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Administrations of autochthonous probiotics altered juvenile rainbow trout *Oncorhynchus mykiss* health status, growth performance and resistance to *Lactococcus garvieae*, an experimental infection

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ABSTRACT

The present study was tested how *Oncorhynchus mykiss* can respond to dietary supplementations of autochthonous probiotics, including *Lactobacillus delbrukei* subsp. *bulgaricus* and *Lactobacillus acidophilus* and *Citrobacter farmeri* by measuring different parameters. To address that, 300 fish weighing 19.08–32.9 g were fed by probiotics-enriched diets, containing 5×10^7 CFU g^{-1} for 60 days. Our results indicated that probiotics, especially *L. acidophilus* and *L. bulgaricus* are involved in enhancing the growth performance of this species as compared with the control group. Blood profile (Hemoglobin and Hematocrit) showed significant ($P < 0.05$) increases in probiotic fed groups compared with the control. Serum lysozyme and complement activities were higher in probiotic-fed fish while similar changes were not observed in the case of bactericidal activity and Nitroblue Tetrazolium (NBT) reduction. Better colonization of lactic acid bacteria in fish intestine was observed following *L. acidophilus* and *L. bulgaricus* administrations ($P < 0.001$). Digestive enzyme activities of intestine, including amylase, trypsin, lipase and alkaline phosphatase were elevated either significant or insignificant while protease activity did not act the same. All probiotic treatments led to mild or strong ($P < 0.001$) up-regulation of cytokine and growth gene expressions of intestine in comparison with the control group. Higher *in vitro* antagonist activities of *L. acidophilus* and *L. bulgaricus* against the *Lactococcus garvieae* were coincident with *in vivo* challenge test. The relative percentage of survival (RPS) was obtained 63.71 and 51.56 for *L. bulgaricus* and *L. acidophilus*, respectively, which were higher in those treated fish as compared to control fish. Our results may suggest that the probiotics, applied here, can promote growth performance by improving digestive enzyme activity, gut micro flora and growth gene expression. Up-regulation of immune regulatory proteins may increase the non-specific immune responses and bacterial resistance in this species as well.

1. Introduction

As intensive aquaculture is grown exponentially during the last decades, the risk of disease outbreak as a result of increased susceptibility to pathogens will be more prominent; this leads to the consequent increase in the necessity of monitoring fish health and also improvement of the fish immunity system [1,2]. According to the FAO report, the rainbow trout, *Oncorhynchus mykiss* production may cost between 1.20 and 2.0 USD kg^{-1} BW and medicinal treatments costs 50 USD $tones^{-1}$; these expenses are accounted as a major challenge to its sustainability [3]. Besides the costs made by drug treatments in large aquaculture system, bacterial resistance and lack of efficiency (in some cases) constrain their application and also further development of fish industry [4]. Moreover, the total costs caused by economic losses of trout as a result of mortality by disease did not estimate very much, i.e., some local or regional commercial losses for finfish are now available, for example see Refs. [5–7] while the total economic value of the production was estimated to be about 3 billion USD in 2015 [3], making trout-culture activity as an important sector of food production and even more fragile to disease outbreak. However, in contrast to therapeutics, other strategies, including vaccination, immunostimulants and probiotics applications were recommended to control or prevent the incidence of infectious disease of fish [8–10].

Probiotics were made as a commercial additive in aquaculture

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industry due to their beneficial impacts on fish health and growth performance [11]. Over the past few years, probiotic administrations are more desired as a preventive agent against disease outbreak instead of traditional antibiotic therapy [12] owing to detrimental effects of the latter on environments and human and animal health [13]. Given that, advance in probiotic applications to prevent and control pathogenic bacteria in animal farms, particularly in aquaculture are not too out-of-mind [14,15], these biologically-active compounds not only boost the quality of water and sediments in the aquaculture ponds, but can also be applied as food additives to enhance aquatic organisms' immunity and disease resistance [16–19]. Therefore, this might be more effective in the economic point of view, owing to general belief regarding to the cost-effective prospective of disease prevention in the aquaculture industry.

Beyond the much clear role of probiotics on gut microflora balance and feed efficiency, there are other positive effects on immunity, digestive enzymes, growth performance and pathogen resistance which were investigated among different aquatic species [12,19–22]. In *O. mykiss*, several studies was oriented towards the effect(s) of potential probiotics, including *Aeromonas* sp., *Bacillus* sp., *Carnobacterium* sp., *Enterococcus* sp., *Kocuria* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Lactococcus* sp., *Micrococcus* sp., *Pseudomonas* sp., *Vibrio* sp., *Saccharomyces* sp. [15,17,19,23–25]; these were limited to only one or two effects such as immune stimulation, blood profile alteration, cytokine gene expression, survival and pathogen resistance, and growth performance, for more details, see the review by Pérez-Sánchezet al. [26]. By whatever impacts, the underlying mechanism(s) of action for each probiotic bacterium might show a single or combined ability, from colonization in the gut to compete with pathogens to improve the immunity stimulation of host [10,27,28].

O. mykiss is considered as a commercial important fish species for aquaculture in many countries, including Iran, they suffer from some bacterial infections [29–31]. Amongst them, *Lactococcus garvieae*, the causative agent for lactococcosis, accounted as reputational losses in the trout industry [31,32], is estimated to be responsible for 50–80% of losses in trout farms [33]. Reports on increasing prevalence of antibiotic-resistance of this pathogen strain are well available now [34], signifying the application of an alternative option such as probiotics to treat the infected fish. Although the current use of probiotics in diets to improve different aspects of fish health is investigated to some extent, more urgent studies on autochthonous probiotics in fish are needed on this area [16]. Previously, it has been reported that higher adaptive capacity of some lactobacillus species to localize within the intestinal mucus of fish [21]. The autochthonous bacteria like *C. farmeri* are able to provide better nutrient digestibility for the host and digest higher dietary protein/amino acids when compared to the allochthonous bacteria [20]. In this approach, putative bacterial strains could be isolated from an ambient environment, becoming a suitable probiotic candidate for improving fish health [22]. As mentioned above, there are several studies investigating the role of probiotics on different aspects of *O. mykiss* but studies which integrated all responses, from molecular to cell to organism, following different autochthonous probiotics are completely rare. Therefore, this study was designed to evaluate the effects of diet supplemented by some previously isolated bacterial strains on gut microbiota, digestive enzymes activities, immune responses, hematology indices and growth performance of *O. mykiss*. The growth and cytokine gene expressions were also determined in the same fish. The survival of *O. mykiss* fed with above-mentioned probiotics was also examined following experimental infection with *L. garvieae*.

