Probiotics and Antimicrobial Proteins (2018) 10:616–628
https://doi.org/10.1007/s12602-017-9373-8

Changes in Immunity, Expression of some Immune-Related Genes of Shabot Fish, *Tor grypus*, Following Experimental Infection with *Aeromonas hydrophila*: Effects of Autochthonous Probiotics

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Published online: 27 December 2017

Abstract
In this study, the effects of orally administrated two native probiotics (*Lactobacillus plantarum* and *Lactobacillus delbrueckii* ssp. *bulguricus*), isolated from the intestine of Shabot fish, *Tor grypus*, on some immune response parameters and immune-related genes expression against *Aeromonas hydrophila* in *T. grypus* were evaluated. Four hundred and eighty juveniles weighing 45 ± 10 g were randomly divided into four groups (with three replications) and fed with the experimental diet containing 5×10^7 cfu g^{-1} of *L. plantarum* (G1), *Lactobacillus bulgaricus* (G2), *L*actobacillus *casei* (G3), and a control diet (without probiotics) for 60 continuous days. At the end of the dietary treatments, fish were challenged with a lethal concentration of *A. hydrophila* (5×10^8 CFU ml^{-1}) via intra peritoneal (i.p) injection. Blood and head kidney samples were taken from six fish in each treatment before challenging and 6, 12, 24, and 48 h and also 7 days after injection. The results showed that lysozyme, complement, bactericidal, and NBT activity of probiotic-treated groups were significantly elevated ($P < 0.05$). The IL-8, IL-1β, and TNF-α gene expressions were significantly higher in all probiotic-treated groups ($P < 0.05$). Meanwhile, a high direct correlation was observed between serum immune parameters and expression of immune-related genes ($P < 0.0001$); furthermore, the highest correlation ($R^2 = 0.634$, $P < 0.0001$) was recorded between IL-1β expression and NBT activity. It can be concluded that not only two native probiotics strains stimulate serum immune responses parameters and immune-related gene expression in *T. grypus*, but also a high correlation was seen among these indices. The study suggests that gastrointestinal colonization is preferred for host specificity as the strain previously derived from shabot fish displayed better colonization than the non-indigenous bacteria strain such as *L. casei*. Therefore, these native probiotics bacteria can be accounted as suitable candidates to immune stimulation in fish.

Keywords *Tor grypus* · Probiotic · *Aeromonas hydrophila* · IL-1b · IL-8 · TNF-α

Introduction

In traditional aquaculture systems, bacterial disease outbreaks are typically treated with antibiotics; however, the intensive use of antibiotics is being discouraged due to the emergence of antibiotic resistance. Therefore, it is very essential to find new methods for preventing infectious diseases. In addition, there is a growing interest in the beneficial use of microorganisms to prevent or control pathogenic microorganisms as an alternative to traditional treatments [5, 18]. Probiotics are microorganisms that beneficially affect the host by selectively stimulating beneficial gastrointestinal microbial communities. The use of probiotics have also been suggested to be an alternative method to reduce pathogenic organisms in the gastrointestinal tract of fish due to their antagonistic activity in colonization site on the host's intestine, resulting in prevention and control of diseases [4, 28]. The use of probiotics in aquaculture

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is rather new [39]. Various microorganisms have been evaluated as probiotics in aquatic animals [6, 7, 27]. In an autochthonous approach, many putative probiotic strains have been isolated from their immediate aquatic environment in which fish exist; the historical safety status of these isolated microbial strains may not be similar and the historical safety status of the common counterparts likewise [1]. However, lactic acid bacteria (LABs) are the most commonly used probiotics in aquaculture [2, 36, 59, 61].

Discovery of a vast number of immune relevant genes during the recent years facilitated the study of many immune processes in more detail. Hence, studies on the expression of immune-related genes of teleost, infected with various pathogens, are increasing [12]. Remarkable progress has been achieved in isolating and characterizing cytokine genes of fish in recent years [51, 55]. In addition, the identification and characterization of various immune-regulatory genes have promoted the study of gene expression during disease processes. There are a large number of studies reported the expression of immune-regulatory genes in fish infected with bacterial pathogens [8, 40, 43, 45, 47].

Shabot fish (*Tor grypus*) is one of the most important fish species in southwest Asian countries (i.e., Iran, Iraq, Turkey, and Syria) since it has excellent biological characteristics such as fast growth rate and high resistance against natural stressors, good marketing, and high economic value. These species, which have been artificially propagated, were introduced to cyprinid farms as a new species in cyprinid polyculture systems during the last decade. The lack of knowledge is a major obstacle to the establishment of effective preventive measures against a wide range of infectious agents. With intensification of aquaculture production, problems with opportunistic pathogens, such as *Aeromonas hydrophila*, are increasing. This pathogen is an important common freshwater pathogen of fish in temperate and tropical regions, leading more disease control programs should be developed to reduce economic losses following this kind of disease outbreaks [33]. However, there is no information available on immune responses of *T. grypus* against *A. hydrophila* infection, and it is not clear how systemic immunity is modulated by probiotics after bacterial infection. Because of a lack of information on using *Lactobacillus* probiotic in *Barbus* species fish and few reports on immune response of *Barbus* fish, this study has focused on the effects of two lactic acid bacteria (LABs), *Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, isolated from the intestine of *T. grypus* and a standard *Lactobacillus* strain (*Lactobacillus casei* ATTC1608®) on some non-specific immune parameters and immune-related gene expression as well as their correlation in juvenile *T. grypus* infected with *A. hydrophila*.

Materials and Methods

Bacterial Strains

Lactobacillus plantarum subsp. *Plantarum* and *Lactobacillus delbrukei* subsp. *Bulgaricus* were used in food supplementation. These strains were chosen from over 30 LAB obtained from the intestine of healthy wild *T. grypus*, according to their high in vitro probiotic characteristics [33]. These strains were primarily identified based on colony and cell morphology, gram staining, biochemical characteristics, and 16S rRNA gene sequencing [3, 33]. The positive control strain (*L. casei*, PTCC 1608) was obtained from Pasteur Institute, Tehran, Iran. They were cultured in the DeMan Regosa and Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 30 °C. Bacterial strains were preserved in skim milk at − 80 °C until used. The fish pathogens *A. hydrophila* strain ATCC AH04 was obtained from marine laboratory, Institute of Aquaculture, University of Stirling, Scotland.

