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Changes in Intestinal Microflora and Humoral Immune Response Following Probiotic Administration in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: The aim of this study was to evaluate the effects of probiotic bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* (PTCC 1332) on the humoral immune response of the rainbow trout, *Oncorhynchus mykiss*. The bacterium was administered at 3 different doses, 5×10^5 (LAB5), 5×10^7 (LAB7) and 5×10^9 (LAB9) CFU g^{-1} of feed to the rainbow trout (110 g initial mean weight) for 4 weeks and the feed was changed to un-supplemented diet for 2 weeks after. During the trial, water quality parameters such as temperature, dissolved oxygen, pH, NH_4^+ and NO_2^- was measured. Blood and intestinal samples were taken from the onset of feeding supplemented diets at 10, 20, 30 days after LAB feeding and 2 weeks after feeding withdrawal (45 days). The humoral immune response of the fish was evaluated during the experimental period. During the LAB feeding period, *L. delbrueckii* sp. *bulgaricus* persisted in the fish intestines in the high numbers, but the number of LAB rapidly decreased in the intestines after changing to the un-supplemented diet. In comparison to untreated control fish, the alternative complement activity in the serum was found to be significantly ($p<0.05$) greater in all LAB groups especially in the LAB7 group 20-30 days after feeding. The lysozyme activity of the treatment groups were increased significantly ($p<0.05$) 20 days after feeding and the LAB9 group had higher lysozyme activity than all groups. The total plasma immunoglobulin level was increased significantly ($p<0.05$) in the fish groups that received the *L. delbrueckii* sp. *bulgaricus*, especially in the LAB7 group 10 days after feeding. The results showed that the humoral immune response was enhanced by the using of probiotic bacteria.

Key words: Probiotic, rainbow trout, colonization, complement activity, lyzozyme activity, total immunoglobulin

INTRODUCTION

The increased intensify of aquaculture has led to a high number of disease outbreaks with an increasing range of pathogens. Consequently the extensive use of broad-spectrum antibiotics in aquaculture has led, as in other fields, to antibiotic resistance problems. In order to improving health and welfare in the rearing of these animals, several alternatives including improved husbandry, nutrition, water quality, lower stocking densities, use of vaccines, non-specific immunostimulants (Villami *et al*., 2002; Sakai *et al*., 2001; Peddie *et al*., 2002; Bricknell and Dalmo, 2005) and bacterial probiotics such as Lactic Acid Bacteria (LAB) (Gatesoupe, 1999; Ringo and Gatesoupe, 1998; Vazquez *et al*., 2005; Kim and Austin, 2006) have been proposed.

The term probiotic, a relatively new word means "for life" refers to dietary supplements or foods that contain beneficial, or "good," bacteria, which beneficially affect the host by producing inhibitory compounds, improving the microbial balance, competition for chemicals and adhesion sites, immune modulation and stimulation (Fuller, 1989; Verschuere *et al*., 2000; FAO/WHO, 2001). Indeed, there has already been intensive research on probiotics for use in aquaculture (Kim and Austin, 2006). Although probiotics in human and terrestrial animals are dominantly lactic acid bacteria, many different genera, including *Aeromonas*, *Pseudomonas*, *Bacillus*, *Carnobacterium*, *Clostridia*, *Bifidobacterium*, *Enterococcus*, *Roseobacter* and *Vibrio* have been evaluated as probiotics in fish and shellfish (Irianto *et al*., 2003; Nikoskelainen *et al*., 2003; Planas *et al*., 2006; Song *et al*., 2006).

The use of probiotics as farm animal feed supplements dates back to the 1970s (Farzanfar, 2006). The beneficial affect of the application of certain beneficial bacteria in human, pig, cattle and poultry nutrition has been well documented (Farzanfar, 2006).

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However, the use of such probiotics in aquaculture is a relatively new concept. With interest in treatments with friendly bacterial candidates increasing rapidly in aquaculture, several research projects dealing with the growth and survival of fish larvae, Crustaceans, Oysters and Artemia have been undertaken (Mrquez *et al*., 2005; Farzanfar, 2006).