2. Materials and methods

2.1. Bacterial isolation and culturing

The bacterial strains, including *Lactobacillus delbrukei* subsp. *bulgaricus* and *L. acidophilus,* which were previously isolated from gastrointestinal tract of the healthy wild *Tor grypus* (see Mohammadianet al. [22]) were recovered initially. The *Citrobacter farmeri* was also applied as a phytase bacterial strain (isolated from *Cyprinus carpio* intestine). Each gram-positive or -negative strain was grown on either duplicate plates of de Man, Rogosa and Sharpe (MRS) agar (BD, Sparks, MD, USA) media or Trypticase Soy Agar (TSA, Sigma–Aldrich) media and incubated at 30 °C for 24 h and at 37 °C for 48 h, respectively. Their identities were confirmed by gram staining which was followed by complementary biochemical identification tests and also 16S rRNA gene sequencing [22,35]. Briefly, the bacterial DNA was extracted by using commercial kit (SinaClon, Iran). The PCR was carried out on a PC 707 thermal cycler (Thermocycler, Mastercycler Gradient, Eppendorf, Germany), in a final volume of 25 μl. The PCR was performed following 2 min of initial denaturation at 92°C, and 35 cycles of 30 s for denaturation at 95 °C, 45 s for the annealing at 57°C, 45 s for primer extension at 72 °C and 5 min of final extension. Amplification products were analyzed by electrophoresis in 1.5% (w/v) Agarose gel containing Ethidium Bromide (1 mg ml^{-1}). Aliquots 15 μl of PCR product was sequenced (BioEdit, 7.2) and then obtained sequence was blasted (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Probiotic characteristic of isolates

All selected strains were subjected to the thermal-growth experiment. To perform that, three dilutions from each colony (similar to McFarland No. 0.5, OD = 0.132 at 600 nm) were inoculated in MRSB or TSB for 72 h at different temperatures, including 10, 15 and 20 °C. The bacterial growth rate was then recorded at 24, 48 and 72 h through turbidity measured by optical density (spectrophotometer, Jenway, 6400, UK) at 600 nm. Simultaneously, the number of colonies in each corresponding plate was also measured [36].

All probionts were examined *in vitro* to check their antagonist effects against common pathogenic bacteria of trout, including *A. hydrophila* (AH04), *L. garvieae*, *Y. ruckeri*, *S. iniae* (previously isolated). These pathogenic bacteria were isolated form previously-infected *O. mykiss* in our lab. To do this, small amounts of fresh (18 h-old) cultural media of each probiotic strain (colonies were between 20 and 30) were poured onto either MRS or TSA plate, incubated at 37 °C for 48 h. Similarly, of 18 h-old cultural medium of each above-mentioned pathogen (on TSB), a peripheral bacterial culture was streaked in crossly over the test bacteria inoculum in three triplicates. The plates were then incubated at 25 °C for 24 h and finally, the inhibition zone of growth were measured (if any) and accounted as antagonist activity of each probiont [37–39].

2.3. Diet preparation

To prepare food containing probiotics, we applied the recommend methods by Planaset al. [40] and Vine et al. [41]. Each bacterial strain was grown in an aerobic condition (on MRSB in shaking incubator, at 25 °C) and then harvested by centrifuge (at 3000 rpm for 5 min), washed twice with normal saline and re-suspended in the same solution; therefore, the bacteria concentration was adjusted to 3×10^9 CFU ml^{-1} according to McFarland standard grade. Finally, 1.66 ml of each suspension, containing 5×10^7 CFU probiont g^{-1}, was gently added to a 100 g diet. This was according to the previous recommended dose of probiotic in the fish diet [1]. The same amount of normal saline was added to the control diet. The control fish was fed with bacteria-free diet at the same duration. Food preparation was performed under sterile conditions. All prepared diets were packed in sterile propylene containers and stored at 4 °C for weekly use. The viability of each probiont in the diet at the above-mentioned final concentrations was confirmed by suspending 1 g of food in 9 ml sterile PBS and preparing serial diluted food suspensions in MRS media. Counted bacteria in the food were almost same as added probiotic bacteria in their corresponding diets.

2.4. Treatments

Juvenile rainbow trout, *O. mykiss* were purchased form a local supplier. Ten fish were subjected to routine bacteriological test to ensure there is no previous infection among the experimental population. The healthy specimens were kept in the recirculation system for at least two weeks prior to the experiment commenced. Fish were fed daily with standard diet (*ad libitum*) for trout (35.3% crude protein, 7.6% crude fat, 11.9% ash). The water temperature was kept at 16 ± 2 °C, dissolved oxygen: 8.4–9.0 mg l^{-1}, salinity: 0.5–0.7% and pH < 7.0, even during the whole experiment. After acclimation, 300 fish (19.08–32.9 g, 14.3–18.2 cm for TL) were divided into 4 groups with three experimental units for each group (i.e., 12 tanks). Therefore, we stocked 25 fish in each reservoir tank (300 l). To determine the effects of different feeding treatments on parameters studied below, the probiotic-supplemented diets, including *L. delbrukei* subsp. *bulgaricus*, *L. acidophilus* and *C. farmeri* (contained 5×10^7 CFU bacteria ml^{-1}) were conducted for 60 continuous days. A group with no added probiont was served as the control and administrated at the same diet intergradient. The feeding rate (1.5%) and water quality parameter were checked daily and kept similar to the acclimation period. All procedures on animals in this experiment were carried out according to the guide for the care and use of laboratory animals by the National Academy of Sciences (National Institutes of Health publication No. 86–23).

2.5. Growth performance and survival rate

All fish body weights were recorded prior to stocking (control-0d) and at the end of feeding trial (i.e., 60d). To do this, all fish were starved for 24 h before sampling or biometry and each individual fish was then weighed. All growth performance and feed utilization parameters, including body weight gain (BWG%), condition factor (CF, g cm^{-3}), specific growth rate (SGR%), feed conversion ratio (FCR), protein efficiency ratio (PER), daily weight gain (DWG), relative weight gain (RGR) and feed efficiency ratio (FER) were calculated [42]. The survival rate was also evaluated for the whole experimental period.

2.6. Sample collection

At the end of experimental time (= day 60), three starved fish were randomly withdrawn from each tank (totally = 9/treatments) and blood samples were then taken under sterile conditions. Approximately 2 ml of whole blood was collected from the caudal vein. Aliquot of collected blood was transferred into the heparinized microtube and kept on ice for further hematological assay and the residue was allowed to clot at the room temperature (for 60 min) and then subjected to centrifugation (3000 × g, 10 min, 4 °C) to separate serum. The sera were then stored at −80 °C until use. Thereafter, the fish was immediately dissected out under sterile conditions and fresh tissue (posterior intestine) samples were used for bacterial counts and the residues were homogenized (Heidolph instruments, Germany) for intestine enzyme using cold homogenizing buffer containing 100 mM Trise–HCl, 0.1 mM EDTA and 0.1% Triton X-100 pH = 7.8 (1:9 v/w) followed by centrifugation (13,500 × g, 30 min, 4 °C). The supernatant was collected and kept at −80 °C in small portions for further measurements [43]. The same number of fish (n = 9) was also sampled at day 0 (prior to experiment commenced) as the control-0d.

2.7. Intestinal flora

Samples of intestine were analyzed to quantify total and Lactobacilli counts. Nine samples from each treatment were taken just prior to starting the experiment (control-0d) and following 60-day probiotic feeding trial. One gram of the samples was then homogenized by 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH = 7.0) and stirred into 1 min in the stomacher (Heidolph instruments, Germany). Serial dilutions of each were then prepared under the sterile condition and spread on MRS and TSA plates. Following the 48 h incubation at 30 °C, the number of colony on each plate was counted and reported as colony-forming units (CFU) per gram of tissue [19].

