (Matsuyama *et al*., 1992). The phagocytic rate (PR) and phagocytic index (PI) were calculated as follows:

$$PR = (\text{Phagocytosis cell/Total cell}) \times 100$$

$$PI = (\text{Total phagocytosed beads/Phagocytosis cell}) \times 100$$

Total bacteria count and probiotic bacteria of intestinal tract

After 60 days of feeding with probiotic containing dosages of *L. brevis* (10^8 and 10^{10} CFU /g) to *Sander lucioperca*, from each treatment 4 fish were chosen. After scarification, abdominal sections were cut under sterile conditions and the intestine was homogenized in saline serum (9%) and various dilutions (10^{-1}-10^{-9} CFU mL^{-1}) were prepared and cultured as a pure plate in Tryptone soya agar, TSA (for total bacteria counting) and MRSA (for lactic acid bacteria count) (Bagheri *et al*., 2008).

Histological examination of intestine

To determine the effect of probiotic diets on intestine, *S. lucioperca* specimens from treatments were sampled for histological examination after 60 days of experimental period. They were fixed in Bouin's fluid for 24 hours, washed by 70 percent ethanol and dehydrated through a graded series of ethanol. Paraffinated blocks of fish intestine was sectioned by microtom wih 5µ sections. Each section was stained by Hematoxylin and Eosin blue, then studied under light microscope (Wittekind, 2003).

Challenge test

A. hydrophila was obtained from the European Union Reference Laboratory for fish and crustacean diseases, Denmark. Bacteria were first grown in brain, heart infusion agar (BHI), then incubated at 35°C for 18-24h. After growing the bacteria, the cells were centrifuged at 4000 RPM for 15 min at 4°C for ELISA harvesting. First 10^1 to 10^8 dilutions (total bacterial flora per gram) of *Aeromonas hydrophila* were prepared separately in sterile physiological serum, then inoculated with 0.1 mL of each dilution in 20 fish and kept in aquarium for seven days. The bactericidal activity of *Aeromonas hydrophila* was examined according to the method of Reed and Muench (1938) 4.5×108 CFU /mL (total bacterial flora / mL).

Bacterial suspension (0.5 mL) with 100µL PBS was injected to all treatments (10^8 and 10^{10} CFU /g) and 0.5 mL of saline serum was injected into the control treatment. All treatments were kept under observation for 7 days. Fish were fed according to the basal diet. Then, the results were recorded as the rate of survival and mortality (Andani *et al.*, 2012).

Statistical analysis

The obtained data were analyzed using one-way analysis of variance. The significant differences among treatments were analyzed at 95% confidence level ($p<0.05$). To test significance between pairs of treatments Duncan's multiple range test was conducted. All statistical analyzes were done using the SPSS software package version 17.

Results

Humeral immune parameters

The alternative complement activity (ACH50) and serum IgM level of dietary administered *L. brevis* at (10^8 and 10^{10} CFU /g) were significantly higher than those of the control treatment over 8 weeks ($p\leq0.05$, Fig. 1). The serum lysozyme activities and the Phagocytic did not show significant difference among treatments ($p>0.05$). However, the ACH50 between groups fed with diet containing *L. brevis* (10^8 and 10^{10} CFU /g) did not show significant difference ($p>0.05$, Fig. 1).

Count of gut microbiota

Total bacterial counts and the average number of probiotics in groups fed with diet supplemented with *L. brevis* at 10^8 and 10^{10} CFU g^{-1} in the intestine of *Sander lucioperca*, were significantly

higher than those in the control treatment after feeding for 60 days. In the control treatment, s showed no probiotic in the intestine of the fish (Table 2).