There are 2 main procedures for evaluating the efficacy of an immunostimulant: *In vivo*, such as protection test against fish pathogens; *in vitro*, such as the measurement of the efficiency of cellular and humoral immune mechanisms. There are many reports that some bacterial compounds act as an immunostimulant in fish and shrimp. Such immunostimulants enhance the defense system of host against pathogens by enhancement of antibody production, lysozyme and complement activity (Taoka *et al*., 2006).

The aim of the current study was to investigate if the probiotic bacteria, *L. delbrueckii* sp. *bulgaricus* (PTCC 1332) prepared for human use (Nikoskelainen *et al*., 2001), can stimulate the humoral immune response of rainbow trout (*Oncorhynchus mykiss*) and to determined its capacity for binding intestinal mucus in these fish.

MATERIALS AND METHODS

Bacterial strain and culture: Lactobacillus delbrueckii sp. *bulgaricus* (PTCC 1332) which was from Persian type culture collection was kindly provided by, institute of biotechnology, Iranian research organization for science and technology in a freeze-dried form. The viability of the freeze dried bacteria, 1.6×10^{11} CFU g^{-1}, was determined by plate counting on MRS agar (de Man, Rogosa and Sharpe; MRS, Merck, Darmstadt). A pure colony was inoculated in 10 mL test tube containing 5 mL MRS broth and incubated at 37 °C for 24 h. Mass cultures were carried out in a shaking incubator (200 rpm at 37 °C) using 250-mL Erlenmeyer flasks with the optimum volumes (150 mL) of MRS broth (Planas *et al*., 2004) for 24 h. After 24 h, cultures were harvested by centrifuging at 2000 ×g for 10 min at 4°C (Eppendorf 5810 R, Germany) and washed twice using sterile Phosphate-Buffered Saline; 0.1 M, pH 7.2 (PBS). The bacterial pellet was re-suspended in sterile PBS and cell density was adjusted to 5×10^5, 5×10^7 and 5×10^9 CFU mL^{-1} (Vine *et al*., 2004).

Preparation of the feed: Commercial pelleted feed (BTA®, Hormozdam Co., Hormozgan, Iran) used as a basis in witch the various concentrations of bacteria were gently sprayed on the feed and slowly mixed part by part in a drum mixer, after that it was air dried in an oven at 45-50°C for 2 h. The amount of *Lactobacillus delbrueckii* sp. *bulgaricus* in feed was determined by plate counting on MRS agar by homogenizing 10 g of feed in 90 mL sterile PBS and spreading appropriate dilutions from 10^{-1}-10^{-10} on MRS plates and the plates were incubated at 37°C for 24 h. Feeds were prepared daily and stored at 4°C until use. Commercial pelleted feed without adding any LAB was used as control diet.

Fish and experimental conditions: Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial farm (Rahmani, Urmia, West Azerbaijan, Iran). The fish were feed with commercial pelleted feed at a rate of 1.53 % of the biomass per day. The fish had neither been vaccinated against any diseases nor exposed to any diseases. The fish were acclimated to laboratory conditions for 3 weeks in 3, 1000-liter-tanks before starting the trial.

After the acclimation period the average weight of the fish were 110 g. The fish were randomly put into 12 tanks (300 L, Poly Vinyl Chloride), comprising 4 groups. Each group was repeated in triplicates and each tank was stocked with 45 fish. The feeding experiment was conducted using a flow-through system. The flow rate of water was approximately 4 L min^{-1}. The water temperature was measured 15±1°C during the whole trial.

One group served as the control and was fed with un-supplemented feed during the entire trial period. The other 3 groups were fed for 4 weeks (1st, 2nd, 3rd and 4th week) with feed containing different amount of Lactic Acid Bacteria (LAB) twice daily. LAB doses in feed were 5×10^5 (LAB5), 5×10^7 (LAB7) and 5×10^9 (LAB9) CFU g^{-1} of feed, for treatment groups 1-3, respectively. After 4 weeks of LAB-feeding, the feed were changed to un-supplemented feed (5th and 6th week).

During the trial, water quality parameters such as temperature, dissolved oxygen, pH (HANNA instruments, USA), NH_4^+ and NO_2^- (kit Visocolor®, Germany) was measured each day.

