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Research Article

Effect of dietary supplementation of potential probiotic *Lacticaseibacillus casei* on immune-related genes expression, intestinal microbiota and gut histology of zebrafish (*Danio rerio*) during *Aeromonas hydrophila* infection

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Abstract

The present research was conducted on 600 zebrafish in four parallel groups, including two control and two experimental groups. The experimental groups (T1, T2) were fed commercial fish feed, along with probiotic *Lacticaseibacillus casei*, while the other two groups (T3, C) only received commercial feed. At the end of four weeks of the feeding, Fish in the groups T2 and T3 were divided and exposed to *Aeromonas hydrophila* at two concentrations included 1.5×10^8 (T2 and T3) and1.5×10^4 (N2 and N3). In this study, the colonization of Lactobacillus in the gastrointestinal tract in the first and last days of the feeding, weight and length in days 0, 15 and 30, and intestinal histology and expression of interleukin-1β (*IL-1β*) and tumor necrosis factor-α (TNF-α) genes using real-time PCR method on days 0, 15, 30 and 35 were done. Based on the results of the present study, feeding with *L. casei* led to the improved the expression of immune-related genes, enhancement epithelial integrity and goblet cells in the intestine, and weight and length of zebrafish ($p<0.05$). While it had no significant effect on the rate of colonization of Lactobacillus in the gastrointestinal tract ($p>0.05$). This results revealed that probiotic feeding led to the reduction of the mRNA levels of IL-1β and TNF-α genes before exposure to the *A. hydrophila* ($p<0.05$). while, after exposure, there was a significant increase in the expression level of genes. This elevation was significantly higher in T3 and N3 than in T2 and N2 ($p<0.05$). As the results indicated, dietary supplementation of *L. casei* can be effective in enhancement of growth and protection of zebrafish against *A. hydrophila* by improving their mucosal immunity and modulating inflammatory responses. Since zebrafish is an animal model with genetic compatibility with humans and *A. hydrophila* pathogenicity in humans, the results obtained can be generalized to humans.

Keywords: *Lacticaseibacillus casei*, IL-1β, TNF-α , *Aeromonas hydrophila*, Zebrafish, Goblet cells

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Introduction

Fish diseases are among the most important causes of economic loss and a barrier to aquaculture development (Lin *et al.,* 2019; Kazempoor *et al.*, 2021). Today, such factors as climate change, problems with water supplies, fish diseases, and overuse of antibiotics on farms have declined aquaculture (Hossain *et al.,* 2014; Halim *et al.,* 2017; Hossain *et al.,* 2017). One of the most important pathogenic agents accounting for dramatic losses in aquaculture is *Aeromonas hydrophila* (Yi *et al.,* 2019; Alavinezhad *et al.*, 2021).

Antibiotics, which are chemical compounds, are used by fish farmers for the prevention and treatment of fish diseases. Nonetheless, since the excessive use of these compounds has led to the development of antibiotic resistance, alternative compounds, such as probiotics, have gained considerable importance (Yi *et al.,* 2019). Probiotics are microorganisms that contribute to health promotion. Numerous studies have examined the positive effects of probiotics on growth performance, food conversion, microbial flora, intestinal morphology, immune status, and disease resistance in fish (Alavinezhad *et al.,* 2020; Mollanourozi *et al.*, 2021; Kazempour *et al.,* 2022; Loghmani *et al.,* 2022). One of the important effects of probiotics is their impact on the immune system of fish and the modification of cytokine gene expression in their gastrointestinal tract (Wang *et al.,* 2016; Yi *et al.,* 2018). Multiple studies have investigated the effect of probiotics on immune factors, especially interleukin-1β (*IL-1β*) and tumor necrosis factor-α (*TNF-α*), in zebrafish (Wang *et al.,* 2016; Lin *et al.,* 2019; Yi *et al.,* 2019; Zang *et al.,* 2019).

One of the probiotics found to be effective in the improvement of the immune system and cytokines is *Lacticaseibacillus casei* in the Lactobacillaceae family, which is regarded as valuable probiotics (Chiba *et al.,* 2010; Kuebutornye *et al.,* 2020). *Lactobacillus* is one of the most genera in the group of probiotic microorganisms, which was first studied in 1780 (Aryana *et al.,* 2017; Kuebutornye *et al.,* 2019). Accordingly, in 2016, *L. casei* was proposed as a safe microorganism to be used in foods in the qualified presumption of safety framework (EFSA, 2017). In this regard, several studies have reported the positive effects of *L. casei* on improving the immune function of fish, including zebrafish (Chiba *et al*., 2010; Qin *et al.*, 2017; Qin *et al.*, 2018).

Zebrafish is a valuable model for the investigation of the evolution, disorders, and diseases of vertebrates since it has several advantages for biological studies. Some of these merits include the possession of developmental genes, small size (allowing to keep a large number of this type of fish in a relatively small space), external fertilization, transparent embryos (facilitating the direct examination of the development), easy use of drugs and water-soluble chemicals, and potential to be used in mutant studies (Keller *et al.*, 2018). Zebrafish have a well-developed immune system and an emerging

developmental and acquired immunity among the jawed fish; as a result, they are a good model for microbiological studies (Falcinelli *et al.*, 2016). Regarding this, they are good options to be used for the study of adaptive immunity, in addition to the innate immune response, when investigating the effect of a pathogen on host immune function (Kazempour *et al.*, 2022).

