Research Article

The effect of different concentrations of *Lacticaseibacillus casei* on the growth performance and intestinal morphology of zebrafish (*Danio rerio*)

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Abstract

Considering the increasing rate of antibiotic resistance and consequently the need for using alternative compounds to increase immunity and prevent diseases, the present study aimed to investigate the effects of *Lacticaseibacillus casei* on the growth indices and intestinal morphology of adult zebrafish. This study was conducted on 80 zebrafish (mean weight: 0.25±0.05 g and mean length: 2.5±0.05 cm), which were assigned to four groups with two replications. Three of the groups received *L. casei* at each of the concentrations of 1.5×10^4, 1.5×10^7, 1.5×10^8 CFU/ml, and one served as the control, which was fed with the basic diet. Samples were collected to examine the weight (W), length (TL), condition factor (CF), and intestinal morphological changes of the fish at the end of the study. The results showed that the probiotic diet boosted the weight and length of the fish compared to the control group ($p>0.05$). Based on these results, feeding with *L. casei* probiotic exerted the most potent and least impact on the growth of the fish at the concentrations of 1.5×10^8 CFU/ml and 1.5×10^7 CFU/ml, respectively. There was also a significant change in intestinal villous length after receiving the probiotic diet compared with the control group ($p<0.05$). However, intestinal villous length was not significantly different comparing the groups receiving different concentrations of the probiotic ($p>0.05$). Based on the results of this study and observed increased length of intestinal villous after being fed with *L. casei*, which will subsequently increase the nutrient absorption and growth of fish, it is recommended to use this probiotic at the indicated concentrations (1.5×10^8 CFU/ml) as a dietary supplement.

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Introduction

Probiotics are living microbial cells used as dietary supplements to improve the host's health (Nguyen *et al*, 2017). In recent years, the use of probiotics in aquaculture has expanded significantly. The importance of using probiotics in aquaculture lies within the ability of these bacteria in colonizing in the gastrointestinal tract, improving the host's health in various ways such as preventing the entry of pathogens, increasing survival, and enhancing the immune response. (Lazado *et al*., 2011; González-Félix *et al*., 2018). In addition, these bacteria have been noted to affect the morphology of the intestine (e.g., increasing the length of villous, increasing the number of intestinal folds, enhancing nutrient uptake, and facilitating digestion) (Jang *et al*., 2019; Yang *et al*., 2019; Najafabad *et. al*., 2016). Due to the increased prevalence of therapy-resistant pathogenic bacteria and because of the accumulation of antibiotics in the tissues of living organisms, the use of antibiotics to treat bacterial infections in aquaculture has decreased in recent years; therefore, using alternative methods and agents, such as probiotic bacteria, seems to be inevitable (Peredo *et al*., 2015).

Among valuable probiotics are the bacteria belonging to the Lactobacillus (LAB) family, covering a number of most important probiotic microorganisms, which was first studied in 1780 (Aryana and Olson, 2017). Most probiotic microorganisms are lactic acid bacteria, which are Gram-positive cocci (sphere-shaped) or bacilli (rod-shaped) and do not produce spores. Lactic acid is the main and characteristic compound produced by these bacteria (Bhavani and Sundar, 2014). In recent decades, Lactobacillus has been used as a valuable species in the aquaculture industry (Kuebutornye *et al*., 2020). Lactobacillus can increase the host's growth and resistance against pathogens by boosting the intestinal structure and microbial flora (Dawood *et al*., 2016; Mirabdollah Elahi *et al*., 2020).

Lacticaseibacillus casei, formerly known as *Lactobacillus casei*, is a member of the Lactobacillus family, which was introduced by the QPS report in 2016 as a safe microorganism for being used in foods, and its positive effects on intestinal structure have been shown in various studies (EFSA, 2016; Xu *et al*., 2020).

Zebrafish (*Danio rerio*) is one of the freshwater fish belonging to the *Cyprinidae* family, which in recent years has been used in many studies as a valuable model for investigating the developmental stages and diseases of vertebrates. This fish has several advantages such as evolutionary genes, small size, which allows keeping a large number of fish in a relatively small space, having external fertilization, transparent embryos (which make it possible to directly study the evolutionary process), the ease of use of water-soluble drugs and chemicals, and finally their applicability in studying mutations (Keller and Keller, 2018). According to the above-mentioned and the effects of probiotic bacteria on the growth

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and structure of fish intestine, this study was performed to investigate the effects of feeding with *L. casei* probiotic on the functional growth and morphology of zebrafish intestine.