Preparation of the Experimental Feed

Three *Lactobacillus* bacterial strains were grown after 48 h in MRS broth in a shaking incubator at 25 °C. After incubation, the cells were harvested by 10 min centrifugation (2000*g*), then bacteria were washed twice with phosphate-buffered saline (PBS, 0.1 M, pH = 7.2) and re-suspended in the same bacterial solution. The concentration of bacteria was adjusted to 3×10^9 CFU ml^{-1} using a spectrophotometer. The proximate analysis of the basal diet according to the AOAC method was 37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal 100 g^{-1} gross energy. The probiotic-enriched diets were prepared by gently spraying the required amount of bacterial suspension on the control diet (16 ml bacterial suspension per kilogram diet) and mixing, bit by bit, in a drum mixer to obtain a final probiotic concentration of 5×10^7 CFU g^{-1}. They were packed in sterile propylene containers and stored at 4 °C for viability studies. This dose was selected based on a previously recommended probiotic concentration in food [31, 33].

Fish and Experimental Design

T. grypus fish weighing 45 ± 10 g (mean ± SD) were obtained from a commercial fish farm, Ahvaz, Khouzestan province, Iran, and then transferred to the fish room of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The fish were acclimatized for 2 weeks in indoor 300 L fiberglass tanks and were fed with a standard diet. All fish were then randomly divided into four treatments, including control, *L. plantarum* (G1), *Lactobacillus bulgaricus* (G2), and *L. casei* PTTC1608 (G3, as a positive control) with three aquaria as replication for each treatment (totally 12 aquaria, including 40 fish/aquarium). The final concentration of each

probiotics was about 5×10^7 CFU g^{-1} of the diet. The aquaria were supplied with filtered water at a temperature of 26 ± 1 °C. The fish were fed with probiotic diets for 60 days (twice a day). Daily feeding rate was about 2% of body weight. The study was carried out in accordance with EC Directive 86/609/EEC for animal experiments.

Resistance to *A. hydrophila* Infection

At the end of the experiment, on day 60, fish in each group were challenged with *A. hydrophila* (strain AH04). AH04 was grown for 24 h in the tryptic soy broth (TSB, FlukaBiochemika) at 37 °C in a shaking incubator at 200 rpm. Bacteria were washed twice with PBS and re-suspended in the same buffer. The concentration of bacteria was adjusted to bacterial LD_{50} (3.7×10^8 CFU ml^{-1}) using a spectrophotometer and the plate counting method. The concentration of the bacterial suspension was determined using a bacterial counting chamber. The fish were anesthetized with eugenol (1:10,000) (Shanghai Reagent, China) before injection. The concentration of 3.7×10^8 CFU ml^{-1} live *A. hydrophila* was injected into fish (LD_{50} resulted in previous study). All fish in each group were intraperitoneally injected with 0.2 ml of *A. hydrophila* suspensions using 1 ml sterile syringe. The control group was divided in two subgroups, one injected with 0.2 ml of *A. hydrophila* suspensions and another group injected intraperitoneally with 0.2 ml PBS. Mortalities were recorded every day during 2-week post-challenge, and all of the dead *T. grypus* were examined bacteriologically to ensure the presence of the pathogen [43].

Sample Collection

On day 60 of the experiment, six fish from each group were bled through the caudal vein after anesthesia with Eugenol (100 ppm) in six sampling intervals: before challenge and also 6, 12, 24, 48 h, and 7 days after challenge of *A. hydrophila*. An aliquot of the blood was heparinized (50 IU ml^{-1}) and the remaining part was used for collecting serum. The collected serum samples were stored at − 80 °C until further analysis for various immune parameters.

Head kidneys from thoroughly bled fish (six samples from each group in each sampling time point) were aseptically dissected and immediately stored in cold PBS, pH =7.2, and stored in 1 ml Trizol at − 80 °C.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from tissues using the TriPure isolation reagent according to the manufacturer's procedure (Roche, Canada). The concentration of extracted RNA was calculated at a wavelength of 260 nm using nanodrop 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).

Real-Time Quantitative RT-PCR

To evaluate the expression levels of IL1-β, IL-8, and TNF-α mRNA in head kidneys, real-time PCR was performed using qPCRTM Green Master Kit for SYBR Green I® (Jena Biosciense, Germany) on a Lightcycler® 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 *Cyprinus carpio* (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 of 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 Lightcycler 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 [44]. All qPCR analysis was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline [11].

Non-specific Immune Responses

A turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich) was employed to determine lysozyme activity in serum [57, 33]. One hundred thirty-five milliliters of *M. lysodeikticus* at a concentration of 0.2 mg ml^{-1} (*w*/*v*) in 0.02 M sodium phosphate buffer (SPB) (pH = 5.8) (Sigma–Aldrich) was added to 15 μl of serum sample. As a negative control, SPB was replaced instead of serum. Results were expressed in the unit of lysozyme ml^{-1} serum. A unit of lysozyme activity was defined as the amount of serum causing a reduction of 0.001 per minute at 450 nm at 22 °C.

Serum bactericidal activity was determined using the previously described method by Kajita et al. [26]. Sera samples were diluted three times with 0.1% gelatin-veronal buffer (GVBC2) (pH = 7.5). *A. hydrophila* was suspended in the same buffer to make a concentration of 10^5 CFU ml^{-1}. The diluted sera and bacteria were mixed at 1:1 ratio and incubated

Table 1 Primer sequences used to quantify gene expression by qPCR

Gene	Forward primer	Reverse primer
TNF-α (GenBank: AJ311800)	5′- GGTGATGGTGTCGAGGAGGAA -3′	5′- TGGAAAGACACCTGGCTGTA -3′
IL1-β (GenBank: AB010701)	5′-ACCAGCTGGATTTGTCAGAAG-3′	5′-GCACATACTGAATTGAACTTTG-3′
IL-8 (GenBank: KC184490)	5′-TGAGTCTTAGAGGACTGGT-3′	5′-ATGTCAGATGTGGCCATATC-3′
β-actin (GenBank: M24113.1)	5′-AGGGTGGCAATGATCTCTGT-3′	5′-GTCTCAAACATGATCTGTGTCAT-3′

for 90 min at 25 °C. The number of viable bacteria was then calculated by counting the colonies from the resulting mixture incubated on TSA plates for 24 h in duplicate. The bactericidal activity of test serum was expressed as a percentage of the ratio of colony forming units in the experimental group to those in the control group.