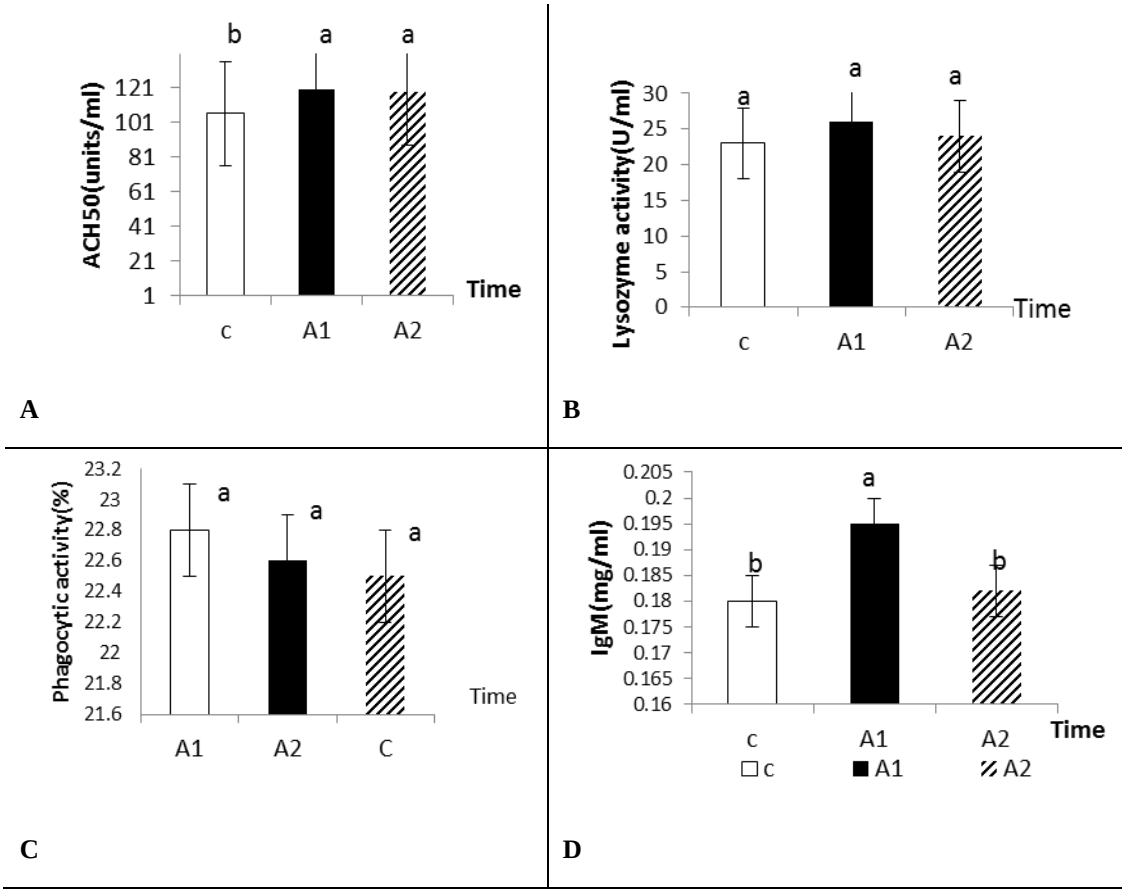

Figure 1: Alternative complement activity (A), Lysozyme activity (B), Phagocytic activity (C), and IgM levels (D) of *sander lucioperca* fed a control diet (C) and *L. brevis*-containing diets at [(A1)10^{-10}, (A2)10^{-8}] CFU g^{-1} after 8 weeks feeding. Data means at the same sampling day with different letters were significantly different.

Table 2: Total bacterial counts and the average number of probiotic cells (±standard error) in the intestine of *Sander lucioperca* during the feeding period. Columns with different letters were significantly different.

Treatment	Probiotic cells	Total microflora	Ratio (%) (pro biotic/total
A1	$1.6\times 10^{6}\pm 0.1^{a}$	$2.4\times 10^{6}\pm 0.1^{a}$	75
A2	$1.05\times 10^{5}\pm 0.3^{b}$	$2.2\times 10^{6}\pm 0.3^{a}$	72.7
Control	-	5×10^{4b}	-

However, two weeks after stopping the probiotic feeding, there was a reduction in the rate of probiotic and total bacterial in fish digestive track (Table 3).

Histology of the intestine

To determine the effect of probiotic diets on intestine, *S. lucioperca* specimens in each treatment were sampled for preparing histological sections after 60 days of experimental period. Samples were fixed in Bouin's fluid for 24 hours, washed by 70 percent ethanol and dehydrated through graded series of ethanol (Kelly, 1984). Fixed fish intestine was paraffinated and then 5 µ sections were prepared by microtome. Sections were then stained by Hematoxylin and Eosin blue and studied under light microscope (Wittekind, 2003) (Fig. 2).