The positive features mentioned above enticed the researchers to choose this fish in the present study. There is a paucity of studies on the mechanism of action of probiotics. Therefore, the current research was aimed to assess the effect of *L. casei* as a food additive on the mucosal and cellular immunity of zebrafish by the histopathologic study of their intestinal tissue and changes in the gene expression of *IL-1β* and *TNF-α*. This study was also targeted toward investigating the results of Modify the immune system in reducing the complications of *A. hydrophila* infection in zebrafish.

Materials and methods

Experimental fish and husbandry conditions

This study was performed on 600 zebrafish (average body weight: 0.25±0.05 g and length: 2.5±0.05 cm) obtained from an ornamental fish farm in Tehran, Iran. The fish were transferred to the RAZEF Research Center of Islamic Azad University, the Science and Research Branch, Iran. After 2 weeks of acclimatization to laboratory conditions, the zebrafish were randomly divided into 12 tanks (length×width×height of 28×86×50 cm; three tanks for each treatment; 50 fish in each tank). The tank conditions included the water temperature of 27±1ºC, light/dark cycle of 12:12 h, NO_2-N of <0.05 mg/L, NH_4^+-N of <0.5 mg/L, pH: 7.64±0.04, Conductivity<1,550 µS/cm, range of salinity: 0.35±0.10 ppt and dissolved oxygen level of > 5 mgO/l. Furthermore, 30% of the tank water was replaced on a daily basis.

Bacterial strains and culture conditions

Lacticaseibacillus casei ATCC 393 was prepared from the Microbial Bank of the Iranian Biological Resource Center. *L. casei* was cultivated in MRS broth (De Man, Rogosa, Sharpe) at 37°C for 24 h. The pellets were washed with sterile water three times and then resuspended in sterile water at a final concentration of 0.5 McFarland (1.5×10^8 CFU/ml). In the next stage, they were poured into sterile tubes, and after adding 20% glycerol, they were stored at -20ºC until consumption (Barbour and Pries,1986).

Aeromonas hydrophila was obtained from the bacterial culture collection of the Microbiology Department of the Faculty of Veterinary Medicine, University of Tehran. *Aeromonas hydrophila* was first cultured in soy broth medium and incubated at 37ºC for 18 h under constant shaking. It was then centrifuged at 1,000 g for 5 min, and the pellets were washed twice in phosphate buffer saline (PBS) at a pH of 7.4. Finally, it was resuspended in PBS at a final concentration of 0.5 McFarland (1.5×10^8 CFU/mL) (Patel *et al.*, 2016).

Feed preparation and experimental trials

The basal diet used in the present study was supplied from the BioMar (size 0.5 mm; proximate composition of 35% crude protein, 12% total lipid, 10% ash, 4% moisture; 20.5 kJ/g energy). The probiotic diet was prepared daily by supplementing the suspension of *L. casei* (1.5×10^8 CFU/mL) with commercial feed and Incubate in ice for 15 minutes to absorb bacteria and then mix with 1% skim milk (as a protective agent). in the control diet, a similar volume of sterile PBS was added to the basal diet (Wang *et al.,* 2016). The fish were fed twice a day (i.e., at 9:00 and 16:00) equal to 2% of their body weight for 4 weeks (Yi *et al.,* 2019).

In the present study, the fish were assigned into four groups, including two experimental (i.e., T1 and T2) and two control groups (i.e., T3 and C). In this regard, the fish in the T1 group were fed a probiotic diet, while those in the T2 group were subjected to a probiotic diet and pathogen exposure. With regard to the control group, the T3 group (i.e., positive control group) were fed a basal diet and exposed to the pathogenic agent, whereas the C group (i.e., negative control group) received a basal diet without any pathogen exposure (Fig. 1).

Aeromonas. hydrophila exposure test

At the end of four weeks of the feeding intervention, the remaining fish in the T2 and T3 tanks were divided into two equal groups (15 fishes in each tank) and then transferred to new tanks (T2 to N2 and T3 to N3). Finally, the four groups (i.e., T2, T3, N2, and N3) were exposed to *A. hydrophila* for 5 days. The T2 and T3 groups were exposed to *A. hydrophila* at a concentration of 1.5×10^8 CFU/ml, while N2 and N3 were exposed to *A. hydrophila* at a concentration of 1.5×10^4 CFU/mL (Fig. 1) (Qin *et al.*, 2018).

Lactobacillus colonization in zebrafish digestive tract

To estimate the bacterial colonization in the digestive tract of the zebrafish at the baseline and end of the feeding intervention, five fish were randomly collected from each replicate and then euthanized with clove oil according to Wong *et al.* (2014). In the next stage, the intestines were dissected and then weighed separately. Subsequently, they were homogenized and serial dilutions with distilled saline solution up to 10^{-7} dilutions were prepared (0.9%, w/v). Afterward, 100 µl of different dilutions were spread on MRS agar and then incubated at 30ºC for 24 h. Finally, the grown colonies were counted (Sahandi *et al.,* 2019).

Histopathological examination

At the baseline, 2 and 4 weeks post-feeding, and after exposure, three fish from each group were randomly collected and euthanized (Wong *et al.,* 2014). Subsequently, the fish were cut along the ventral line and placed in buffered formalin 10%. After 24 h, the fixative was replaced with buffered 10% formalin, and the samples were stored. In the next stage, the gastrointestinal tract of the fixed samples was removed, embedded in paraffin wax, and stained with hematoxylin-eosin (HandE).