NBT reduction: Blood (0.1 ml) was placed in micro titer plate wells; an equal amount of 0.2% NBT solution was added in each well and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.1 ml) was added to a glass tube containing 2 ml N, N-dimethyl formamide and centrifuged for 5 min at 3000*g*. The optical density of supernatant was measured in a spectrophotometer (Biophotometer, Eppendorf, Germany) at 620 nm [21, 60].

The complement activity was assayed using Rabbit Red Blood Cells (RaRBC) as a target. RaRBC were prepared in 1.5% agarose (pH = 7.2) containing 0.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$. The RaRBCs in agarose were washed with PBS (0.1 M pH = 7.0) and centrifuged at 750×*g* for 5 min, and the cell concentration is adjusted to 1×10^8 cell ml^{-1}. Agarose containing RaRBC was dispensed into plates, incubated at 4 °C and hole punched (3 mm in diameter). Subsequently, each hole was filled with 15 μl of serum of *T. grypus* and was incubated at room temperature. After 24 h of incubation, the zone of lysis was measured and expressed in AU/ml [9, 34].

$$\textit{Arbitrary unit}\ (\mathrm{AU/ml}) = (\textit{Zone of lyses}/\textit{Volume of the sample loaded}) \times 1000$$

Statistical Analysis

All treatment data were checked for normality (Shapiro-Wilk) and Leven's test for homogeneity of variance. If normality and homogeneity were achieved, general linear model, Univariate ANOVA was used. When interaction effects between different independent parameters were not significant, one-way ANOVA and Tukey's multiple comparison test were used to determine the significant variation ($P < 0.05$) in the immune response between the control and experimental groups. All statistics were performed using SPSS for version 19.0 (SPSS, Chicago, USA). All experimental data were presented as the mean ± SE, and the level of significance for all tests was set at $P < 0.05$. The Pearson correlation test was used to find any correlation between immunological parameters and the respective immune gene expression.

Result

Challenge Test

Analysis of mortalities after challenge-testing with *A. hydrophila* showed significant differences in the resistance to bacterial infection among probiotic-treated and control groups ($P < 0.05$). Although there were no significant differences among the probiotic-treated groups ($P > 0.05$), the *A. hydrophilla* caused the first mortalities at 20 h post-infection (hpi) in all infected groups. The mortality rate of fish fed with diet supplemented with *L. bulgaricus*, *L. plantarum*, and *L. casei* was significantly higher than that of fish fed with the control diet. Mortalities continuously occurred until 48 hpi. The highest mortality rate was recorded at 30 hpi in all groups (Table 2).

Relative mRNA Expression of Immune-Related Genes

The results of expression of immune-related genes (IL-1β, IL-8, and TNF-α) in the head kidneys of *T. grypus* have been shown in Figs. 1–3.

As shown in Fig. 1, in all probiotic-treated groups, a mild upregulation in TNF-α gene expression was observed at 6 and 12 h post *A. hydrophila* injection ($P > 0.005$). A significant upregulation in the level of expression of TNF-α gene in fish fed diet supplemented with *L. plantarom*, *L. bulgaricus*, or *L. casei* was seen in both 24 and 48 h post-injection ($P < 0.05$). A slight increase occurred in the expression of TNF-α gene in all probiotic-treated groups 7 days after challenge ($P = 0.085$).

Moreover, the level of IL-1β gene expression was low in the head kidneys of all groups before infection at day zero. A statistically significant increase in the expression level of IL-1β was observed in all three probiotic-treated groups at 24 and 48 h after infection *A. hydrophila* ($P < 0.05$). The highest upregulation (3.27 ± 1.32) belongs to *L. plantarum* group at 24 h after challenge (Fig. 2).

An insignificant increase in IL-8 gene expression of probiotic-treated groups at 6, 12, and 48 h post-challenge

Table 2 Percent of mortality rate of *T. grypus* after challenge with *A. hydrophila* in experimental groups. Values are shown as means ± standard error. ($n = 3$). Different lowercases superscripts denote significant differences within groups ($P < 0.05$)

Treatments	Control	*L. plantarum*	*L. bulgaricus*	*L. casei*
Number of challenged fish	45	45	45	45
Relative mortality (%)	70 ± 12^{a}	33.3 ± 5^{b}	23.3 ± 7^{c}	36.6 ± 3^{b}
P value		**0.017**		

was reported ($P > 0.05$). IL-8 gene expression in the *plantarum* and *bulgaricus* groups was significantly ($P < 0.05$) higher at 24 h after infection in comparison to the control group (Fig. 3).

There was no significant difference in the levels of IL-1β and IL-8 gene expression among the groups 7 days after challenge ($P > 0.05$).

Non-specific Immune Responses

The results of two-way ANOVA of non-specific immune response parameters of fish fed with a diet containing different probiotic contents have been presented in Table 3.

Serum lysozyme activity gradually increased at 12, 24, and 48 h post-infection with *A. hydrophila* in all probiotic-treated groups (Table 4). Meanwhile, the highest increase in serum lysozyme activity in different sampling intervals was observed in the *L. plantarum*, followed by *L. bulgaricus* and then *L. casei* groups which were statistically higher than the control group ($P < 0.05$).

Serum bactericidal activity increased in all three probiotic-treated groups compared to the control group in sampling intervals except on day 7 post-challenge ($P < 0.05$). The probiotic species did not affect serum bactericidal activity after the challenge with *A. hydrophila* of *T. grypus* in different sampling intervals ($P > 0.05$).