Table 3: Total bacterial counts and the average number of probiotic cells (±standard error) in the intestine of *Sander lucioperca* two weeks after stopping the feeding. Columns with different letters were significantly different.

Treatment	Probiotic cells	Total microflora	Ratio (%) (pro biotic/total
A1	$1.3\times10^{5}\pm0.1^{a}$	$5.4\times10^{5}\pm0.6^{a}$	24.07
A2	$1.1\times10^{5}\pm0.2^{a}$	$4.5\times10^{5}\pm0.78^{b}$	24.4
Control	-	$4.2\times10^{4}\pm0.8^{b}$	-

A1=*L. brevis* (10^{10}) CFU /g and A2 =*L. brevis* (10^{8}) CFU /g were significantly different ($p<0.05$) among treatments.

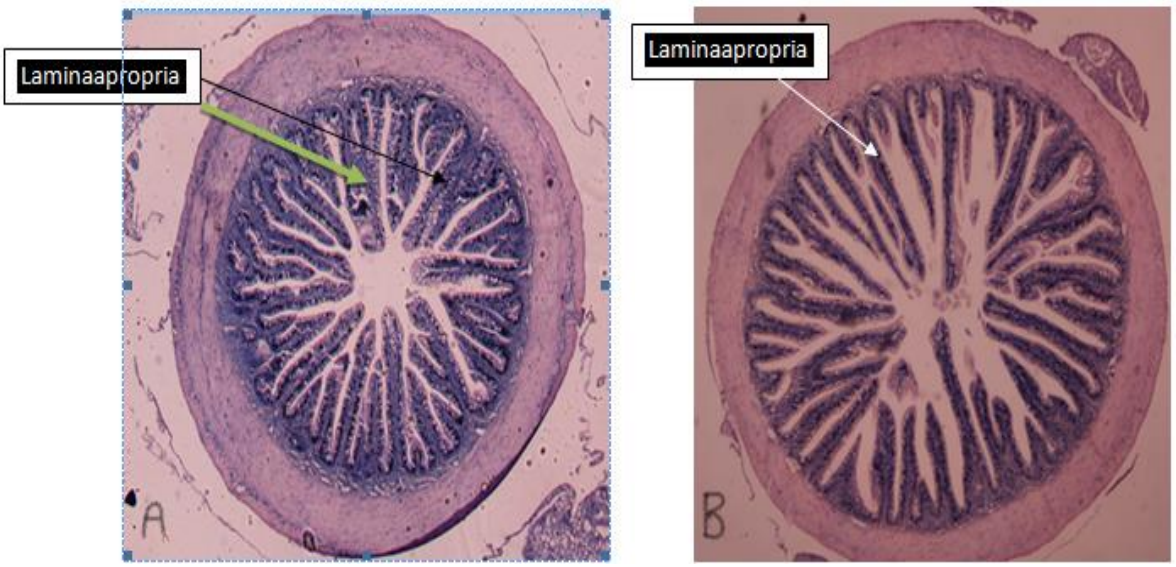

Figure 2: Comparative histology of the epithelial layer of the villi of middle intestine of *sander lucioperca* (A). Treatments were supplemented with probiotic(10^{10} CFU mL^{-1})(B) and control treatments. The layer thickness in probiotic treatment(A) is more than the control treatment(B).
***Note the difference in the thickness of the lamina propria at the base of the folds.**

After challenge with A. hydrophila

The survival rate of *Sander lucioperca* challenged with *A. hydrophila* after 8 weeks of feeding with different doses of *L. brevis* (10^{8} and 10^{10} CFU mL^{-1}) diets were significantly higher than that of the control (Table 4). The survival rate of *Sander lucioperca* challenged with *A. hydrophila* in A1, A2 and control treatments were 86.6%, 86.6% and 60%, respectively.

Table 4: Survival rate of *Sander lucioperca* challenged with *A. hydrophila* after 8 weeks feeding with different doses of *L. brevis* (0, 10^8, 10^{10} CFU mL^{-1}) diets.