Following staining, histopathological changes were examined by an optical microscope (Olympus BX51; Olympus, Tokyo, Japan) (Pressley *et al.*, 2005).

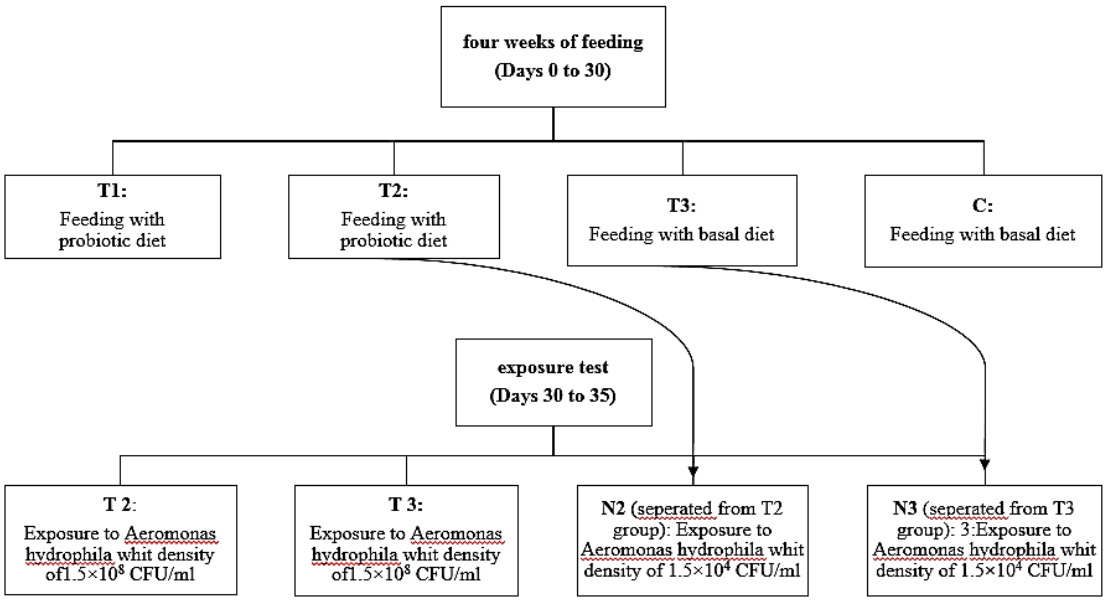

Figure 1: Experimental trials of the study.

Total RNA isolation, cDNA synthesis, and real-time polymerase chain reaction

At the baseline, 2 and 4 weeks post-feeding, and after exposure, three fish from each tank were randomly collected. After euthanization (Wong *et al.*, 2014), they were put in RNAse-free microtubes and stored at -80ºC. Total RNA was isolated from the whole-body samples using Wizol Reagent in accordance with the manufacturer's specifications. The cDNA synthesis was accomplished using the thermo scientific kit. Finally, real-time polymerase chain reaction (PCR) was performed by means of the WizPure kit in accordance with the manufacturer's specifications (Qin *et al.*, 2018). In addition, in order to quantify and normalize the results of gene expression, $2^{-\Delta\Delta C}{}_t$ formula was employed. The primer sequences were synthesized using Gene Runner software designed by SINACOLON (Table 1).

Statistical analysis

Data analysis was performed using SPSS 21 and Microsoft Office Excel 2013 software. The normality of the data was determined using the Kolmogorov-Smirnov test. Significant differences between treatments were considered by One-way ANOVA. Duncan's test was used at a significant level of 0.05 to compare means. The statistical analysis of real-time PCR was performed in the Relative Expression Software (version18.). The data were presented as mean±SD, and a p-value less than 0.05 was considered statistically significant.

Table 1: Primer sequences used in this study.

Primer name	Sequence	Length (amplicon)
Forward primer IL.1 β	CGTGAAGTGAACGTGGTGGA	160 bp
Reverse primer IL.1 β	CTTTCAAGTCGCTGCTTCCG	
Forward primer Beta Actin	CCTTCTTGGGTATGGAATCT	194 bp
Reverse primer Beta Actin	GATCTTGATCTTCATTGTGCTA	
Forward primer TNF-α	AGAGTCGGGCGTTTTTGGAT	130 bp
Reverse primer TNF-α	AGGCCCACAGCCAAAATGGT	

Results

Effect of a probiotic L. casei diet on Lactobacillus colonization in zebrafish digestive tract

Based on the findings, *L. casei* diet had no significant effect on the colonization of *Lactobacilluses* in the digestive tract (Table 2). In this regard, the cultivation and counting results of *Lactobacillus* colonies in the intestines of the zebrafish after 30 days of probiotic feeding revealed no significant difference with those obtained for the basal diet groups (p>0.05).

Table 2: *Lactobacillus* colonization in the digestive tract.