2.8. Intestinal enzyme activities

The homogenized intestine sample was subjected to centrifugation as mentioned above. The diluted supernatant was used to determine the activity of total protein content in the intestine according to Bradford [44], using bovine serum albumin as a standard. The α-amylase activity of intestine was also determined using of soluble starch solution (Sigma–Aldrich) as the substrate according to the Bernfeld method, with some modifications as described by Areekijsereeet al. [45]. Amylase specific activity was expressed as μmol maltose produced by mg $protein^{-1}$ min^{-1}. Trypsin activity was measured using Nα-Benzoyl-L-arginine ethyl ester (BAEE) as a substrate in the presence of 0.1 mM HCl at room temperature [46,47]. Lipase activity was assayed based on the measurement of fatty acids release as a result of enzymatic hydrolysis of triglycerides to glycerol in the stabilized emulsion of olive oil, Fluka ™ [48]. Total ALP activity in the homogenized tissue was measured at 410 nm and 37 °C using P-nitrophenyl phosphate as substrate and 2-amino-2-methyl-1-propanol buffer (0.84 mM, pH = 10.3) according to a modified method [49]. Protease activity was measured using casein (Sigma–Aldrich) as the substrate and then the product will react with Folin's reagent [50], with modification). A spectrophotometer (UV-2802S; Unico, Shanghai, China) was used to determine absorbencies of each individual sample and enzyme activities which were recorded as absorbance were changed and then expressed as specific activity (U mg^{-1}protein min^{-1}) [51].

2.9. Immunological-hematological parameters

Hemoglobin concentration (Hb) was measured spectrophotometrically (JENWAY 6400, UK) at 540 nm by the cyanomethemoglobin method [48]. Hematocrit percentage (Hct%) was measured with the microcentrifuge method (Micro-hematocrit centrifuge, 346, UNIPAA, Poland) for 10 min in duplicate. Red blood cells (RBC) and white blood cells (WBC) were counted manually using a Neubauer hemocytometer (Boeco, Germany) after diluting the blood by adding Daice solution. Blood indices, including Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated according to the method of Schalm et al. [52].

Serum lysozyme activity was determined by the turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich). According to the method of Sharifuzzaman and Austin [13] described previously, 0.02 M sodium phosphate buffer (SPB) (pH = 5.8, Sigma–Aldrich) was used. The SPB-free serum sample was applied as a negative control. Results were obtained at 450 nm and expressed in the unit of lysozyme per ml serum when causing a reduction of 0.001 per min at 22 °C. Serum bactericidal activity was determined by incubating (90 min at 25 °C) the mixture of the diluted sera and *L. garvieae* as previously described by Kajitaet al. [53]. The bactericidal activity of serum was expressed as a percentage of the ratio of CFU in the experimental group to those in the control group. Respiratory burst activity (NBT) assay was performed spectrophotometrically by adding N, N-dimethyl formamide (Sigma–Aldrich) to heparinized blood sample and was centrifuged at 3000 × g for 5 min [54]. The activity of the alternative complement pathway in serum (ACH_{50}) was assayed using Agarose containing rabbit red blood cells as a target. The plates were incubated at 4 °Cand hole punched (3 mm in diameter) was filled with 15 μl of serum. After 24 h of incubation at room temperature, the zone of lysis was measured and expressed as an arbitrary unit per ml of serum [55].

2.10. Gene expression

2.10.1. RNA isolation and cDNA synthesis

Total RNA was isolated from 9 intestine tissues for each treatment on day 60, using the Tri Pure isolation reagent according to the manufacturer's procedure (Roche, Canada). The concentration of extracted RNA was calculated at a wavelength of 260 nm using nano-drop spectrophotometry (Eppendorf, Germany). To detect the purity of RNA, the optical density (OD) absorption ratio at 260/280 nm was determined and samples having a ratio more than 1.8 were used for the cDNA synthesis. Possible DNA contamination was removed by the treatment of RNA (1 μg) with DNase I (2 U μl^{-1}) for 1 h at 37 °C(Vivantis, Malaysia). Reverse transcription was carried out with the Rocket Script RT PreMix Kit using 1 μg of RNA and oligo dT based on the manufacturer's protocol (Bioneer Corporation, South Korea).

2.10.2. Real-time quantitative PCR

To evaluate the expression levels of insulin-like growth factor-1(IGF-1), fatty acid transport protein (FATP), gamma-glutamyl transpeptidase (γ-GTP), interleukin-1-β (IL-1β), interleukin-8 (IL-8) and interleukin-10 (IL-10) mRNA in the intestine, real-time PCR was performed using qPCRTM Green Master Kit for SYBR Green I® (Jena Bioscience, Germany) on a Light cycler® Detection System (Roche, USA). Relative expression levels of the all transcripts were compared to β-actin as a housekeeping gene. Specific sets of primers (Bioneer, South Korea) were designed based on *O. mykiss* (Table 1). Reactions were performed in a 12.5 μl mixture containing 6.25 μl qPCRTM Green Master Mix (2X), 0.25 μl of each primer (10 μM), 3 μl (100 ng) cDNA, and 2.75 μl nuclease-free water. The PCR protocol consisted of a 5 min denaturation at 94 °C followed by 45 cycles at 94 °C for 15 s and 60 °C for 30 s. Reactions were performed in triplicate. Two separate reactions without cDNA or with RNA were performed as control groups in parallel with experimental groups. According to the comparative $2^{-\Delta\Delta Ct}$ method, the relative quantification was performed using Light cycler 96® software. Validation of assay to check that the primers for the chβ-actin and chCASQ2 had similar amplification efficiencies was carried out as described previously [21]. All qPCR analysis was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline [56].

2.11. In vivo bacterial challenge and LD_{50} measurement

At the end of the experiment (day 60), fish in each group were challenged with *L. garvieae*. Prior to its use for the challenge, we investigated whether the applied probionts are able to protect the fish against pathogenic bacteria. Therefore, we initially determined the lethal dose (LD) of *L. garvieae* (previously isolated) to *O. mykiss*. To address that, the serial i.p. doses of this bacterium, i.e., 10^5, 10^7–10^9 CFU ml^{-1} were injected to the 45 fish (15/dose), weighing 21.2–30.4 g. The mortality rate was recorded at each dose during 4 continuous days and then subjected to probit analysis (SPSS, 18, USA) to determine the exact LD_{50} of *L. garvieae* to *O. mykiss*.

Table 1
Primers used for detection of target genes.

Gene	Primer (5-'3′)	Product size (bp)
IGF-1	Forward TAACCGTGGTATTGTGGACG Reverse GTTCTTGGCATGTCTGTGTG	135
FATP	Forward TGAAAATGGCAACCTTGTGC Reverse CCATCACGCATTTCGCTATC	104
γ-GTP	Forward ACAATCCTCACGACCAAGTG Reverse GCTGAACCTCTCGCACTAAA	124
IL-1β	Forward TGAGTCTTAGAGGACTGGGTGT Reverse AGGGTGGCAATGATCTCTGT	239
IL-8	Forward TGAGTCTTAGAGGACTGGGTGT Reverse: AGGGTGGCAATGATCTCTGT	136
IL-10	Forward GGATTCTACACCACTTGAAGAGCCC Reverse: GTCGTTGTTGTTCTGTGTTCTGTTGT	157

The L. *garvieae* was prepared for challenging test in following manner; the bacterium was grown for 48 h in the TSA at 37 °C. Bacteria were washed twice with PBS and re-suspended in the same buffer. The concentration of bacteria was adjusted to bacterial LD_{50} (4.7×10^5 CFU ml^{-1}) using a spectrophotometer. The concentration of the bacterial suspension was determined using a bacterial counting chamber to verify the challenge dose. A total of 45 fish (15 from each treatment) were anesthetized with 2-phenoxyethanol (1:10,000) (Shanghai Reagent, China) before injection. All fish in each group were i.p. injected with 0.2 ml of *L. garvieae* suspensions using 1 ml sterile syringe. The control group was also injected with 0.2 ml of *L. garvieae* suspensions. Mortalities were recorded every day during 4 days post-challenge, and all of the dead *O. mykiss* were examined bacteriologically to ensure the presence of the pathogen [25,57].