Materials and methods

Experimental fish and husbandry conditions

In this experiment, 80 pieces of male and female zebrafish (average weight: 0.25 ± 0.05 g and average length: 2.5 ± 0.05 cm) were purchased from an ornamental fish breeding center in Shahriar, Tehran (Iran). For adaptation, the fish were transferred and maintained in the research complex of the Islamic Azad University, Science and Research Branch, Shahriar, Tehran, for two weeks before the study. During the study, the condition of maintaining the fish was 26 ± 0.05° C, pH = 7-7.5, and dark/light cycle of 12:12 hours. The fish were fed based on 2% of body weight, twice a day. On a daily basis, 30% of the water of aquariums was removed and replaced with chlorine-free water.

Bacterial strains and culture condition

Lactobacillus casei ATCC 393 was obtained from the Microbial Bank of Iranian Biological Resource Center. To prepare the required logarithm, an initial lyophilized suspension of the bacterium was poured into a test tube containing MRS broth (Merck, Germany) and incubated under aerobic condition for 24-48 hours at 35° C and 150 rpm. Then the desired growth turbidites (1.5×10^4, 1.5×10^7, and 1.5×10^8 CFU/ml) were prepared using a spectrophotometer and poured into sterile tubes. After adding 20% glycerol to the tubes, they were kept at -20° C until use (Barbour & Priest, 1986).

Feed preparation

The probiotic diet was prepared daily by supplementing 2 ml of the suspension of *L. casei* (1.5×10^4, 1.5×10^7, and 1.5×10^8 CFU/ml) with commercial feed (BioMar, France) and Incubate in ice for 15 minutes to absorb bacteria. The diet of the control group was prepared by combining the commercial feed with an equivalent volume of PBS (i.e., 2 ml). The diets were given based on 2% of fish body weight twice daily at 9 AM and 16 PM (Wang *et al*. 2016).

Experimental trials

After the adaptation period, the fish were randomly divided into four groups with two replications (20 fish per aquarium) and then fed with different diets for 25 days. The groups included P1 (feeding with the probiotic diet at the concentration of 1.5×10^4 CFU/ml), P2 (feeding with the probiotic diet at the concentration of 1.5×10^7 CFU/ml), P3 (feeding with the probiotic diet at the concentration of 1.5×10^8 CFU/ml), and C as the control group which was fed by the basic diet.

At the beginning and end of the experiment, five fish were randomly caught from each aquarium to ascertain the weight (WG) and total length (TL) of the fish using a scale with the accuracy of 0.01 g and a standard ruler with the accuracy of 1 mm, respectively. Also, the effects of different concentrations of the probiotics on intestinal morphology were assessed at the end of the study period by randomly catching two fish from each group

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and Euthanized using clove powder according to the method of Wong *et al*. (2014). Then, an incision was made along the abdominal line, and the fish were placed in a tube containing 10% formalin (in a quantity of five times of the sample mass). To complete the fixation process, formalin was replaced after 24 hours. The histological slides were prepared by placing the fixed intestine specimens in alcohol solutions with ascending concentrations for dehydration and in xylene for clarification. Then the specimens were immersed into melted paraffin to penetrate into the tissue. Tissue sections (5-micron diameter) were prepared using a microtome device and then placed on a slide. Finally, hematoxylin-eosin (H&E) staining was performed, and histological examinations were conducted under an optical microscope (Olympus BX51; Olympus, Tokyo, Japan) (Pirarat *et al*., 2011). Measurement of Villi was done using Image-j application.

Statistical analysis

After checking the normality of data distribution using the Kolmogorov-Smirnov test, statistical analyses was performed in SPSS software version 18 using one-way analysis of variance (ANOVA). Finally, the results were reported as mean ± standard deviation (Mean ± SEM), and $p<0.05$ was considered as the statistical significance level.

Results

Growth performance

The results of growth performance have been summarized in Table 1, according to which growth indices on day 0 were the same in all the groups, and on day 25th, no significant differences were observed between the groups. Regarding weight gain at the end of the experiment, the highest increase was observed in the P3 group; however, no significant differences were noticed between the groups (P = 0.07). The highest increase in TL was related to the P3 group, but again, no significant difference was reported between the groups (P= 0.17). Regarding the condition factor, the largest decrease was observed in the P3 group; however, the differences between the groups were statistically insignificant (P = 0.31).