Day/Treatment	T1	T2	T3	C
	(CFU/ml)			
0	16×10^7	20×10^7	270×10^7	300×10^7
30	155×10^7	115×10^7	249×10^7	317×10^7

Effect of L. casei on growth of zebrafish

Based on the results of weight changes in this study, on 15 and 30 days of experiment, the highest weight gain was recorded in the probiotic diet groups (i.e., T1 and T2). On 30th day, this value showed a considerable difference, compared to those obtained at the baseline and day 15 in the same groups and also day 30 in the basic diet groups (i.e., T3 and C; p<0.05). In addition, the highest mean weight was observed after 15 days of probiotic feeding. However, it did not show a significant difference, compared to the values estimated at the baseline and 15 days post-intervention in the control groups (p>0.05). The basal diet after 30 days did not have a significant effect on zebrafish weight gain (approximately 0.02 g per month). On the other hand, the use of a probiotic diet significantly increased fish weight gain, especially in the 30th day (p<0.05). Regarding the total length of the fish, the highest amount was observed in probiotic diet groups (T1 and T2) after 30 days of the experiment. This variable was significantly different in the experimental groups at the mentioned time point, compared to those at the baseline and day 15 post-intervention and also the 30th day of basal diet groups (T3, C) (p<0.05; Table 3).

Table 3: Weight (g) and total length (cm) in zebrafish after 0, 15 and 30 days of the experiment. Values are expressed as the mean±SD.

Treatments	Weight (g)			Total length (cm)		
	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30
Basal diet	0.13±0.00 ^{aA}	0.13±0.00 ^{aB}	0.15±0.15 ^{aC}	2.34±0.10 ^{aA}	2.5±0.12 ^{aA}	3.72±0.15 ^{bB}
Probiotic diet	0.14±0.00 ^{aA}	0.19±0.00 ^{bA}	0.28±0.01 ^{cA}	2.34±0.21 ^{aA}	2.68±0.10 ^{aA}	4.24±0.20 ^{bA}

Different uppercase letters in each row indicate significant differences between treatments; Different lowercase letters in each column indicate a significant difference between different days in treatment.

Probiotic effect of L. casei on histopathology of zebrafish intestine

Based on the obtained micrographs of the intestine, the villi length in the T1, T2, T3 and control groups was same at the baseline. The histopathological evaluation of the intestine in the T1 and T2 groups on day 15 revealed that the mentioned groups had longer intestinal villi than the T3 and control groups. In addition, the intestinal structure all groups on day 30 of the intervention was intact, with the intestinal villi being still significantly longer in the T1 andT2 groups than in the T3 and control group (p<0.05; Table 4).

Table 4: The hight of villi in different experimental groups on 0, 15 and 30 days (Mean±SD, µm).

Days	T1	T2	T3	C
0	59±1.7	60.3±2.5	59.6±2.5	59±2.6
15	75.3±3.2**	74.0±3.6**	65.3±1.1	63.3±1.5
30	78.6±1.5***	76.3±2.0**	68.6±3.2	66.6±1.5

, *: values indicate treatment group versus group C; ** $p<0.01$, *** $p<0.001$.

On day 35, the micrographs of the intestinal samples obtained from the T1 group showed a normal structure without any histopathological changes. However, the evaluation of the T2 group in terms of the attenuated intestinal damages revealed variable degrees of epithelial cell necrosis in this group. Moreover, the number of goblet cells was significantly increased in this group as compared to those in the T1 and T3 groups (p<0.05). The micrographs of the intestine in the T3 group were suggestive of significant pathological changes 35 days post-intervention (p<0.05). The pathological variations included severe necrosis, submucosal edema, infiltration of inflammatory cells into submucosa, and detachment (shedding) of the epithelial cells on day 35. The histopathological analysis of the intestinal samples in the N3 group showed a close similarity to those of the T2 group. However, the number of goblet cells was significantly increased in the N3 group in comparison to those in the control group (p<0.05). The mentioned group also had the evident signs of necrosis at the villi tip. However, the structure of the villi in the N2 group was intact, and the number of goblet cells was increased in this group in comparison to those of the control. However, this difference was not statistically significant (p>0.05; Fig. 2).

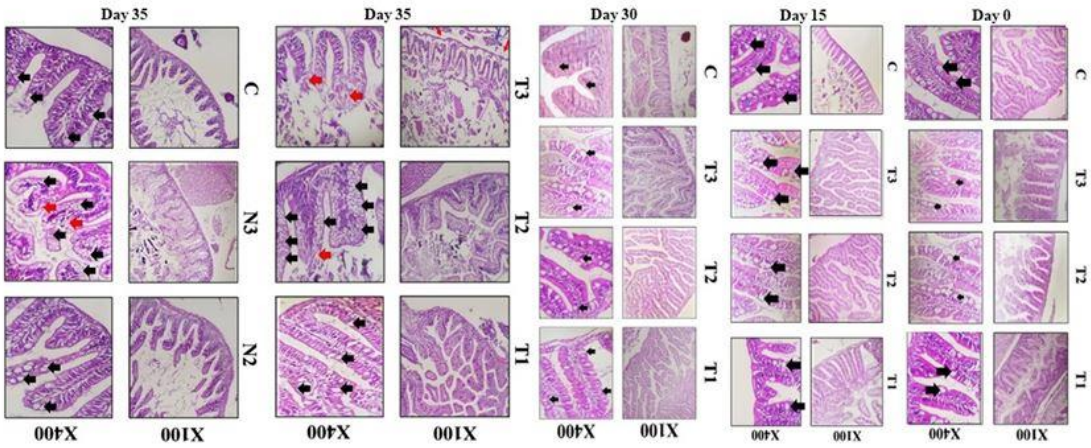

Figure 2: Histopathologic sections of the harvested intestine samples in different experimental groups on different days, thick arrows: goblet cells, red thick arrows: necrosis, red thin arrows: submucosal edema, and blue arrows: infiltration of inflammatory cells (H&E staining).