Microbiological aspects: The microbiological analyses were done at 5 time points: Before the trial and at the end of the weeks 1-6. The fish were starved for 24 h before sampling and were sacrificed with a blow to the head. They opened aseptically and their whole intestines were removed. The intestines were dissected and their contents were collected by carefully scraping using a rubber spatula. Each time 5 fish were sampled and the intestine content of each fish was weighed. One gram of the intestine content was homogenized with 9 mL of sterile PBS and vortexed for 1 min in stomacher. Subsequently, dilution series were prepared from the homogenate in sterile PBS from 10^{-1}-10^{-12} and plated in the MRS agar medium. The plates were incubated anaerobically at 37 °C for 48 h.

The microbial analyses of skin mucus were undertaken at the same time points as described above before the fish were surface disinfected and dissected.

The skin mucus was collected from the whole skin area of 5 fish from each group by scraping using a sterile rubber spatula. The microbial analyses of epidermal mucus were performed as described above for intestinal *Lactobacillus* enumeration.

The presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the tank water was determined 24 h after LAB-feeding. To determine the wash out of the LAB from the tank water, the samples were taken just before feeding on the 5th week of the trial. The samples were analyzed by the plate counting as described above.

Sample collection and preparation: Sampling was scheduled initially, at 10, 20 and 30 days after probiotic feeding and at 45 days (15 days after withdrawing the probiotic diet). At each time point, from one of the triplicate tanks of each treatment, 3 fish were taken randomly each day and, consecutively, all 4 groups were sampled. Thus, a total of nine fish were collected per treatment at the end of each sampling term. Blood was collected from the caudal vein of individual fish after anaesthetization with 200 ppm (200 mg L^{-1}) clove oil (Keene *et al*., 1998; Holloway *et al*., 2004; Cunha and Rosa, 2006). Plasma samples were collected after spinning down the heparinised blood at 1500 ×g for 5 min at 4°C. The whole blood collected using the non-heparinised syringes were left for 1 h at room, allowing to clot in microtubes then they left for 5 h at 4°C. Both the samples were preserved at -80°C prior to analysis. The plasma samples were used for total immunoglobulin analysis and the serum samples for determining lysozyme and alternative complement activity.

Alternative complement activity: The complement activity (alternate pathway) was assayed following the procedure of Yano (1992) based on the hemolysis of Rabbit Red Blood Cells (RaRBC) as described earlier (Amar *et al*., 2000) and briefly mentioned bellow. The rabbit RBC were washed 3 times in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01 M-EGTA-Mg-GVB, pH 7, prepared as described by Yano, 1992) and the cell numbers were adjusted to 2×10^8 cell mL^{-1} in the same buffer. At first, the 100 % lysis value was obtained by exposing 100 μL of the above RaRBC stock to 3.4 mL distilled water. The hemolysate was centrifuged and Optical Density (O.D) of the supernatant was determined at 414 nm by using spectrophotometer against distilled water. Following this, the test sera were diluted (×100) and different volumes ranging from 100-250 μL (total volume was adjusted to 250 μL with the buffer) were allowed to react with 100 μL of RaRBC in small test tubes. This mixture was incubated at 20°C for 90 min with intermittent mixing, following which 3.15 mL of 0.85 % NaCl solution was added and tubes were centrifuged at 1600 ×g for 10 min at 4°C and the O.D of the supernatant was measured as mentioned above. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added on a log-log graph. The volume yielding 50 % hemolysis was used for determining the complement activity of the sample as follows:

ACH50 value (units mL^{-1}) = 1/K × (reciprocal of the serum dilution) × 0.5.

Where, K is the amount of serum (ml) giving 50 % lysis and 0.5 is the correction factor since the assay was performed on half scale of the original method.

Lysozyme activity: The turbidometric assay method, originally described by Parry and modified for micro titer assay by Demers and Bayne (1997) was used based ob the lysis of the lysozyme sensitive Gram positive bacterium *Micrococcus lysodeikticus* (Sigma) and employing hen egg white lysozyme (Merck) as standard. The standard egg lysozyme and undiluted serum sample (25 μL) were placed in triplicate into wells of a 96-well plate, following by 175 μL of the bacterial suspension (75 mg mL^{-1} in 0.1 M-Phosphate buffer with 0.09 % (v v^{-1}) NaCl, pH 5.8). After the plate had been shaken, the decrease in absorbance at 450 nm was recorded for 5 min. lysozyme activities were converted to lysozyme concentrations using hen egg white lysozyme as a standard.