Although the NBT reduction was enhanced in *L. plantarum* and *L. casei* groups 24, 48 h, and 7 days after challenge ($P < 0.05$), no significant change occurred in other sampling intervals and other groups compared to the control ($P > 0.05$).

In comparison to the controls, complement activity was significantly higher in the *L. plantarum* and *L. bulgaricus* supplemented groups in all sampling intervals ($P < 0.05$). The highest complement activity was recorded in the *L. plantarum* supplemented group at 48 h after *A. hydrophila* infection. A graduate increase pattern was observed in the complement activity almost in all probiotic-treated groups (Table 4).

Discussion

The involvement of probiotics in nutrition, disease resistance, and other beneficial activities in fish has been proven beyond any doubt. Among the numerous health benefits attributed to

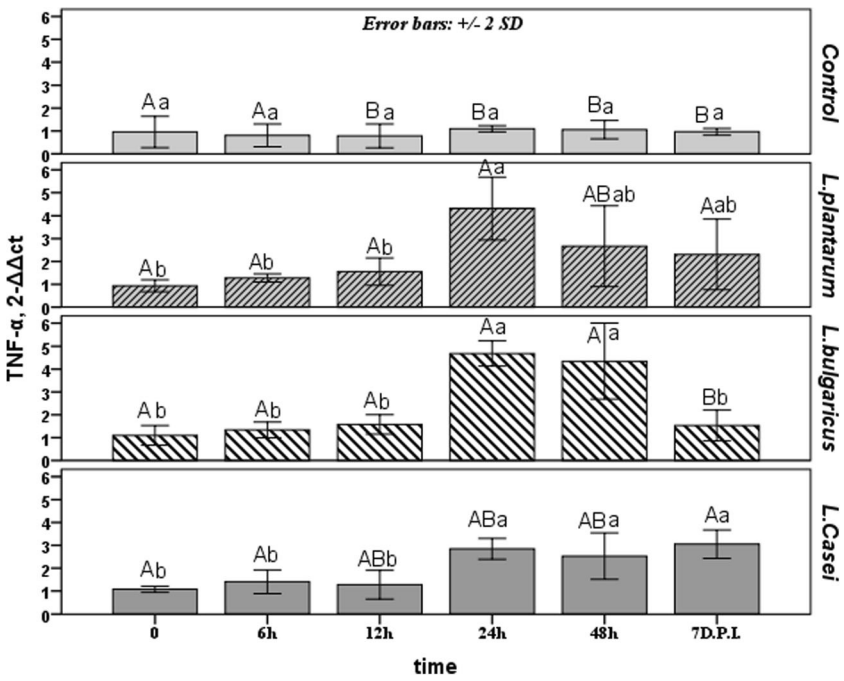

Fig. 1 Gene expression of TNF-α from juvenile *T. grypus*. Fish were fed with diet containing *L. plantarum*, *L. bulgaricus* subsp. *Bulgaricus, L. casei*, and control (diet/diets without probiotics). Samples were taken at different time after challenge with *A. hydrophila*. Values are shown as means ± standard error ($n = 3$). Different lowercase superscript letters denote a significant difference between values in each row ($P < 0.05$). Different capital superscripts denote significant differences within columns ($P < 0.05$)

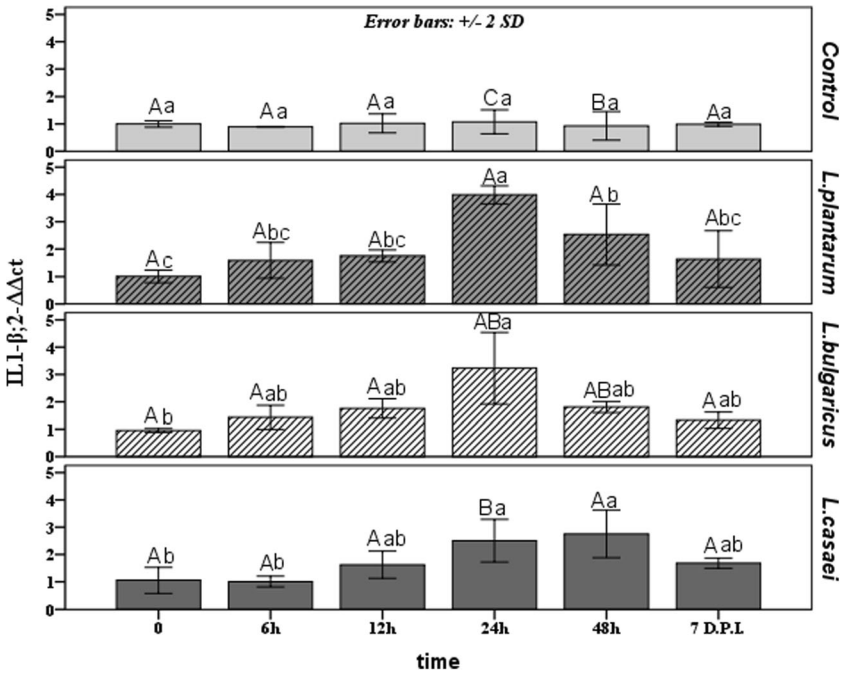

Fig. 2 Gene expression of IL1-β from juvenile *T. grypus*. Fish were fed with diet containing *L. plantarum*, *L. bulgaricus* subsp. *Bulgaricus*, *L. casei*, and control (diet/diets without probiotics). Samples were taken at different time after challenge with *A. hydrophila*. Values are shown as means ± standard error ($n = 3$). Different lowercase superscript letters denote a significant difference between values in each row ($P < 0.05$). Different capital superscript letters denote significant differences within columns ($P < 0.05$)

probiotics, modulation of immune system is one of the most commonly purported benefits of the probiotics and their potency to stimulate the immunity under in vitro and in vivo conditions is noteworthy [20, 35].