2.12. Statistical procedure

The normality of data and the homogeneity of variances were analyzed by applying Shapiro-Wilk and Levene tests, respectively. In order to determine the effects of treatments on different parameters, the analysis of variance (ANOVA) was applied. The Multiple comparisons (Bonferroni) were followed if the *p value* on this variable was statistically significant (SigmaPlot, 12). All experimental data were presented as the mean ± SD, and the level of significance for all tests was set at $P < 0.05$. Logarithmic transformations of bacterium doses were used to calculate the LD_{50} at exact time durations using the probit regression analysis (SPSS, 18).

3. Results

3.1. Overall response of fish to the experimental diets

Over the 60 days feeding trial, there was no mortality observed due to the probiotic administrations. The *O. mykiss* fed with different probiotics showed significant increases ($P < 0.001$) in BWG, SGR, and RGR in all treated fish as compared with the control group in which the higher value was observed in *L. acidophilus* group. Furthermore, the maximum FER, DWG and PER were observed when fish were fed with *L. bulgaricus* diet while other probiont groups had also higher value rather than control untreated fish ($P < 0.001$). Although there was no significant difference in CF between *L. bulgaricus* and the control group, two other treatments had lower CF as compared with control ($P < 0.001$). The FCR was significantly ($P < 0.01$) declined following all probiotic-fed groups; in other words, the best FCR was obtained following *L. bulgaricus* administration and to some extent, as a result of *L. acidophilus* (Table 2).

3.2. In vitro antibacterial activity

The antibacterial characteristic of each probiont, including *L. bulgaricus*, *L. acidophilus and C. farmeri* was examined against pathogenic bacteria of trout (i.e., *L. garvieae*, *A. hydrophila*, *Y. ruckeri*, *S. iniae*), by using an *in vitro* test in which the inhibition zone of the crossly culture medium was measured. Table (A) in supplementary data shows the higher inhibition zone for *L. bulgaricus* and *L. acidophilus* rather than *C. farmeri* in most cases ($P < 0.001$).

3.3. Gut flora

Under control conditions, the total viable count of bacteria and lactic acid bacteria (LABs) in fish intestine was elevated ($P < 0.001$) during 60 days of feeding as compared with the control fish at the beginning of experiment (control-0d). At 60 days of probiotics feeding, the *C. farmeri* treatment led to an increase ($P < 0.001$) in total viable

Table 2
The effects of different probiotic diets on growth performance of *O. mykiss* following 60d feeding trial.

	Control-60d	*L. bulgaricus*	*C. farmeri*	*L. acidophilus*	P value
BW_{Int}	22.61 ± 2.08^{b}	22.76 ± 2.07^{b}	23.86 ± 2.57^{b}	31.88 ± 3.79^{a}	< 0.001
BW_{Fin}	49.76 ± 4.00^{c}	70.44 ± 3.96^{b}	71.16 ± 4.84^{b}	125.05 ± 11.47^{a}	< 0.001
BWG (%)	45.24 ± 11.61^{c}	79.48 ± 2.02^{b}	80.50 ± 14.88^{b}	155.27 ± 12.40^{a}	< 0.001
CF (g cm^{-3})	1.04 ± 0.01^{a}	0.90 ± 0.02^{a}	0.45 ± 0.02^{b}	0.33 ± 0.17^{b}	< 0.001
SGR (%)	0.27 ± 0.01^{c}	0.42 ± 0.01^{b}	0.42 ± 0.05^{b}	0.67 ± 0.03^{a}	< 0.001
FCR	2.27 ± 0.13^{a}	0.67 ± 0.01^{d}	1.26 ± 0.14^{b}	0.75 ± 0.03^{c}	< 0.001
PER	1.08 ± 0.12^{c}	2.02 ± 0.04^{a}	0.94 ± 0.10^{c}	1.47 ± 0.09^{b}	< 0.001
DWG	0.19 ± 0.04^{c}	0.42 ± 0.01^{a}	0.19 ± 0.02^{c}	0.31 ± 0.01^{b}	< 0.001
RGR	45.24 ± 11.61^{c}	79.48 ± 2.02^{b}	80.50 ± 14.88^{b}	155.27 ± 12.40^{a}	< 0.001
FER	45.37 ± 9.43^{d}	150.35 ± 3.31^{a}	79.98 ± 8.92^{c}	132.61 ± 8.49^{b}	< 0.001

BW_{Int}: Initial body weight, BW_{Fin}: Final body weight, BWG: Body weight gain, CF: Condition factor, SGR: Specific growth rate, FCR: Feed conversion ratio, PER: Protein efficacy rate, DWG: Daily weight gain, RGR: Relative growth rate and FER: Feed efficiency ratio. All data appear as mean and SD (n = 9). Significance between treatments is indicated by different letters in each row.

Table 3
The effects of different probiotic diets on total viable and lactic acid bacteria (LABs) of *O. mykiss* intestine. MRS plates are related to LABs and TSA plates are for total counts. All data were expressed as mean ± SD for 9 samples. Significance between treatments is indicated by different letters in each row.

	Control-0d	Control-60d	*L. bulgaricus*	*C. farmeri*	*L. acidophilus*	P value
TSA (CFU g-1)	14000 ± 3000^{d}	2800000 ± 150000^{b}	500000 ± 50000^{c}	3500000 ± 86000^{a}	526000 ± 25000^{c}	< 0.001
MRS (CFU g^{-1})	192.67 ± 72.94^{d}	2402.34 ± 100.28^{c}	26300.00 ± 2000.00^{a}		8816.17 ± 175.59^{b}	< 0.001

bacteria of *O. mykiss* intestine ($3.500 \times 10^{6} \pm 0.866 \times 10^{5}$ CFU g^{-1}) whilst while two other lactobacilli treatments led to decreases ($P < 0.001$) in total bacterial counts as compared with control after 60 days (they have been significantly ($P < 0.001$) raised in comparison with control-0d). Total LABs in fish intestine were enhanced ($P < 0.001$) following *L. bulgaricus* and with lower intensity following *L. acidophilus* feedings as compared with control group (Table 3).

3.4. Digestive enzyme activities

There was no significant changes in the activity of all digestive enzymes among control fish intestine (day 0 *vs.* 60). The specific amylase activity of intestine, measured for the control group, was obtained 0.0752 ± 0.0127 U mg protein^{-1} min^{-1}. The lactobacilli treatments led to either a significant (*L. acidophilus*) or an insignificant (*L. bulgaricus*) increase in amylase activity as compared with control-60d ($P < 0.001$) while this parameter had a little reduction (but not significant) in the *C. farmeri* treatment in comparison with the control. Administration of *O. mykiss* by probiotic treatments led to a significant increase ($P < 0.001$) in the activity of trypsin by 2.09-fold, 4.24-fold and 29.5% for *L. bulgaricus*, *C. farmeri* and *L. acidophilus*, respectively. The L. *acidophilus* treatment did not cause any significant change in the lipase activity of intestine while both *L. bulgaricus* (11.156 U mg protein^{-1} min^{-1}) and *C. farmeri* (11.701 U mg protein^{-1} min^{-1}) treatments resulted in higher lipase activity as compared with control-60d ($P < 0.001$). The L. *acidophilus* and *L. bulgaricus* groups showed higher ($P < 0.001$) activity of intestine ALP as compared with control-60d whereas *C. farmeri* did not cause the same change. Among the probiotic treatments, there was only a significant (26.9%, $P < 0.001$) decline in the protease activity of intestine following *C. farmeri* feeding as compared with the control group (Fig. 1).