Effect of L. casei on TNF-α in zebrafish

The results of TNF-α gene expression are shown in Figures 3 and 4. Based on these results, gene expressions were almost the same on days 0 and 15 (p>0.05). On day 30 showed an decreasing trend in T1 and T2 (p<0.05), but No significant differences were reported between T1 and T2 and between T3 and C groups (p>0.05; Fig. 3).

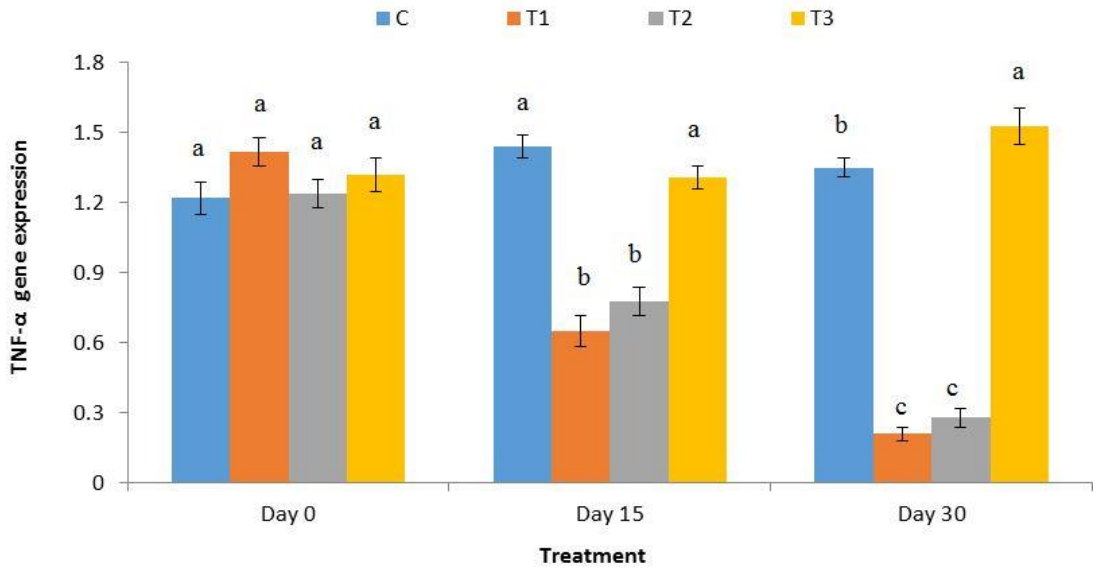

Figure 3: The comparison of TNF-α gene expression between Treatment on different days. The same letters mean no difference (p>0.05) and different letters mean a significant difference at the 5% level (p<0.05).

On day 35 significant decreases were reported in T1, T2 and N2, so that the lowest values were recorded in group T1 (p<0.05). On day 35, an increase was also reported in T3 and N3 groups, so

that the highest values were recorded in group T3 (p<0.05; Fig. 4).

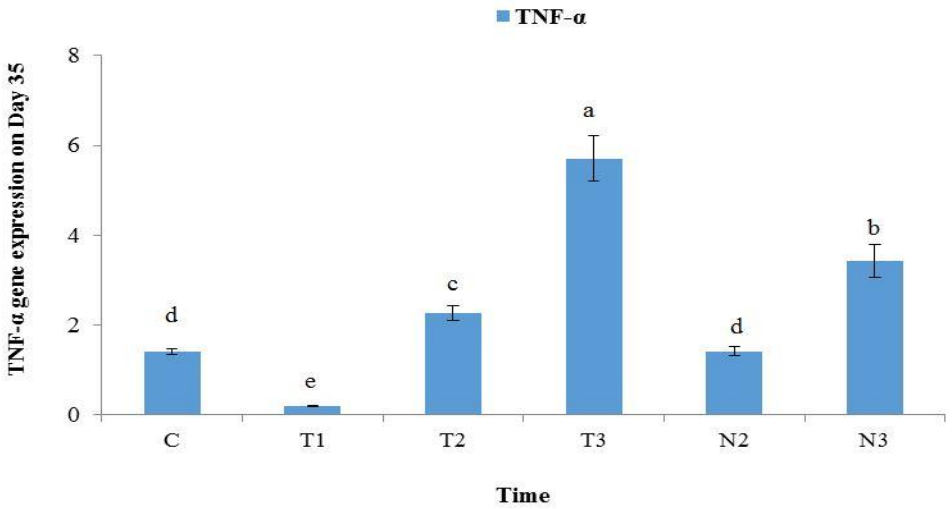

Figure 4: The comparison of TNF-α gene expression between Treatment on 35 day. The same letters mean no difference (p>0.05) and different letters mean a significant difference at the 5% level (p<0.05).

Effect of L. casei on IL-1β in zebrafish

The results of IL-1β gene expression are shown in Figures 5 and 6. Based on these results, gene expressions were almost the same on days 0 (p>0.05). On days 15 and 30 showed an decreasing trend in T1 and T2 (p<0.05), but No significant differences were reported between T1 and T2 and between T3 and C groups (p>0.05; Fig. 5).