Results of present study showed that both native isolated Lactobacilli were not only successful in increasing the expression of immune-related gene in head kidney of *T. grypus*, but they also deeply impacted on selected serum immune parameters and their resistance to bacterial infection of *A. hydrophila*. Besides, a high positive correlation was recorded in expression of immune-related gene in head kidneys and serum non-specific immune response parameters which confirmed the stimulation of primary immune organs in the gene level and humeral immune response in serum. These findings may be very useful and promising for commercial aquaculture and may help to protect the fish against bacterial infection because aquatic animals are continually vulnerable to numerous opportunistic pathogens [22]. Our results were in agreement with results of previous studies, demonstrating that oral administration of probiotics impacted on

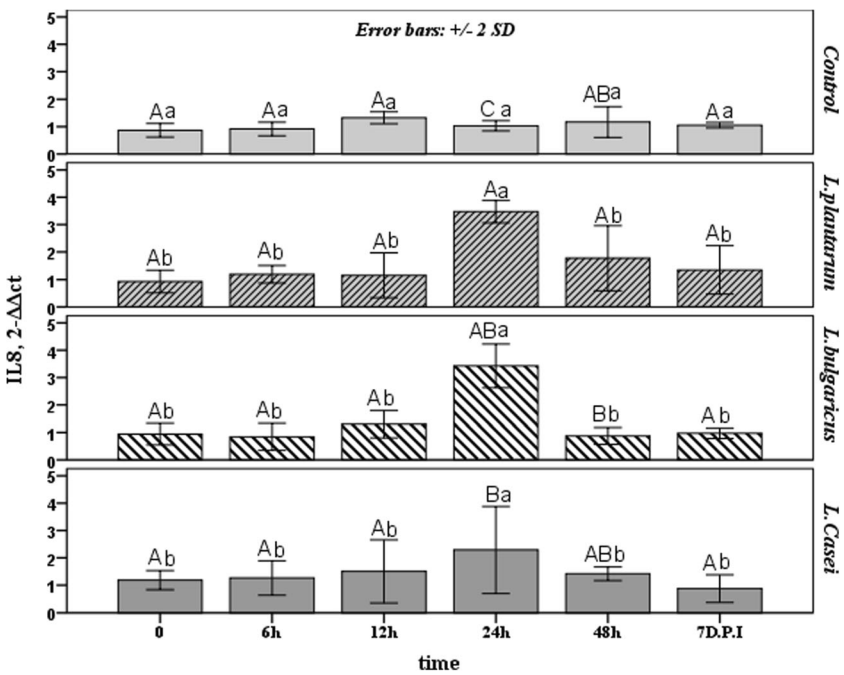

Fig. 3 Gene expression of IL-8 from juvenile *T. grypus*. Fish were fed with diet containing *L. plantarum*, *L. bulgaricus* subsp. *Bulgaricus*, *L. casei*, and control (diet/diets without probiotics). Samples were taken at different time after challenge with *A. hydrophila*. Values are shown as means ± standard error ($n = 3$). Different lowercase superscript letters denote a significant difference between values in each row ($P < 0.05$). Different capital superscript letters denote significant differences within columns ($P < 0.05$)

Table 3 Multivariate analysis of variance (two-way ANOVA) performed for each parameter with its exact *P* value

Parameters	*P* value			
	Complement	Lysozyme	NBT	Bactericidal
Time	< 0.0001	< 0.0001	< 0.0001	<0.001
Treatments	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Time × treatments	0.002	< 0.0001	< 0.0001	0.015

immune regulatory proteins which resulted in enhanced protection against pathogens [36, 62].

Probiotics are sometimes unable to colonize viably and predominantly in the host's intestine and relatively ineffective as transient flora [33] but probably indigenous *Lactobacillus* sp. can colonize in the intestine of fish. At this study, *T grypus* fed with a diet containing the indigenous probiotic bacteria *L. bulgaricus* and *L. plantarum* showed immune stimulation after challenge with *A. hydrophila*, possibly due to the promotion in the immune-related protein level in immune organs [49]. We predict that these bacteria are highly adhesive to GI tract of *T. grypus* because these bacteria are autochthonous. In previous studies, the beneficial effects of probiotic administration against *A. hydrophila* infection in fish have been demonstrated with dietary probiotic supplementations of *Aeromonas sobria* GC2 [10], *Leuconostoc mesenteroides* LFP 196 and *L. plantarum* CLFP 238 [61], *L. plantarum* subsp. *plantarum* CLFP 3, *Lactococcus lactis* subsp. *cremoris* CLFP 25 and *L. mesenteroides* CLFP68 [43], *L. acidophilus* and *L. brevis* [30], and *L. acidophilus* [2]. As far as we are aware, this study is the first report of a high positive correlation between immunological parameters and the respective immune-related gene expression against bacterial infection in fish treated with probiotics.

Cytokines are the most important modulators for initiation and developing a perfect immune response; moreover, the investigation of cytokines functions through studying their expression profile may provide valuable data that can clarify the immunostimulatory mechanisms of probiotics in aquaculture [58]. Probiotic benefits, more specifically the use of autochthonous probiotics as immune enhancers, have not been tested in the *Tor* (*Barbus*) genus fish; however, extensive studies regarding other fish species have been done. In the current study, a series of immune-related genes was used as a primary biomarker to characterize the effects of indigenous probiotics on innate immune responses of *T. grypus* after intraperitoneal injection (ip injection) of *A. hydrophila*. Besides, the correlation between expression of these cytokines' genes and humeral non-specific immune defense parameters was evaluated. Involvement of probiotics in upregulating the gene expression of immune relevant cytokines, the first line of immune defense mechanism, is already recorded [58]. The bacterial mono-association studies on gnotobiotic fish also indicated the upregulation of serum amyloid A1, C-reactive protein, and complement components [48].