3.5. Hematology profile and immunity response

The hematological parameters of fish fed with different probiotic treatments are given in Table 4. It can be seen that there were no significant differences between control groups in two different times, on day 0 and day 60. The Hct percentage and Hb concentration were elevated significantly ($P < 0.001$ and $P = 0.033$, respectively) following all probiotic treatments with the highest value observed for *L. bulgaricus*. Although there was significant increase ($P < 0.001$) in the WBC counts as a result of different probiont administrations, the RBC value did not change significantly as compared with control. There were significant increases in MCV and MCH as a result of *L. bulgaricus* and the *C. farmeri* over the course of 60 days but *L. acidophilus* did not increase significantly as compared with the control (Table 4).

By comparing the data of the control on day 0 and 60, we found out all measured immunity responses were not changed. The lysozyme activity of serum was elevated significantly ($P < 0.001$) following all probiotic treatments as compared to the control group. Similarly, the serum ACH_{50} was also observed to be higher in all probiotic treatments ($P < 0.001$). Bactericidal activity of serum was totally reduced ($P < 0.001$) in fish fed by probiotics for 60 days. Probiotic treatments led to either a significant (*C. farmeri* and *L. acidophilus*) or an insignificant (*L. bulgaricus*) decrease in the NBT reduction of serum as compared with the control (Fig. 2).

3.6. Relative mRNA expression of immune-related genes

Relative gene expressions of immune-related genes were studied here in *O. mykiss* intestine. As it can be seen from Fig. 3, all probiotic treatments led to mild or strong ($P < 0.001$) up-regulation in all immunity gene expressions of intestine. Among the probiotic treatments, the highest up-regulated gene expressions, i.e., IGF-1 and FATP was observed in *L. acidophilus* group while two other probiotics did not show any significant difference to each other. The increases in the level of γ-GTP and IL-1B gene expression of intestine were in the following order: *L. acidophilus* > *C. farmeri* > *L. bulgaricus*, they were significantly different ($P < 0.001$). In the contrary, the IL-8 and IL-10 gene expressions did not differ between *L. bulgaricus* and *L. acidophilus* treatments (Fig. 3).

3.7. Challenge and survival rate

When fish were subjected to the challenge test with bacterial infection, the mortality rate was recorded daily and compared as the cumulative mortality rate for 4 days. The cumulative mortality rate of control as a result of *L. garvieae* infection was 73.3%. The relative percentage of survival (RPS) was significantly ($P < 0.05$) higher than that recorded for *L. bulgaricus* (63.71%) or *L. acidophilus* (51.56%). The

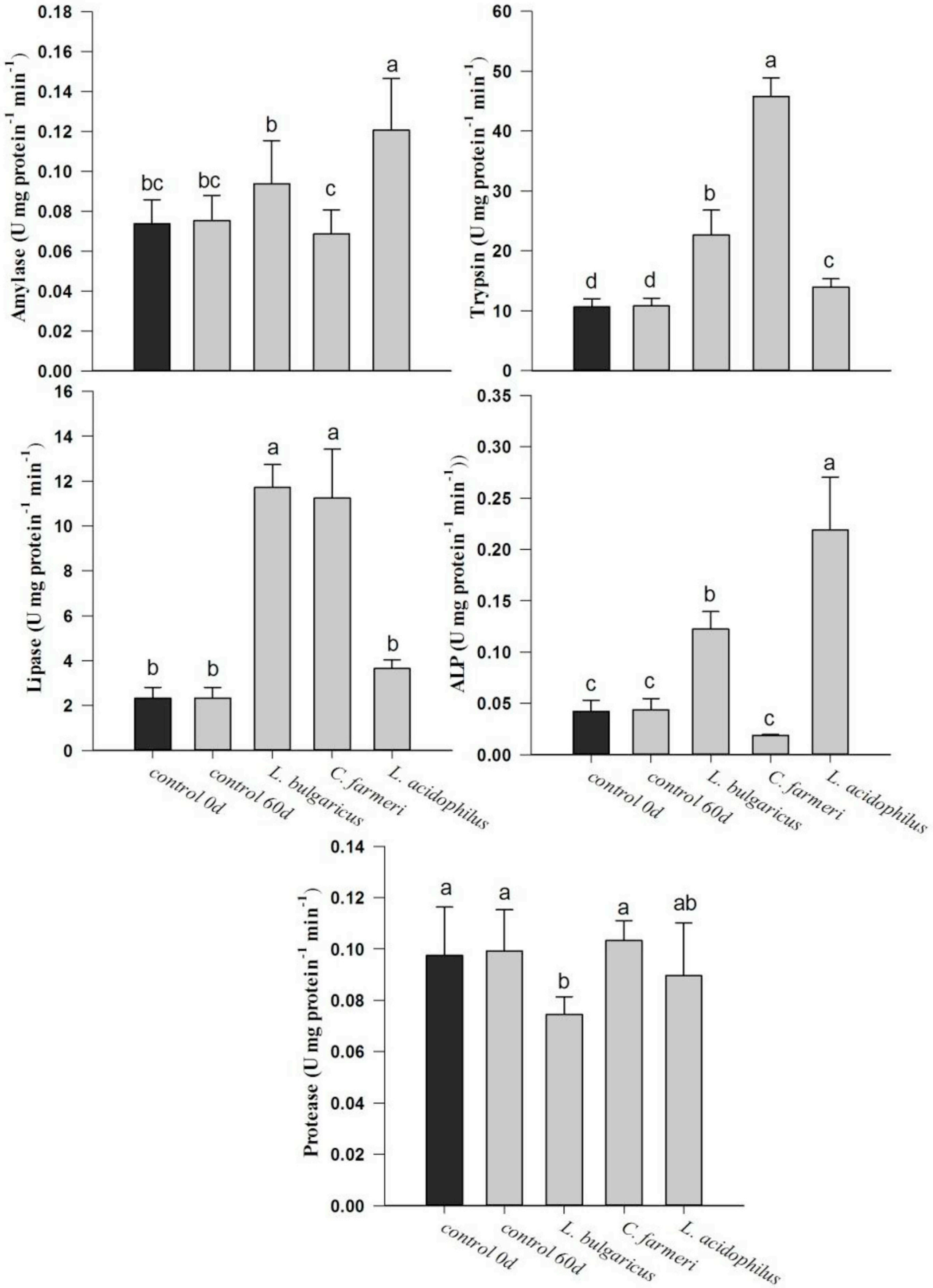

Fig. 1. The effects of 60 days different probiotic diets on digestive enzyme activity of *O. mykiss* intestine. All values were obtained from 9 individual fish (3/replicate) and expressed as mean ± SD. Different alphabetic letters on each bar indicate significant difference ($P < 0.001$).

RPS for *C. farmeri* was 9.14%, indicating a similar mortality rate to the control unfed group. The highest mortality was observed at the day 3 post-infection in all treated groups.