Total immunoglobulin: Plasma total immunoglobulin was measured by the method of Panigrahi *et al.* (2005). Shortly, the plasma sample was diluted 100 times with 0.85% NaCl and the Bradford method was employed for determining the protein content, the Bovine Serum Albumin (BSA) and reagents being sourced from Sigma. On the other hand 100 μL of each plasma sample was mixed with an equal volume of 12% solution of Polyethylene Glycol (PEG, 10,000 MW, Sigma) and incubated for 2 h that helped in bringing down the Ig molecules that were removed upon centrifuging at 5000 ×g at 4°C. The supernatant was diluted 50 times with 0.85% of NaCl and the protein content was determined as mentioned before. The differences between the protein values of the untreated and PEG treated sample corresponds to the total Ig content and is expressed as mg per mL.

Statistical analysis: One-way analysis of variance was used (SPSS, ver. 11.5, Chicago, IL, USA) to determine the significant variation between the treatments.

RESULTS

Physiochemical analysis: Data relating to physiochemical parameters of water at the whole trial have been presented

Table 1: The physiochemical parameters of the tank water in the whole trial

Treatment	Temp/°C	pH	Dissolved oxygen (mg L^{-1})	NH_4^+ (mg L^{-1})	NO_2^- (mg L^{-1})
LAB5*	15.25±0.271	7.64±0.133	8.73±0.771	0.25±0.054	0.129±0.0455
LAB7*	15.25±0.239	7.65±0.129	8.82±1.011	0.25±0.054	0.129±0.0455
LAB9*	15.23±0.206	7.70±0.171	8.64±1.372	0.25±0.054	0.129±0.0455
Control*	15.22±0.199	7.65±0.151	8.89±1.119	0.25±0.054	0.129±0.0455

* Mean±S.D., n= 135

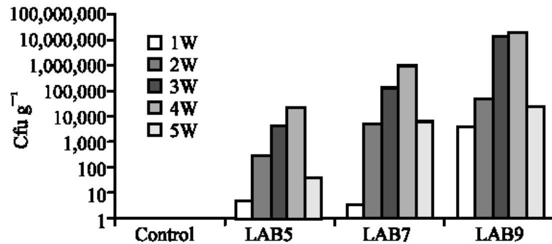

Fig. 1: The number of lactobacilli in the fish intestine in the different experimental groups (n = 5)

in Table 1. There was no significant difference in physiochemical values of tanks, as all values were within the optimum range for rainbow trout juvenile rearing (Kelly, 1998).

Microbiological observation: Before the trial, the fish had no detectable lactic Acid bacteria in the intestine. The number of viable Lactobacilli increased significantly ($p<0.05$) in all LAB groups from below the detection limit levels (<10 CFU g^{-1}) at the end of the 1st week of the trial to 1.9×10^3-1.7×10^6 CFU g^{-1} at the end of the 4th week (Fig. 1). Following the replacement of the LAB-feed with the un-supplemented feed, the number of Lactobacilli decreased rapidly in all LAB groups and no lactobacilli were detected by the end of the 6th week.

The number of *Lactobacillus* in the skin mucus increased which it was in harmony with the Lactobacilli dose in the feed, ranging from 1.1×10^4-2×10^5 CFU mL^{-1} (Table 2). Upon changing to un-supplemented feed, no lactobacilli were detected at the end of 5th week.

The numbers of viable lactobacilli in the tank water samples were related to the dose of LAB in the feed ranging from 89-1.3×10^2 CFU mL^{-1}. After changing the feed to the un-supplemented diet, the numbers of lactobacilli decreased to the bellow detection level after 3 days. Lactobacilli had the highest concentration in the LAB9 group (Table 3).

Complement activity: The alternative pathway complement activity (ACH50) exhibited a trend with respect to days of probiotic feeding. The complement activity increased in all the LAB groups corresponding to the feeding. The rate of increase was highest in the LB7 group followed by LAB9 and LAB5 (Fig. 2). At 10 days, there was significant ($p<0.05$) difference between the

Table 2: The numbers of *Lactobacillus* in the skin mucus at the different time points