IL-1β is a prototypic pro-inflammatory cytokine which accelerates additional inflammatory processes by inducing other inflammatory molecules like TNF and IL-8 [30, 53, 63]. In the present study, a significant upregulation in IL-1β expression was observed in all probiotic-treated groups, but the highest expression of IL-1β was seen in *L. plantarum*-treated group followed by *L. bulgaricus*- and then *L. casaei*-treated groups, respectively ($P < 0.05$). The subsequent decline in these transcripts at day 7 post-infection may be well correlated with the reduction in activity of serum immune defense parameters. Probably, probiotics induced more expression of IL-1β in leukocytes and macrophages of head kidneys [64, 65]. The lymphocytes and macrophages have binding sites for peptidoglycan (a cell wall component of LAB) which can stimulate the secretion of IL-1β. Although no significant differences were observed in all probiotic-treated groups at 6 and 12 h post-injection, upregulation in probiotics groups (after 6 h) is possibly related to IL-1β which is one of the initiators and drivers of cytokines released during inflammatory response in fish to this bacterium [19]. An increased level of IL-1β expression was reported in carp injected with bacterial LPS [23] and in Atlantic cods injected with IPNV after 24 and 48 h, respectively [56]. On the other hand, Zebrafish and *Puntius sarana* injected with *A. hydrophila* showed upregulation in IL1-β expression at 1 h post-challenge and it began to decline to control levels at 6 hpi [16, 50]. Significant upregulation of IL1-β in fish treated with probiotics provides evidence that early inflammatory immune response is more stimulated in probiotic-treated groups infected with *A. hydrophila*, and this stimulation of IL-1β showed a high positive correlation with other serum innate immune defense parameters like lysozyme ($R^2 = 0.572$; $P < 0.0001$), complement ($R^2 = 0.596$; $P < 0.0001$), and NBT reduction ($R^2 = 0.634$; $P < 0.0001$) which seem to play a key role in controlling bacterial pathogenesis (Table 5).

In this study, the highest upregulation in TNFα expression was obtained in *L. bulgaricus*-treated groups and afterward in *L. plantarum* group both at 24 and 48 h post-infection with *A. hydrophila*, compared to the control group, but a gradual reduction in TNFα expression occurred in all groups treated with probiotic after 24 hpi. The pleiotropic cytokine TNFα has been shown to be an important component of the innate immunity and pro-inflammatory response of fish [24]. In this regard, several immune gene expression studies have shown the increased levels of expression of TNFα in relation to different probiotic diets, such as *Canrobacterium maltaromaticum*, *Carnobacterium divergens* [27], *L. plantarum* [43], and *Lactobacillus rhamnosus* [41].

As other researchers suggested, this study also revealed that pro-inflammatory cytokines such as IL-1β, and TNFα

Table 4 Non-specific immune responses of *T. grypus* fed with diet containing *L. plantarum*, *L. bulgaricus*, and *L. casei* in different times following challenge with *A. hydrophila*. Different capital superscripts indicated significant differences between values in each row ($P < 0.05$). Different lowercase letters represented significant differences within the column ($P < 0.05$)

	Treatment	b.i	12 h	24 h	48 h	7 days	*P* value
Serum lysozyme activity (μ/ml)	*Plantarum*	$38.05 \pm 7.1^{A,a}$	$68.4 \pm 6.4^{B,a}$	$70.3 \pm 7.5^{B,a}$	$77.3 \pm 2.6^{B,a}$	$68.3 \pm 9.4^{B,a}$	< 0.001
	Casei	$25.4 \pm 4.8^{A,b}$	$41.2 \pm 7.1^{B,ab}$	$46.3 \pm 5.7^{B,ab}$	$58.4 \pm 3.9^{B,ab}$	$50.4 \pm 6.4^{B,ab}$	< 0.001
	Bulgaricus	$29.97 \pm 3.9^{A,b}$	$41.3 \pm 3.9^{B,ab}$	$52.7 \pm 7.1^{B,ab}$	$73.5 \pm 3.9^{C,a}$	$59.7 \pm 3.9^{B,ab}$	< 0.001
	Challenged control	$26.45 \pm 3.7^{A,b}$	$36.2 \pm 2.9^{AB,b}$	$35.2 \pm 9.4^{AB,b}$	$47.2 \pm 7.1^{B,b}$	$31.2 \pm 3.9^{A,b}$	0.015
	Control	$24.97 \pm 3.9^{A,b}$	$28.4 \pm 3.9^{A,b}$	$31.3 \pm 7.1^{A,b}$	$30.2 \pm 3.9^{A,b}$	$32.6 \pm 3.9^{A,b}$	0.119
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	
Serum bactericidal activity (CFU)	*Plantarum*	$67.66 \pm 7.1^{A,a}$	$71 \pm 4.8^{A,ab}$	$73 \pm 6.3^{A,a}$	$77 \pm 9.4^{A,a}$	$79 \pm 3.4^{A,a}$	0.055
	Casei	$65.4 \pm 2.4^{B,a}$	$61 \pm 6.4^{B,a}$	$68 \pm 2.8^{AB,a}$	$72 \pm 9.8^{AB,a}$	$77 \pm 6.5^{A,a}$	0.004
	Bulgaricus	$62.63 \pm 9.4^{B,a}$	$64 \pm 9.4^{B,a}$	$68 \pm 5.8^{A,a}$	$61 \pm 5.4^{B,a}$	$71 \pm 7.4^{A,a}$	0.001
	Challenged control	$91.75 \pm 5.3^{A,b}$	$99.4 \pm 6.6^{B,b}$	$87 \pm 6.2^{A,b}$	$79 \pm 6.4^{A,a}$	$81 \pm 4.4^{A,a}$	0.007
	Control	$87.6 \pm 6.4^{A,b}$	$83 \pm 6.9^{A,b}$	$82 \pm 7.4^{A,b}$	$89 \pm 2.4^{A,b}$	$85 \pm 6.8^{A,a}$	0.797
P value		< 0.001	< 0.001	0.017	< 0.001	0.306	
NBT reduction (OD)	*Plantarum*	$0.98 \pm 0.13^{A,a}$	$1.12 \pm 0.19^{AB,a}$	$1.5 \pm 0.16^{B,a}$	$1.63 \pm 0.17^{B,a}$	$1.16 \pm 0.26^{AB,a}$	< 0.001
	Casei	$0.97 \pm 0.21^{A,a}$	$1.02 \pm 0.12^{AB,a}$	$1.2 \pm 0.12^{B,a}$	$1.23 \pm 0.23^{B,a}$	$1.08 \pm 0.21^{AB,a}$	0.045
	Bulgaricus	$0.83 \pm 0.17^{A,ab}$	$0.93 \pm 0.21^{AB,a}$	$1.04 \pm 0.17^{B,a}$	$1.09 \pm 0.13^{B,a}$	$0.93 \pm 0.089^{AB,ab}$	0.014
	Challenged control	$0.73 \pm 0.09^{A,b}$	$0.81 \pm 0.14^{A,ab}$	$0.87 \pm 0.13^{A,b}$	$0.84 \pm 0.16^{A,b}$	$0.79 \pm 0.13^{A,b}$	0.194
	Control	$0.69 \pm 0.16^{A,b}$	$0.72 \pm 0.12^{A,b}$	$0.71 \pm 0.08^{A,b}$	$0.66 \pm 0.21^{A,b}$	$0.73 \pm 0.16^{A,b}$	0.715
P value		< 0.001	< 0.001	< 0.001	< 0.001	0.046	
Compelment activity (AU/ml)	*Plantarum*	$3801 \pm 80.4^{A,a}$	$4113 \pm 10.4^{A,a}$	$4733 \pm 74.4^{B,a}$	$4933 \pm 59.4^{B,a}$	$4400 \pm 65.4^{AB,a}$	0.002
	Casei	$2838 \pm 61.4^{A,ab}$	$3113 \pm 65.4^{A,ab}$	$4086 \pm 98.4^{B,ab}$	$4493 \pm 47.2^{B,ab}$	$3400 \pm 57.4^{AB,ab}$	< 0.001
	Bulgaricus	$3491 \pm 42.66^{A,a}$	$3693 \pm 49.8^{A,a}$	$4373 \pm 62.4^{AB,a}$	$4800 \pm 93.4^{B,a}$	$3866 \pm 66.7^{A,a}$	< 0.001
	Challenged control	$2360 \pm 43.8^{A,b}$	$2866 \pm 39.2^{A,b}$	$3466 \pm 68.4^{B,bc}$	$3866 \pm 66.7^{B,bc}$	$2800 \pm 69.4^{A,bc}$	< 0.001
	Control	$2533 \pm 48.4^{A,b}$	$2800 \pm 68.4^{A,b}$	$2600 \pm 67.8^{A,c}$	$2600 \pm 61.4^{A,c}$	$2066 \pm 64.4^{A,c}$	0.307
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Table 5 The correlation between immunological parameters and the respective immune gene expression showing r and its relevant p value