4. Discussion

In this study, we examined the effects of two autochthonous probiotic strains, *L. bulgaricus* and *L. acidophilus*, which were previously isolated from *T. grypus* intestine and compared their effects to those of *C. farmeri* as a bacterial strain which can improve fish gut ability to utilize diets by phytase activity [58] during 60 days feeding trial.

The LABs, *L. bulgaricus* and *L. acidophilus* showed higher (*in vitro*) inhibitory effect against the growth of some pathogenic bacteria of *O. mykiss* on cultural media rather than *C. farmeri*. Although the antagonistic activities of each probiotic against fish pathogens could possibly suggest their role to improve fish health or even prevent and control infectious disease outbreaks [59], their functional ability should not be expected *in vivo* [60,61], needing further examinations. In this study, among *in vitro* tested pathogens, the lowest inhibition zone was related to *L. garvieae* in all probiotic cultural media; this suggests lower

Table 4
Changes in hematological profile of *O. mykiss* following 60d feeding trial with different probiotic diets.

	Hct (%)	Hb (g dl^{-1})	WBC (10^3 cells mm^{-3})	RBC (10^6 cells mm^{-3})	MCV (fl)	MCH (pg)	MCHC (g dl^{-1})
Control-0d	24.67 ± 2.80^c	5.21 ± 0.82^b	2.45 ± 0.50^c	0.99 ± 0.30	271.71 ± 101.68^b	54.95 ± 11.01^c	21.56 ± 5.25
Control-60d	25.00 ± 2.75^c	5.38 ± 0.72^b	2.51 ± 0.32^c	1.01 ± 0.15	251.99 ± 49.80^b	53.41 ± 5.97^c	21.85 ± 4.46
L. bulgaricus	43.00 ± 4.60^a	6.89 ± 1.12^a	11.9 ± 2.09^a	1.00 ± 0.18	436.09 ± 129.77^a	70.33 ± 16.65^{ab}	16.54 ± 3.06
C. farmeri	38.25 ± 1.50^b	7.14 ± 0.74^a	7.02 ± 0.74^b	0.90 ± 0.10	424.86 ± 43.18^a	79.14 ± 8.54^a	18.66 ± 1.50
L. acidophilus	36.80 ± 4.32^b	5.87 ± 1.40^{ab}	6.82 ± 0.82^b	1.02 ± 0.30	387.22 ± 153.16^{ab}	61.02 ± 10.80^{bc}	17.96 ± 6.48
P value	< 0.001	0.033	< 0.001	0.948	0.021	0.008	0.332

All values were obtained from 9 individual fish (3/replicate) and expressed as mean ± SD. Means with the different letter is significantly different.

antagonist activity of these probiotics against the pathogen. Therefore, we conducted a 60d feeding trial with diet supplemented by all above-mentioned probionts and thereafter, probiont-fed fish were challenged with *L. garvieae* to investigate their efficiencies against pathogen. The mortality rate declined in *L. bulgaricus*- and *L. acidophilus*-fed treatments as compared with the control. However, the higher survival rate occurred *in vivo*, proving the stronger bacteriostatic activity of these probiotics and therefore, *in vitro* inhibition experiment can be used as criteria for examining the potency of both strains to enhance *O. mykiss* survival against *L. garvieae* infestation. Although the inhibiting role of other LAB strains (i.e., *L. rhamnosus*) was also examined against *Edwarseilla tarda*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in *O. mykiss* [62,63], the studies on *L. bulgaricus* protective effect was limited to *T. grypus*, in those studies the fish was fed with this probiotic-containing diet for 8 weeks and therefore, resulted in lower mortality rate when infected by *A. hydrophila* [22]. In another study on *Oreochromis niloticus*, the authors found some protective effects against *A. hydrophila* and *S. iniae* following the *L. acidophilus* –supplemented group [62,64]. Therefore, the higher survival rate of probiont-fed fish rather than untreated fish is not very surprising.

All probiotic treatments resulted in a significant increase in BWG percentage but *L. acidophilus* improved the growth performance (especially BWG, SGR, FCR, and RGR) more effectively rather than other probiotics. Similarly, Lara-Floreset al. [65] found that when *O. niloticus* was fed with *L. acidophilus*, the significant increase in the BWG and feed utilization efficiency were observed. Furthermore, in the present study, the promotion of the growth rate following *L. bulgaricus* supplementation was comparable to *L. acidophilus* to some extent but this could not be observed in the case of *C. farmeri*, suggesting possible variability in

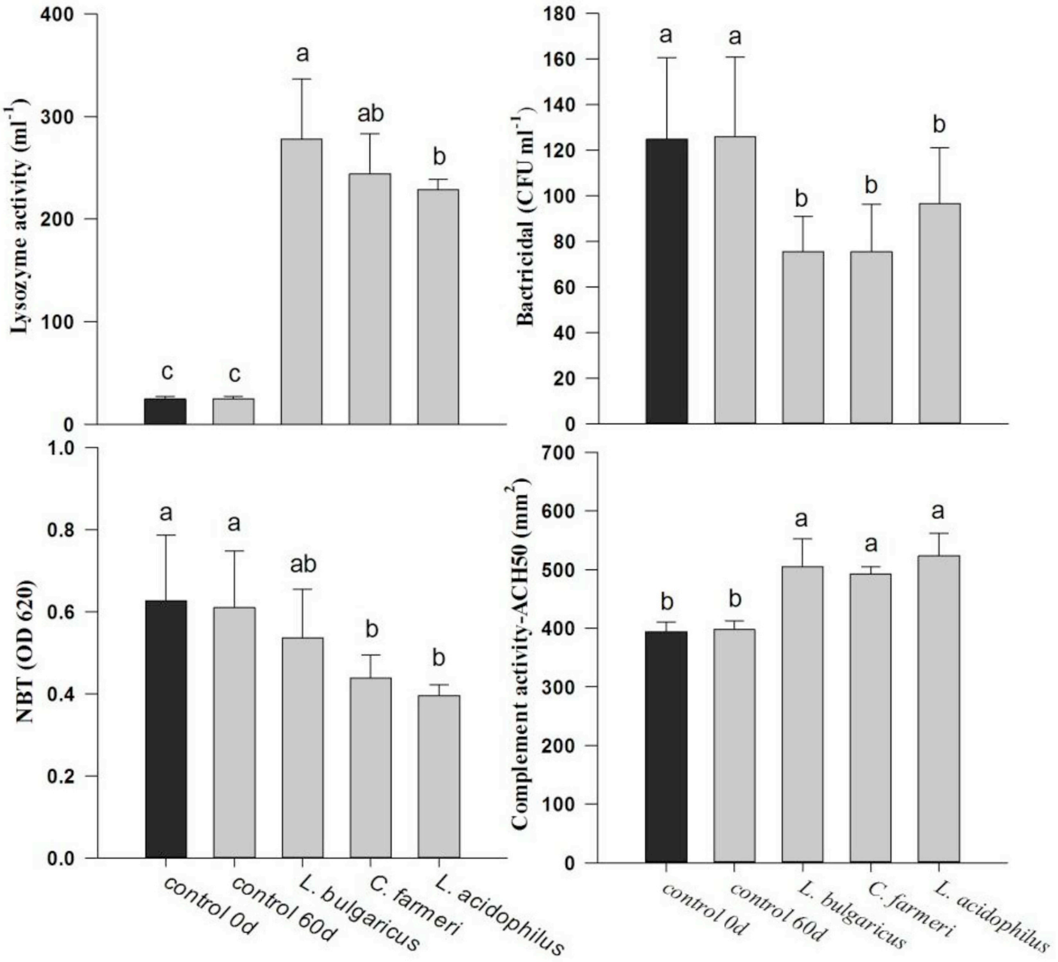

Fig. 2. The effects of 60 days different probiotic diets on serum immune responses of *O. mykiss*. All values were obtained from 9 individual fish (3/replicate) and expressed as mean ± SD. Different alphabetic letters on each bar indicate significant difference (P < 0.001).