Time points (weeks)	Number of *Lactobacillus* (CFU mL^{-1})			
	Control	LAB5	LAB7	LAB9
1 (LAB feeding)	nd	nd	600	1072
2 (LAB feeding)	nd	nd	1.3×10^2	1×10^3
3 (LAB feeding)	nd	84	1×10^3	2.3×10^4
4 (LAB feeding)	nd	135	1.1×10^4	2×10^5
5 (Normal feed)	nd	nd	nd	nd
6 (Normal feed)	nd	nd	nd	nd

nd = Not detected

Table 3: The numbers of viable *Lactobacillus* in the tank water at the different time points

Time points	Number of *Lactobacillus* (CFU mL^{-1})			
	Control	LAB5	LAB7	LAB9
During LAB-feeding period	nd	89	210	1.3×10^2
Days after change of feed to normal	nd	10	10	160
1	nd	10	nd	10
2	nd	nd	nd	10
3	nd	nd	nd	nd
4	nd	nd	nd	nd
5	nd	nd	nd	nd
6	nd	nd	nd	nd

nd = Not detected

LAB7 and all groups. The activities of the LAB groups were significantly ($p<0.05$) higher than the control at 20 and 30 days after probiotic feeding.

At the end of the withdrawal period of 15 days, the elevated values had returned to the same level as in the control group and no significant differences existed among the groups.

Lysozyme activity: The serum lysozyme activity was significantly higher ($p<0.05$) in the all LAB groups at 20, 30 and 45 days compared with the control group (Fig. 3). It is important to point out that the lysozyme activity of the LAB9 was significantly ($p<0.05$) better than all LAB groups and the control group during 20 and 30 days after probiotic feeding and 45 days after changing diet to the un-supplemented feed.

Total immunoglobulin level: The plasma total immunoglobulin level in the LAB groups was found to be higher than that of the control group. The LAB7 group at 10, 20 and 30 days recorded the maximum level of immunoglobulin that was significantly ($p<0.05$) different from the other LAB group and control group (Fig. 4).

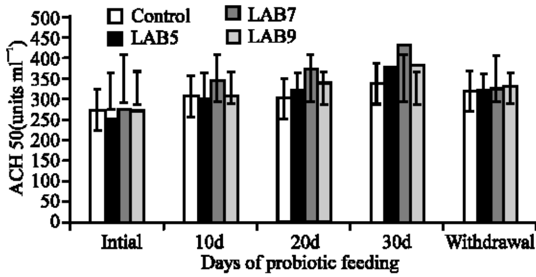

Fig. 2: The mean of the serum alternative complement activity of rainbow trout fed for 3 groups of LAB and the control diet at 10, 20 and 30 days of probiotic feeding and at 15 days after withdrawal (45 days). ($p<0.05$, n = 9)

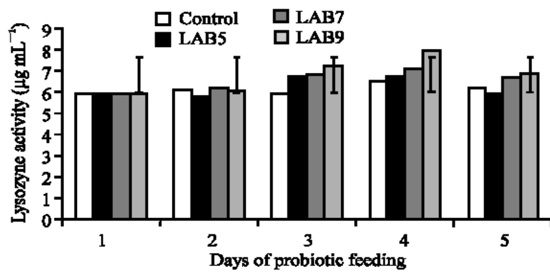

Fig. 3: The mean of the serum lysozyme activity of rainbow trout fed for 3 groups of LAB and the control diet at 10, 20 and 30 days of probiotic feeding and at 15 days after withdrawal (45 days). ($p<0.05$, n = 9)

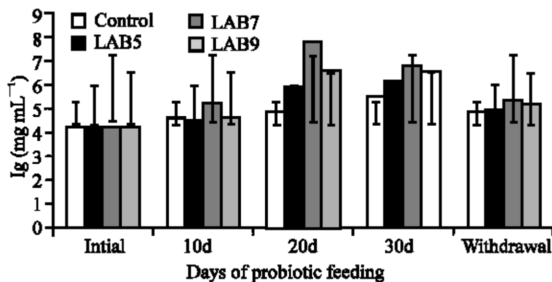

Fig. 4: The mean of the plasma Ig level of rainbow trout fed for 3 groups of LAB and the control diet at 10, 20 and 30 days of probiotic feeding and at 15 days after withdrawal (45 days). ($p>0.05$, n = 9)