Variable	Lysozyme	Bactericidal	NBT	Complement	IL-8	Il-1β	TNF-α
Lysozyme	1	0.202	0.693	0.692	0.269	0.572	0.630
		0.101	< 0.0001	< 0.0001	0.034	< 0.0001	< 0.0001
Bactericidal		1	0.269	0.366	0.061	0.1927	0.233
			0.0383	0.0013	0.639	0.1239	0.056
NBT			1	0.6718	0.3567	0.634	0.629
				< 0.0001	0.0056	< 0.0001	< 0.0001
Complement				1	0.3914	0.596	0.590
					0.0006	< 0.0001	< 0.0001
IL-8					1	0.7353	0.6485
						< 0.0001	< 0.0001
Il-1β						1	0.723
							< 0.0001
TNF-α							1

could reach to the maximum level in systemic immune tissues in comparison to local tissue where pathogens proliferate [51]. Likewise, IL-1β and TNFα transcripts increased in all injected groups and even in the challenge control group, but they slightly increased in non-challenge control, indicating a trend toward upregulation of inflammatory genes after bacterial infection. On the other hand, the higher expression of cytokines genes in the head kidneys of *T. grypus* fed with indigenous probiotics supplemented food was compared to that of *T. grypus* fed with exogenous probiotic (*L. casei*); therefore, this comparison demonstrated the influence of probiotic bacterial origin on its immunomodulatory effects. The results also highly correlated with the increase of IL-1β and TNFα.

The expression of IL-8 in the head kidneys at days 1 and 2 indicates chemotaxis in immune response at the site of infection. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils, then other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. IL-8 production is stimulated by the expression of IL-1β and TNF-α, so it is not surprising to see the simultaneous expression of these three cytokines. A high positive correlation was seen in the expression of IL-8 with two other immune-related genes, IL-1β ($R^2 = 0.736$; $P < 0.0001$) and TNFα ($R^2 = 0.723$; $P < 0.0001$). It shows that whole immune stimulation occurred in probiotic-treated fish and also all immune-related genes in fish head kidneys upregulated in a similar pattern. Interestingly, food supplementation of *T. grypus*, fed with indigenous probiotic bacteria (*L. bulgaricus* and *L. plantarom*), caused higher expression of IL-8 than that of *T. grypus* fed with exogenous probiotic bacteria (*L. caseai*). A more appropriate indigenous bacterial colonization in the intestine may explain this increase in IL-8 [27]. The alteration of the intestinal microflora may explain this increase in IL-8. However, further tests are needed to verify this hypothesis. As reported in this study, the magnitude of the IL-8 transcriptional response to a range of inflammatory stimuli was found to be less than the IL-1β and TNFα transcriptional response [32]. Results obtained in the present research are in agreement with previous studies in rainbow trout (*Oncorhynchus mykiss*) that showed supplemented diet with probiotics increased the expression of proinflammatory cytokines, including IL-8 [43] in haddock (*Melanogrammus aeglefinus*) tissues injected with bacterial LPS [15]. It is interesting to find that upregulation in the expression of IL-1β, TNF-α, and IL-8 genes was observed at early stages of infection (24 and 48 h post-challenge); whereas, downregulation of this gene expression occurred 7 days after challenge. The immune response of fish against *A. hydrophila* depends on the cooperation of both humeral and cellular immune responses which initially are conducted in primary immunocompetent organs by upregulating the expression of immunomudulator cytokines. Autochthonous probiotics indicated that host immunological responses were responsible for mediating high immune responses of these treatments. According to gene expression assay of study, we found not only significant upregulation of immune relevant genes in probiotic-treated fish, but also faster upregulation, more stable expression, and slower decline of three immune-related genes (IL-1β, TNF-α, and Il-8) in fish receiving indigenous probiotics (*L. plantarum* and *L. bulgaricus*) compared with fish fed with exogenous probiotics. Besides, a high positive correlation was seen within expression of three immune relevant genes or between these genes expression and serum humeral immune response parameters. Despite these in vivo studies, some in vitro studies are necessary to better understand the interaction between these probiotic strains and the immune cell and organs in fish.