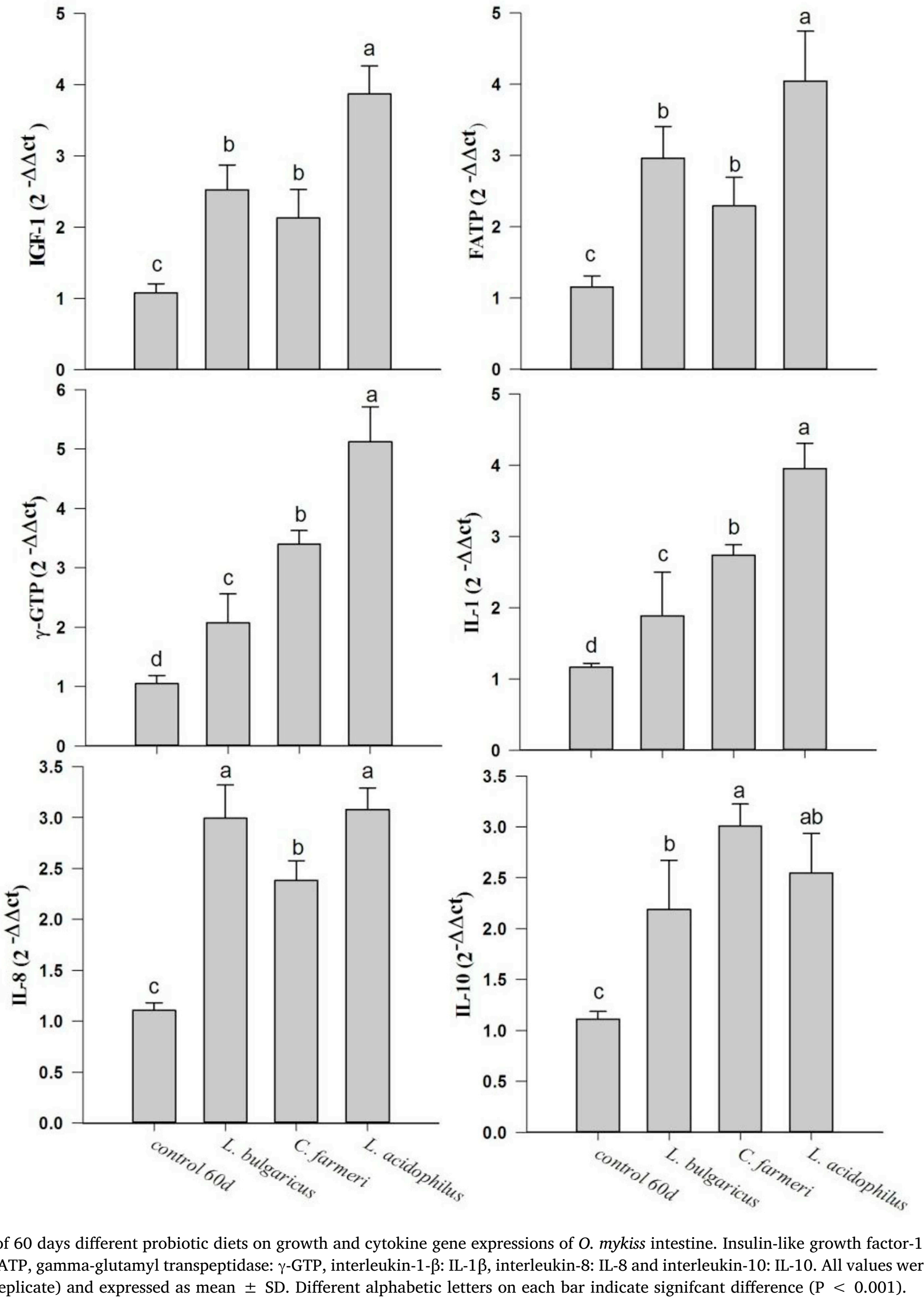

Fig. 3. The effects of 60 days different probiotic diets on growth and cytokine gene expressions of *O. mykiss* intestine. Insulin-like growth factor-1: IGF-1, fatty acid transport protein: FATP, gamma-glutamyl transpeptidase: γ-GTP, interleukin-1-β: IL-1β, interleukin-8: IL-8 and interleukin-10: IL-10. All values were obtained from 9 individual fish (3/replicate) and expressed as mean ± SD. Different alphabetic letters on each bar indicate signifcant difference ($P < 0.001$).

the efficacy of probiotic strain. Another probable reason for this difference can refer to autochthonous characteristic of the latter bacteria species which may provide better nutrient digestibility for the host and digest higher dietary protein/amino acids when compared to the allochthonous bacteria, like *C. farmeri*, this hypothesis was previously suggested for other potential bacterium [20,20]. However, similar improvements of FCR and growth performance were observed in *O. mykiss* fed with *E. faecium*-, *L. plantarum*- and *L. casei*-containing diets [1,19,23], implying the probiotic-fed fish utilized dietary nutrients more efficiently.

The improvement of feed utilization or conversion in probionts supplemented groups could likely be owing to the increase in digestive enzyme activities, induced by probiotics [66,67]. The increase in digestive enzyme activities and therefore, improved feed utilization through the use of probiotics has also been reported in *O. mykiss* as results of other bacterial strains, like *L. casei* and *L. plantarum* or even in other fish species, like *Sparus aurata*, fed with *Lactobacillus* sp [23,66]. Obtained results suggested that higher amylase, trypsin, ALP and to some extent lipase activities, as observed in the *L. bulgaricus*- and *L. acidophilus*-treated fish, might be responsible for better feed utilization and therefore, growth performance. The higher intestine ALP activity indicates the intensity of nutrient absorption in the enterocytes of fish [68], which it can be responsible for more carbohydrate and lipid uptake [69]. Previous studies explained how probiotics (especially *L. bulgaricus*) are able to stimulate this enzyme activity within the brush border of fish enterocyte [20,70].

Moreover, the probiotic effects of bacteria may be affected by other factors, like differences in their survival rates in the gastrointestinal tract and interaction with host microbiota [51]. Considering that, Mohammadian et al. [20] reported higher adaptive capacity of *L. bulgaricus* to localize within the intestinal mucus of *T. grypus* rather than *L. planturum* or *L. casei*. In the present study, the higher LAB colonization was related to *L. bulgaricus* and with less extent to *L. acidophilus* (Table 3). The mechanisms underlying this increase might however be the results of more adhesion ability of probionts to intestinal mucosa [71] or intestinal low pH, induced by LABs [72], inhibiting of the pathogenic microorganisms proliferation [73]. Our results showed that dietary supplementation by probiotics led to either a significant or insignificant increase in the activity of amylase, trypsin and lipase while protease activity of intestine remains steady. Similarly, the higher intestinal lipase and trypsin was accounted as a growth promoter in *O. mykiss* fed by other probiotics [74]. Contrary to our findings, Sun et al. [51] reported higher protease activity of hepatopancrease of *E. coioides* following 60 days feeding of *Lactococcus lactis* or *Enterococcus faecium* but they did not find any significant changes in all digestive enzymes in the intestine. These intestine enzymes are responsible for the digestion of proteins, carbohydrates, cellulose, lipids, and chitin to enhance growth [75]. However, the unchanged intestine protease activity was not in line with the increased PER here, a contradictory finding. However, greater PER in supplemented diets with probiotics supporting higher proteolytic activity of bacteria in those treatments. Nonetheless, such results in this kind of responses cannot be generalized, owing to possible intra-specific difference as well as various experimental procedures [20]. One possible reason for this discrepancy is related to the fact that different probiotics in the diet may affect microbiological and biochemical parameters of the gut independently [17]. Moreover, it seems that some LABs have a deleterious effect on gut morphology [20]. However, this hypothesis should and could be further investigated in detail.