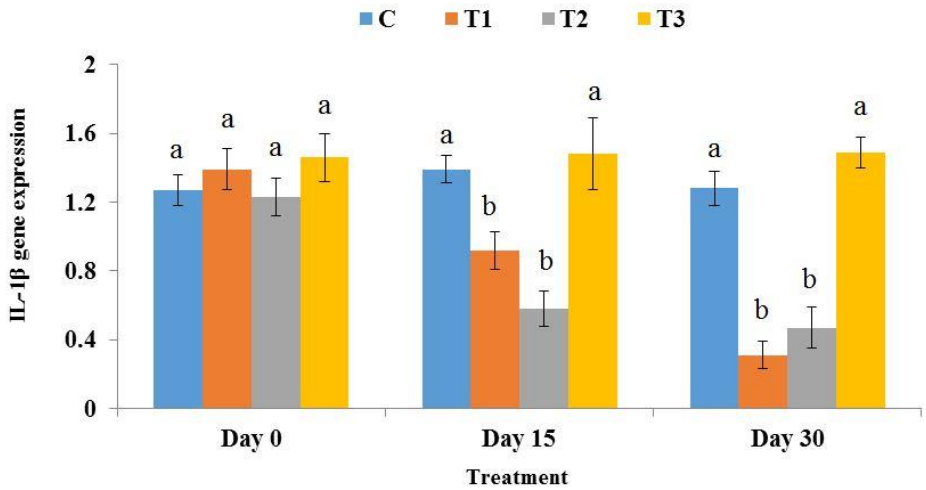

Figure 5: The comparison of IL-1β gene expression between Treatment on different days. The same letters mean no difference (p>0.05) and different letters mean a significant difference at the 5% level (p<0.05).

On day 35 significant decreases were reported in T1, T2 and N2, so that the lowest values were recorded in groups T1 and N2 (p<0.05). On day 35, an increase was also reported in T3 and N3

groups and the highest values were recorded in this groups (p<0.05; Fig. 6).

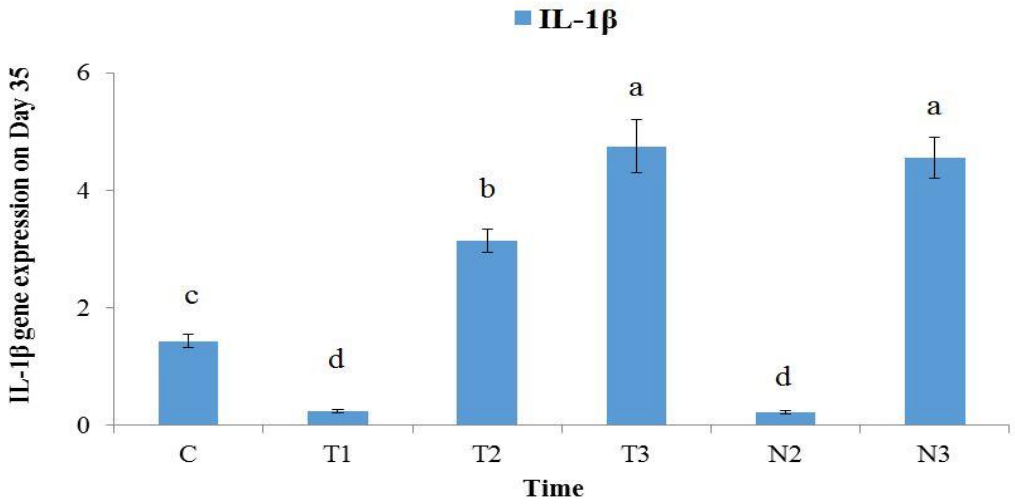

Figure 6: The comparison of IL-1β gene expression between Treatment on 35 day. The same letters mean no difference (p>0.05) and different letters mean a significant difference at the 5% level (p<0.05).

Discussion

Probiotics have been used for decades to control diseases and increase immunity in humans and animals despite showing various levels of efficacy (Sazawal *et al.*, 2006; Kalliomaki *et al.*,2010). However, the precise molecular mechanism of action of these bacteria is not well-understood since their performance has been rarely investigated (Nayak, 2010). One of the studied signaling pathways regarding the functioning of probiotics in the stimulation of the immune system is the production of inhibitory compounds, followed by the inhibition of the expression of virulence genes (Fuente *et al.*, 2015). In addition, probiotics produce detoxifying compounds, help with the digestion of indigestible particles, increase appetite, enhance the ability to absorb minerals and trace elements, and produce important digestive enzymes. In doing so, they increase conversion efficiency and weight gain (improvement in growth function) and stimulate the immune system in some aquatic animals (Gatesoupe, 1999).