Bacterial dosage (CFU g^{-1} fish)	*L. Brevis* in diet (CFU g^{-1})	Survival rate%						
-	10^{10}	24	48	72	96	120	144	168
-	10^8	100	100	100	100	100	100	100
4.5×10^8	0	100	100	100	100	100	100	100
4.5×10^8	10^{10}	93.3	80	73.3	60	60	60	60
4.5×10^8	10^8	100	93.3	93.3	93.3	86.6	86.6	86.6

Discussion

Probiotic may play a different role and affect human and animals in various ways, such as being an adequate alternative to antibiotics and chemicals, making the intestinal microbial balance, stimulation, effects of non-specific defense system in the host, digestive enzyme activities, increase in disease resistance and improved growth rate (Fuller, 1989; Gatesoupe, 1999; Verschuere *et al.*, 2000; Balcázar *et al.*, 2006; Giri *et al.*, 2011; Eissa and Abou-ElGheit, 2014).

The complement system is a non-specific humoral immune response that plays such an essential role as making the immune system alert when pathogens attack the body and can directly inhibit pathogens (Panigrahi *et al.*, 2005; Faeed *et al.*, 2016). Balcázar *et al.* (2007) studied *Salmo trutta* fed with supplemented *Lactococcus lactis* and *Leuconostoc mesenteroides* with dosages at 10^6 CFU /g for 14-21 days and indicated a significant increase of ACH50. Son *et al.* (2009) studied dietary supplemented *Lactiplantibacillus plantarum* at values of 10^6, 10^8, and 10^{10} CFU /g in grouper *Epinephelus coioides* which demonstrated enhanced ACH50 in the 10^8 CFU /g treatment and significantly higher than that of *L. plantarum* at the dosage of 10^{10} CFU /g as compared to other treatments.

In the present study ACH50 was significantly increased in both dosages of probiotic treatments. Serum immunoglobulins constitute the main part of the humoral immune system. IgM is a basic antibody and is the most important immunoglobulin in fish (Wilson *et al.*, 1995). Nikoskelainen *et al.* (2001) noted the probiotic bacteria (*Lacticaseibacillus rhamnosus*) as a stimulus for immune globulin production. Panigrahi *et al.* (2004) showed that dietary supplemented *L. rhamnosus* JCM1136 (live or dead) enhanced serum immunoglobulin in Rainbow trout (*O. mykiss*) for 20 days, after this period, the immunoglobulin level reduced in all probiotic fed treatments and showed an amount similar to that of the control group. These results confirmed findings of Reyes-Becerril *et al.* (2012) obtained from Pacific red snapper *(Lutjanus peru*). These results indicated as well that the immune system of fish fed with a diet containing *L. brevis* were different from the control group.

Lysozyme is a cationic enzyme and a defense factor against invading microorganisms in vertebrates. Its

function is bactericidal and opsonin (Jollès and Jollès, 1984). Sharifuzzaman and Austin (2009) found that feeding of rainbow trout with probiotic diet caused a significant increase in lysozyme activity over 2, 4 and 6 weeks. Panigrahi *et al.* (2004) reported enhanced serum lysozyme activity in *O. mykiss* fed with the probiotic *L. rhamnosus* JCM1136, but Balcázar *et al.*, (2006) did not record significant differences in Rainbow trout after 14 days of feeding with lactic acid bacteria. Giri *et al.* (2013) noted that *Labeo rohita* fed with a diet supplemented with *L. plantarum* VSG3 (10^8 CFU g^{-1}) during a period of 60 days, indicated higher serum lysozyme activity level, in contrast to *L. plantarum* (10^{10} CFU g^{-1}). Kim and Austin (2006) showed that in *O. mykiss* fed *Carnobacterium maltaromaticum* containing diet (10^7 CFU g^{-1}) showed increased lysozyme activity. Reyes-Becerril *et al.* (2012) recorded dietary supplementation of *Latilactobacillus sakei* (10^6 CFU g^{-1}) with marine silages enriched for pacific red snapper for 6 weeks in which post challenge with *Aeromonas veronii* showed a significant increase in the lysozyme concentration of fish blood. The Function of phagocytosis as part of the innate immune response is necessary for the keep up of the organism. The disfeatured of this activity results in bacterial and fungal infections. In the present study on the lysozyme and phagocytic activity there was no significant difference in probiotic and control groups. The results support the findings on grouper *Epinephelus coioides* (Son *et al.*, 2009), *Oreochromis niloticus* (Eissa and Abou-ElGheit, 2014) and *Labeo rohita* (Giri *et al.*, 2013).