DISCUSSION

In the present study, we observed a correction between colonization with probiotic bacteria and non-specific humoral responses such as alternative complement activity and lysozyme activity in rainbow trout. As a prerequisite for bacterial colonization, good adhesion ability to mucosal surface is very important and this has been well demonstrated for the adhesion ability of *L. bulgaricus* to the rainbow trout mucus (Rinkinen *et al*., 2003) and the human mucus (Ouwehand *et* al., 2000; Nikoskelainen *et al*., 2001). The probiotic characteristics of strain ATCC 11842 of *L. bulgaricus* was evaluated earlier by Ouwehand *et al*. (2000), based on the mucus adhesion, mucus penetration and bile resistance (Nikoskelainen *et al*., 2001). After one week of LAB feeding we were able to detect a dose-related amount of *L. bulgaricus* in the intestine and skin mucus which demonstrates *L. bulgaricus* colonization on the intestine and skin mucus which were sampled 24 h after the last feeding. After changing the supplemented feed with *L. bulgaricus* to the un-supplemented feed, the number of *L. bulgaricus* in the intestine and skin mucus and tank water dropped dramatically after one week. This result was in agreement with the observations by Nikoskelainen *et al*. (2003) where they observed a full wash out of probiotic bacteria in 2 days. These results demonstrated that the probiotic supplemented feed must be given to fish continuously in order to retaining the probiotic bacteria in the intestine and skin mucus and tank water. Furthermore, adhesion properties of probiotic bacteria may be a factor to influence the speed of wash out.

The level of alternative complement activity, which is antibody independent, is very high in fish serum compared with mammalian serum (Yano, 1992), suggestion that this pathway is more important in fish than mammals. The complement proteins have multifunctional roles in the defense against micro-organisms, ranging from the opsonisation, lysis and killing of bacteria, to chemotaxis and anaphylaxis (Balcazar *et al*., 2007). In the present work, the activation of alternative complement system pathway after LAB feeding for 20-30 days may be attributed to the supplemented probiotics, confirming the benefit for the non-specific humoral defense system. This result is in agreement with observation by Nikoskelainen *et al*. (2003) and Balcazar *et al*. (2007) that elevated level of complement activity resulted from feeding probiotic to rainbow trout and brown trout (*Salmo trutta*), respectively.

Lysozyme occurs prominently in fish serum and mucus. Although its exact physiological role is not yet understood, there is a general acceptance that lysozyme is involved in the defense against micro-organisms. Lysozyme hydrolyses *N*-acetylmuramic acid and *N*-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls. An increase in the lysozyme concentration in fish blood can be caused by infections or invasion by foreign material (Panigrahi *et al*., 2005).

Balcazar *et al.* (2007) showed significantly higher serum lysozyme activity in brown trout fed with probiotics bacteria at 10^7-10^8 CFU g^{-1} of feed for 2 weeks. In the present study, the groups that had been supplemented with *L. delbrueckii* subsp. *bulgaricus* during the first 10 days exhibited an elevated level of lysozyme activity and it was significantly ($p<0.05$) higher than that the control group during the 20 and 30 days of trial.

Supplementation of LAB in the diet induced greater levels of immunoglobulin in the plasma of rainbow trout (Fig. 4). Immunoglobulins are well recognized to provide disease protection in animals and humans and several studies have demonstrated the effects of LAB on enhancing immunoglobulin. The administration of *L. rhamnosus* at 2.8×10^8 CFU g^{-1} into diet of rainbow trout has been reported to increase the level of immunoglobulin after one week (Nikoskelainen *et al.*, 2003). In our study, we did not examine the specific antibody response, but we observed that probiotic feeding resulted in higher total immunoglobulin level compared to the control group. The clear elevation was record until 30 days, the maximum being for the LAB7 group.

The present research showed that selected probiotic bacteria had an impact on the humoral immune system of fish. Further studies will be needed to identify the new strains of useful probiotic bacteria, detection of their optimal dose and the effect of the combination of different strains in the immune system enhancement.

CONCLUSION

In conclusion, the ability of *L. delbrueckii* subsp. *bulgaricus* to modify the intestinal microflora and stimulate the humoral immune response as potential probiotic bacteria was elucidated in the present study. The *L. delbrueckii* subsp. *bulgaricus* could therefore be a useful alternative to chemotrapeutic treatment to promote fish health, since the high consumption of chemotrapeutic agents in aquaculture can alter the intestinal microflora and induce resistant populations of bacteria, with unpredictable long-term effects on public health.

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