Previously, improvement in fish growth performance as a result of feeding functional diets has been reported (Alavinezhad *et al.*, 2020; Zorriehzahra *et al.*, 2021; Kazempoor *et al.*, 2022). One of these functional diets are probiotics (Bazari Moghaddam and Pourjaafari, 2021; Ramzannejad *et al.*, 2021). Given the abovementioned positive effects of probiotics, the current study assessed the effects of probiotics *L. casei* feeding on the growth rate, bacterial flora, and intestinal structure of zebrafish. This research was also targeted toward investigating the improvement of the immune response to the pathogenesis of *A. hydrophila* in zebrafish as a laboratory model. Multiple studies have revealed the

positive effects of using *Lactobacilli* as an additive in fish feed. The first visible evidence of the positive effects of using probiotics in the diet was the increased growth rate in these animals (Carnevali *et al.*, 2017). Accordingly, the results of the present study were indicative of a significant increase in the weight and longitudinal growth of the zebrafish fed the *L. casei* diet. Similar studies have revealed a more effective absorption of food in the fish fed the diet containing probiotics (Carnevali *et al.*, 2013; Zang *et al.*, 2019). As reported by Qin *et al.* (2018), the function of *L. casei* in stimulating the growth of zebrafish is not dependent on its binding to the gastrointestinal epithelium. The effect is rather attributed to the compounds derived from the metabolism of *L. casei* (Qin *et al.*, 2018). In the present study, the results of the bacterial colonization analysis indicated no changes in the colonization of *Lactobacilli* in the intestinal tract as a result of probiotic feeding. In this regard, Rinkinen *et al.* (2003) stated that the binding of *Lactobacilli* to the intestines was not affected by fish species (Rinkinen *et al.*, 2003). Nevertheless, in a study conducted by Nikoskelainen *et al.* (2001), *L. casei* strain Shirota was reported to have a higher binding affinity to mucus. Accordingly, it can be concluded that fish species and environmental and nutritional conditions can collectively affect the research results (Nikoskelainen *et al.*, 2001). Zhou *et al.* (2012) demonstrated that *L. casei* has lower binding and colonization rates in the digestive tract of zebrafish, compared to other probiotics, such as *L. brevis, L. plantarum,* and *L. r*hamnosus. However, it should be noted that the increase in the colonization of this bacterium was observed only in the first week of feeding (Zhou *et al.*, 2012). This can explain the reasons behind our conclusion regarding the sampling days since sampling in the early days could have yielded more accurate results.

Some of the factors accounting for the improvement of growth as a result of probiotic feeding include the detoxification of feed compounds, digestion of indigestible dietary compounds by hydrolyzing enzymes (i.e., amylase and protease), and production of such vitamins as biotin and B_{12} (Balcázar *et al.*, 2006; Suzer *et al.*, 2008). Furthermore, the use of probiotics in animal feeding reportedly leads to an increase in the length of the intestinal villi, thereby enhancing the level of absorption and efficacy of food resource consumption (Pirarat *et al.*, 2011; Alavinezhad *et al.*, 2020). In the present study, an increase was observed in the length of the intestinal villi as a result of *L. casei* feeding, indicating the positive effect of this additive on fish growth. In this regard, studies using zebrafish larvae have provided new insights into other mechanisms involved in the regulation of growth, feed utilization, and metabolism of probiotic-fed fish. Based on the evidence, feeding *L.* rhamnosus to zebrafish larvae regulates lipid processing in the host by inhibiting the genes involved in cholesterol and triglyceride metabolism (i.e., *FIT2, AGPAT4, DGAT2, MGLL,*

HNF4A, *SCAP*, and *CCK*). Moreover, it leads to a decrease in the levels of cholesterol and triglyceride in the larval body and an increase in fatty acid levels, thereby resulting in an increase in the growth rate of zebrafish larvae (Falcinelli *et al.,* 2015; Falcinelli *et al.*, 2016). When comparing our results with those of the aforementioned studies by considering the differences in fish age and utilized probiotics, the mechanism identified in the present study to be responsible for increasing fish growth seems probable. Fish growth is a complex process that is directly related to muscle growth and is mainly regulated by the growth hormone /insulin-like growth factor (IGF) system (Reinecke *et al.,* 2005). Montserrat *et al.* (2007) indicated a change in the transcription of IGF family members as a result of such a diet. Accordingly, it can be concluded that probiotic feeding can increase growth via this mechanism. Avella *et al.* (2012) and Qin *et al.* (2018) investigated the mechanism of the action of *L. casei* and *L. rhamnosus* in the growth of zebrafish, respectively. They observed that probiotic feeding to zebrafish increased *IGF1* and *IGF2* levels. Based on our results, this pathway could be one of the influential mechanisms of *L. casei* improving the growth of zebrafish. However, to confirm this hypothesis, it is required to perform extensive studies and investigate cellular pathways. Studies have revealed that the majority of the farmed fish subjected to a probiotic diet show an improved immune response, in addition to more efficient food absorption (Tovar-Ramírez *et al.,* 2010; Abid *et al.,* 2013; Gioacchini *et al.,* 2014; Falcinelli *et al.,* 2015). In the present study, *L. casei* feeding reduced the expression of *TNF-α* and *IL-1β* inflammatory factors in fish. These results could indicate that the immune system is modulated by inflammatory and pre-inflammatory cytokines that play a peculiar role in the modulation of the immune system. Likewise, Lin *et al.* (2008) indicated that the regulation of *TNF-α* transcription in humans subjected to *L. reuteri* probiotic diet is one of the main mechanisms of increased immunity by probiotics (Lin *et al.,* 2008). Moreover, Picchietti *et al.* (2009) showed that probiotics (*L. delbrueckii*) reduced *IL1-β* transcription levels.

The histopathological results of the present study were indicative of a significant increase in the length of the intestinal villi. It can be regarded as another mechanism responsible for the positive effect of probiotics on the gastrointestinal tract system of fish. In numerous studies, this effect has been shown to improve the intestinal structure by improving the uniformity, density, and/or length of the intestinal villi (Merrifield *et al.,* 2015; Standen *et al.,* 2015; Alavinezhad *et al.,* 2020). A possible mechanism regarding the increase in the length of the intestinal villi could be that probiotics begin to grow and use glucose to make short-chain fatty acids (SCFAs) after entering the stomach. The SCFAs, especially butyric acid, are the main source of energy for the intestinal epithelial cells.