Intestinal microbiota plays an important role in the health and safety of aquatic animals. In the current study, dietary supplemented *L. brevis* at 10^8 and 10^{10} CFU /g over 8 weeks were able to increase the probiotic reacting bacteria in comparison with the total bacteria in the intestine of *Sander lucioperca* after 60 days. After feeding for 60 days the average number of probiotic bacteria in groups fed with *L. brevis* supplemented in 10^{10} and 10^8 (CFU g^{-1}) in the intestine of fish were 75% and 72.7%, respectively. In the control no probiotic bacteria were found in the intestine. Two weeks after stopping feeding with *L. brevis* supplemented at 10^8 and 10^{10} CFU g^{-1}, not only the number of probiotics but also the total bacterial number in the digestive system reduced markedly (Table 2).

Mohammadian *et al.* (2017) showed *L. plantarum* and *L. delbrueckii subsp. bulgaricus* caused significant changes in gut microbiota in *Tor grypus*. Bagheri *et al.* (2008) reported that concentration of probiotic bacteria in the intestinal contents increased as a result of their continual use. As long as probiotic bacteria were in the fish's diet, they can be dominant in the intestinal tract. When probiotic bacteria were removed from the diet, their numbers in the gut were urgently reduced (Table 3).

The intestine is a digestive and absorbing system, but few studies have been conducted in relation to feeding with probiotics and fish intestinal morphology (Mohammadian *et al.*, 2019; El-Saadony *et al*., 2021). The studies were conducted on assessing the effect of *Lactobacillus delbrueckii* (Salinas *et al.*, 2008) and lactic acid bacteria (Ringø *et al.*, 2010) on the intestinal epithelium of the salmonids.

Nakandakare *et al.* (2013) reported that the average length of the proximal, middle and intestinal distal, the total surface of the intestine in the fishes which received probiotic feed were higher than those in the fishes received control feed. An increase in the thickness of the epithelial layer of the middle intestine of tilapia juveniles, and the morphology of the intestinal microvilli improvements after using for fed probiotics were also observed.

The results showed that the intestinal villi in probiotic treatments were more folding and thicker than those in the control group and thus in interactions with the intestinal microflora, intestinal morphology, the immune system and nutrient absorption they may better participate in the health of the fishes, confirming the results obtained by Sweeteman *et al.* (2008). The highest survival rates were found in fish with dietary administration of *L. brevis* at 10^8 and 10^{10} CFU g^{-1} (86.6%). The results showed that *L. brevis* supplementation provides resistance to *A. hydrophila* disease development by simultaneously triggering the non-specific immune defense in *Sunder lucioperca* (Table 4).

This study supported earlier research on rainbow trout (Nikoskelainen *et al.*, 2001; Panigrahi *et al.*, 2004; Balcázar *et al.*, 2007), tilapia (Pirarat *et al.*, 2006; Aly *et al.*, 2008; Ngamkala *et al.*, 2010), African catfish (Al-Dohail *et al.*, 2009), *Labeo rohita* (Giri *et al.*, 2013) and *Sander lucioperca* (Faeed *et al.*, 2016).

Giri *et al.* (2013) reported that feeding of higher dosages of *L. plantarum* VSG3 (10^{10} CFU g^{-1}) did not result in highest post-challenge survival rate, better growth or immune performance. Although the reason is unclear, it may be related to the period of feeding and dosage, aquatic species and probiotic bacteria strain or isolate. Therefore, the dietary delivery of *L. brevis* clearly showed a positive effect on growth performance, food efficiency, non-specific immune system and survival rate of *Sander lucioperca* after challenge with *A. hydrophila*. The probiotic (10^{10} CFU g^{-1}) can be used as an effective bio-control agent and as an immune stimulant in aquaculture.

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