Hematological and biochemical tests are important tools to assess the health status of fish [76]. It has been suggested that hematological indices can reflect the effects of dietary adequacy or food additives [77,78]. Irianto and Austin [79] reported the slight increase (insignificant) in the number of RBC in *O. mykiss* fed with probiotics for 14 days. Similarly, *Micrococcus luteus* administrate fish showed an insignificant increase in the number of RBC in *O. niloticus* [80,83]. Contrary to our finding, administration of *Astronotus ocellatus* by probiotic mixed diet (protexin) indicates a significant increase in all RBC counts following 60 days experiment [81]. The authors also reported higher levels of other blood indices (Hb and Hct), which were consistent to our findings. They conclude that the increase in hematological parameters can be accounted as a result of higher growth rate, observed in those groups to provide more hematopoiesis and consequently, leading to higher oxygen-carrying capacity. However, their conclusion cannot be generalized owing to the fact that changes in hematological indices in response to nutrient manipulation indicate ion regulatory or respiratory disturbances that imply an increase in the energy demand to restore homeostasis instead of other physiological functions and growth. Notwithstanding this hypothesis, our findings showed the same results. However, studies that combine both metabolic capacity and blood parameters in the framework of probiotic supplementation still remain untested.

Besides the beneficial role of probiotics on fish survival and growth rate, there is an increasing evidence that probiotics, especially LABs, can stimulate the cellular and humoral immune responses; therefore, benefit to the disease resistance of fish [28,82]. Considering that, the present study indicated that the WBC count was significantly elevated in fish fed with *L. bulgaricus*. Similarly, but with less extent, *L. acidophilus- and C. farmeri*-fed fish exhibited a significant increase in WBC at the end of feeding trial. Previous studies demonstrated when *O. mykiss* received dietary probiotics, the WBC will be enhanced [79,83]. The increase in WBC count in the probiotic fed fish seems to be the result of induced activities in the anterior part of the head kidney.

The lysozyme activity in the serum of all probiotic-treated fish was enhanced in comparison with the control fish; this suggests that these applied probiotics can likely provoke the immunity system of *O. mykiss*. In agreement with our finding, higher level of serum lysozyme in *O. mykiss* fed with *L. casei*, *L. plantarum*, *L. rhamnosus*, *Carnobacterium maltaromaticum* and *C. divergens* was reported previously [23,24,84,85].

The alternative complement activity is accounted as another indicator of innate immune response in the case of infectious disease [86]. In the present study, the ACH_{50} was elevated probiotic administrations for 60 days. Consistent to our finding, Andani et al. [23] showed that administration of *L. plantarum* for 30 days slightly increases the serum *O. mykiss*. Similarly, Balcázaret al. [17,36] reported the *L. lactis* and *E. faecium* induced the complement pathway of *O. mykiss*. On the other hand, contradictory findings were also reported [19], attributing the possible difference in experimental procedure and even bacterial strains [51].

Obtained results showed that when fish supplemented by LABs, the NBT reduction was lower than the control untreated group. There was also a significant reduction in serum bactericidal activity in fish fed with probiotics than those of the control. NBT reduction, an indicator for respiratory burst activity of immune-related cells, decreased in indigenous probiotic-treated groups compared to the control groups. The findings of respiratory burst activity following the probiotics treatment in fish are often contradictory, while some studies indicated probiotics did not have any significant impact on this non-specific defense mechanism of fish [13,86]. Several *in vitro* and *in vivo* studies showed a significant increase in respiratory burst activity by various probiotics in many aquatic animals including fish. Probiotics such as *B. subtilis* and certain members of LAB group can stimulate respiratory burst activity in fish [87]. This study further confirmed that the probiotics might be responsible for degrading free radicals production by host phagocytic cells.

Results of present study showed that all probionts, applied here, are able to up-regulate the immune-related gene expression of *O. mykiss* intestine. More appropriate indigenous bacterial colonization (microflora alteration) in the intestine may explain this increase in IL-8, IL-1 and IL-10 [36,41]. Results obtained in the present research are in agreement with previous studies on *O. Mykiss*; these studies showed that supplemented diet with probiotics increased the expression of proinflammatory cytokines, including IL-8 [25]. Furthermore, the IL-10 has an anti-inflammatory effect (contrast to IL-1B and IL-8), which can possibly regulate the inflammatory effect of other cytokines following probiotic administration in this species. Therefore, probiotics could enhance fish immunity system and disease resistance by stimulating pro- and anti-inflammatory cytokines.

The IGF-1 has been considered as a somatic growth promoter of fish [88]. Therefore, the higher BWG in *L. acidophilous* treatment might be related to the higher IGF-1 expression in the *O. mykiss* intestine. Similarly, Carnevaliet al. [89] showed that *L. delbrueckii delbrueckii* could stimulate IGF-1 in *Dicentrarchus labrax* larvae. In addition, the *in vitro* effect of IGF-1 on innate immunity of *O. mykiss* was proved by Yada [90]. IGF-1 has been localized in different immune cell types, particularly macrophages and granulocytes, and in supporting cells [91], therefore it can promote the fish immunity system when probiotics up-regulate those genes, this, however, can be comparable to our serum immune findings.

The FATP and γ-GTP are responsible for long chain fatty acid and amino acid absorption to the cells, respectively [92,93], indicating *functions* of *the enterocyte* for food uptake and therefore, metabolic activity and fish growth rate [94]. Although there are limited studies on the expression of these growth indicators in fish, their regulations in other tissues were exhibited to be altered during dietary manipulation [95]. Our findings confirmed the higher expression of those genes when the higher growth rate was occurred.

5. Conclusion

Given that probiotics may improve digestion by stimulating production of digestive enzymes or through other alterations in the gut environment of fish, this means that probiotic can decrease the amount of feed needed for animal growth resulting in reduction of production cost. In the present study, the highest numbers of LABs concomitant of the highest growth rate were found in the treatment. It suggests that the numbers of viable LABs are more efficient than enzyme activity in enhancing the growth parameters of *O. mykiss*. Up-regulation of immune regulatory proteins along with the stimulation of the immunity parameters could provide the enhanced protection against pathogens. Particularly fish fed diet containing LABs revealed higher growth gene expression, which was coincident with higher growth rate in those groups. In the current study, better resistance against *L. garvieae* was observed in fish fed with the *L. bulgaricus* and *L. acidophilus* diets; this can be mainly explained by a significant increase in non-specific immune responses such as lysozyme and alternative complement activities of serum.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.052.

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