Consequently, as noted in a study conducted by Pelicano *et al.* (2005), this may play an important role in increasing the length of the intestinal villi. Moreover, in the present study, the number of intestinal goblet cells increased as a result of *L. casei* feeding. One of the major benefits of this effect is an increase in mucus production and subsequent reduction in the likelihood of pathogen binding to receptors in the intestinal epithelium. It should be noted that this increase in the number of goblet cells as a result of probiotic feeding has been already examined and confirmed in other studies (Standen *et al.,* 2013; Reda and Selim, 2015; Standen *et al.,* 2016). However, our findings dealing with the post-exposure to *A. hydrophila* agent are the remarkable points of the present study. The obtained results of intestinal histopathology not only revealed the positive effect of probiotic feeding on the fish intestinal structure but also indicated the reduction of pathological effects (e.g., necrosis, edema, and infiltration of inflammatory cells into the intestines) upon exposure to a pathogen. This finding has been also observed in other studies despite differences in the investigated fish species, probiotic type, and pathogen (Ringø *et al.,* 2007; Elahi *et al.,* 2020). In this regard, the mechanisms involved in the assessment of the inhibitory effect of probiotic feeding against the internal microbes in the intestine can be the production of inhibitory compounds (e.g., lactic acid, hydrogen peroxide, and bacteriocins), as well as competition for chemicals, energy, and adhesion sites (Oyetayo *et al.,* 2003; Merrifield *et al.,* 2010; Merrifield and Carnevali, 2014).

In the present study, probiotic nutrition decreased TNF-α and IL-1β gene expression. The observed effect of probiotic feeding on the expression of pre-inflammatory genes can be explained by the effect of NF-κβ regulator and TLR1/TLR2 signaling pathway on inflammatory responses. Accordingly, there are a number of studies suggesting the regulation of pre-inflammatory cytokine responses and NF-κβ activation as a result of *Lactobacilli* feeding (Miettinen *et al.,* 2000). Nevertheless, Qin *et al.* (2017) introduced TLR1/TLR2 signaling pathway as an effective factor in modulating the probiotic-fed fish system. Another influential factor in this regard could be the inhibitory effect of cell wall polysaccharide of *Lactobacilli* on the activation of macrophages. This view was confirmed in a study carried out by Yasuda *et al.* (2008). In contrast to our findings, a number of studies on the effects of probiotics on the immune system of tilapia have shown an increase in *TNF-α* and *IL-1β* levels in probiotic-fed fish (Liu *et al.,* 2013; Standen *et al.,* 2013; Standen *et al.,* 2016). However, in this study, the lowest increase in TNF-α level was detected in the group treated with L. casei. Nonetheless, one of the reasons that can justify the discrepancies between the results of the current study and those of previous studies is that the host species and environmental conditions are very influential in the development of an effective immune response. In addition, as Liu *et al.* (2013)

noted, the effect of a probiotic diet on the expression of TNF-α and IL-1β genes depends on the type of probiotic bacterium and time.

Exposure to *A. hydrophila* resulted in increased TNF-α and IL-1β, although this was lower in the probiotic-fed groups. Qin *et al.* (2017) observed an increase in the expression of *TNF-α* and *IL-β* genes in zebrafish larvae fed *L. casei* and exposed to *Aeromonas veronii* after 24 h. Nonetheless, 48 h after exposure, this rate returned to a level close to that of pre-exposure. Furthermore, in the group that did not receive probiotics, these factors increased slowly, and the increasing trend was maintained for 48 h after challenge. Moreover, in another study on the feeding of zebrafish larvae with *L. casei* in the face of *A. hydrophila*, a significant increase was detected in *IL-1β* levels 8 h after exposure. However, after 72 h, this increase showed a significant difference, compared to the levels obtained at pre-exposure and in the control group (Qin *et al.*, 2018). The aforementioned findings can confirm the results of the current study and generally indicate the modification of the immune system as a result of a probiotic diet and fast response in the face of a pathogenic agent while protecting against inflammation process and the resultant tissue damage. Based on the findings of the present study concerning the expression of fewer *TNF-α* and *IL-1β* genes in the group which received probiotics after exposure to the pathogen, we can refer to the probiotic effect on lipopolysaccharide (LPS) tolerance in the intestinal epithelial cells or macrophages. Accordingly, in a study conducted by Peña *et al.* (2005), the use of a combination of *L. paracasei* and *L. reuteri* in the diet was reported to reduce the expression of *TNF-α* by inducing LPS tolerance in the intestinal epithelial cells or macrophages.

In general, it can be concluded that *L. casei* feeding increases the growth rate by creating mechanisms to improve the immune and mucosal systems of the gastrointestinal tract. Moreover, it increases the resistance to *A. hydrophila*, as a pathogenic agent, by modulating the expression of *TNF-α* and *IL-β* genes and increasing the length of the villi and number of intestinal immune cells. Accordingly, it will lead to the control and reduction of the complications of the disease in zebrafish. It is recommended to investigate the effects of *L. casei* feeding to zebrafish and pathogen exposure on the signaling pathways affecting the immune response.

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