A result of this study showed that food supplementation with *L. plantarum* and *L. bulgaricus* were successful in

stimulating of serum immune parameters of *T. grypus*. The serum lysozyme activity of probiotic-treated groups significantly increased ($P < 0.001$) from 12 h until 7 days post-challenge. Besides, a statistically significant correlation between immune relevant genes expression and lysozyme activity was reported in the probiotic-treated groups. Lysozyme, one of the important bactericidal enzymes of innate immunity, is an indispensable tool for fish to fight against infectious agents. Probiotics are found to trigger the lysozyme level in teleost [6]. Probiotics like *L. rhamnosus*, *Carnobacterium maltaromaticum*, *C. divergens* in *O. mykiss* [38], *L. lactis* ssp. *Lactis*, and *L. mesenteroides* in *Salmo trutta* [6] enhanced the lysozyme level; NBT reduction, an indicator for respiratory burst activity of immune-related cells, increased in indigenous probiotic-treated groups compared to the control groups ($P < 0.05$). Superoxide anion production during the respiratory burst of phagocytes can be induced by a variety of phagocyte activating agents [25, 29, 54]. 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 [17, 57]. 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 like *Bacillus subtilis* and certain members of LAB group can stimulate respiratory burst activity in fish [64]. Heat inactivated *L. delbrueckii* and *B. subtilis* under in vitro condition and also enhanced activity of head kidney leucocytes of gilt-head sea bream [52]. This study further confirmed that the probiotics might be responsible for degrading free radicals production by host phagocytic cells. The NBT level before infection and on day 7 post-infection was almost similar. The highest correlation between the immune relevant genes expression and serum immune parameters was recorded between IL 1β expression and NBT reduction ($R^2 = 0.634$, $P < 0.0001$).

There is a general consensus that probiotics from autochthonous sources have a greater chance of competing with resident microbes and of becoming predominant within a short period of intake and persisting in the colonic environment for a period of time after the withdrawal of probiotics [52]. For instance, Carnevali et al. [13] recorded a significant decrease in larva and fry mortalities using *Lactobacillus fructivorans*, isolated from gut of *S. aurata*. Furthermore, it is assumed that the host's immune cells do not naturally react with bacteria on the surfaces and in nature [52]. Complement, a component of the non-specific immune response, may have effector mechanisms such as the direct killing of microorganisms by lysis. Probiotics can enhance natural complement activity of fish; moreover, it was reported that the diet and water along with probiotics treatments could stimulate fish different complement components.

It is also worth noting that non-viable probiotics can stimulate complement components in fish. Choi and Yoon [14] found an increased complement activity in *O. mykiss* from 4th week of feeding with the heat-inactivated probiotics. The hemolytic activity of serum of *T. grypus*, which increased significantly after 6 h challenge with *A. hydrophila*, was estimated by the total complement activity ($P < 0.001$). Complement activation is usually beneficial to the host. However, persistent activation of complement in severe bacterial infection could lead to adverse effects and immunosuppression to the host [42]. However, it returned to the normal level quickly in the survivors 7 days after challenge. The elevation of immune status in the probiotic fish as demonstrated here might indirectly reflect the effect of indigenous probiotics on outcomes in long-term protection against *A. hydrophila* or on a decrease in bacterial load from the circulation or body in the survivors. The higher serum complement activity and the enhanced serum bactericidal power corresponded with the TNF-α and IL-1β expressions in the probiotic supplemented groups.

In the innate humeral response, bactericidal activity of serum played an important role in the immune system. Additional evidence exists in rainbow trout where the complement activity and IL-1β gene expression were enhanced in fish fed with the *Enterococcus faecium* and *L. rhamnosus* supplemented diet [38, 40, 47]. Das et al. [16] suggested that *Bacillus amyloliquefaciens* was a potential probiotic species and could improve the immune response.

Total mortalities following experimental infection with *A. hydrophila* were significantly lower in fish fed with the probiotic supplemented diet (23 to 36% mortality), compared to the control group (70% mortality). There were no remarkable differences in resistance against bacterial infection in different groups treated with probiotics ($P > 0.05$). Our results were similar to the findings of Giri et al. [22], who recorded a lower mortality rate in fish fed on a diet containing probiotics compared with those fed on normal diet. Dietary supplementation of food with defined probiotics may be effective bio-therapeutic or prophylactic means in aquaculture [37, 39]. Our findings suggested that feeding with probiotics for 8 weeks is effective in increasing the resistance against *Aeromonas* infections in fish. In similar work *L. rhamnosus* was successfully used as a feed additive in tilapia [46] and rainbow trout [14] to prevent Edwardsiellosis. The probiotic bacteria can induce inflammatory responses and increase phagocytosis [6, 28]. It can be expected that the increase of immune relevant gene expression and humeral immune parameters in probiotic-treated fish can simultaneously improve resistance against infection of *A. hydrophila*.

Based on the findings of this work, it can be concluded that food supplementation with autochthonous probiotics (*L. plantarum* and *L. bulgaricus*) not only stimulate non-specific immune parameters and resistance to bacterial infection, but also upregulate expression of immune relevant genes with a high positive correlation. Then, probiotic-treated fish possess more effective immunity status against various pathogens especially *A. hydrophila* infection.

Acknowledgements This work was funded by a Grant from Shahid Chamran University of Ahvaz Research Council (Grant No: 27176, 1393.3.2). The authors of this research followed instructions of the university in Iran and performed experiments based on Ethical Guideline of laboratory animals.

Compliance with Ethical Standards All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest The authors declare that they have no conflict of interest.

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