Table 1. Growth performance in zebrafish fed with different concentrations of probiotics on the first and 25th days of the onset of feeding (data are expressed as mean ± SD)

	Day 0				**Day 25**			
	P1	**P2**	**P3**	**C**	**P1**	**P2**	**P3**	**C**
W	0.27±0.2	0.25±0.03	0.28±0.02	0.24±0.02	0.33±0.02	0.31±0.01	0.35±0.01	0.26±0.04
TL	2.88±0.1	2.74±0.2	2.92±0.1	2.88±0.1	3.32±0.1	3.16±0.1	3.44±0.1	3.04±0.1
CF	1.12±0.1	1.22±0.1	1.10±0.07	0.98±0.1	0.86±0.06	0.96±0.05	0.84±0.05	0.84±0.02

Intestinal morphology

As shown in Table 2, in the experimental groups (P1, P2, P3, and C), intestinal villous appeared normal without any tissue damage in all regions. However, the length of intestinal

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villous in the probiotic groups showed a significant increase compared to the control group ($p<0.05$). Although in the P3 group, intestinal villous were longer compared to other groups, there was no significant difference in the length of villous between the groups fed with different concentrations of the probiotics (i.e., P1, P2, P3) ($p>0.05$). Moreover, the number of goblet cells was increased in probiotic groups when compared to the control group but this difference was not statistically significant ($p>0.05$, Fig. 1).

Table 2. The effects of feeding with *Lacticaseibacillus casei* probiotic on the length of intestinal villous in zebrafish

Treatment	P1	P2	P3	C
Villous length	76.6 ± 1.5[a]	78.6 ± 2.5[a]	79.3 ± 4.7[a]	63.3 ± 1.5[b]

Data presented as mean ± standard deviation. Different small letters show significant differences between treatment groups

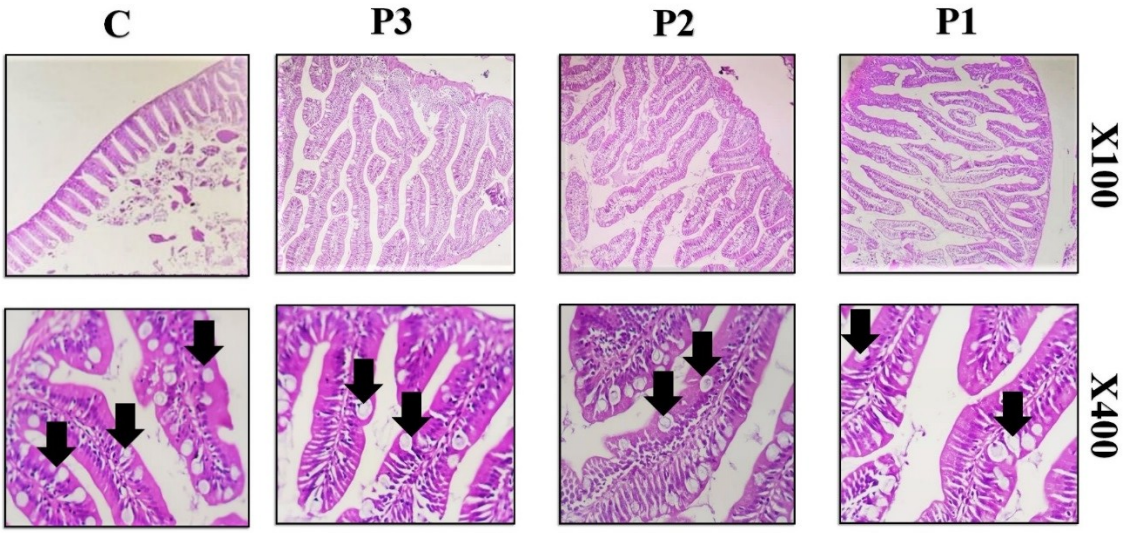

Figure 1. Histological structure of intestine of zebrafish. P1: feeding with the probiotic diet at the concentration of 1.5×10^4 CFU/ml. P2: feeding with the probiotic diet at the concentration of 1.5×10^7 CFU/ml. P3: feeding with the probiotic diet at the concentration of 1.5×10^8 CFU/ml. C: control group. Thick arrows indicate goblet cells (H&E staining).

Discussion

Probiotics have several effects on the biological functions of aquatic animals, among which the most prominent positive effects of dietary probiotics are observable on the growth rate of these animals (Carnevali *et al*., 2017). According to the results of the present study, feeding with different concentrations of probiotics exerted positive effects on the body weight and the total body length of the fish; however, changes in these growth parameters were not statistically significant. In this regard, several studies have proven the effects of probiotics on improving fish growth indices, such as Dawood *et al*. (2016), Avella *et al*. (2012), and González-Félix *et al*. (2018). According to the results of the present study, feeding with *L. casei* probiotic at the concentrations of 1.5×10^8 and 1.5×10^7 CFU/ml had the greatest and lowest effects on the growth rate of the fish, respectively. Nevertheless, we found that these results were statistically

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insignificant. For explanation, and according to a study by Vand *et al*. (2014) investigating the effects of feeding with different concentrations of *L. casei* probiotic (i.e., 10^6, 10^7, and 10^8) for 60 days on the growth parameters of *Barbus grypus* fish, one can note that a relative short duration of feeding with the probiotics in our study.

In general, feeding with probiotics leads to more effective absorption of nutrients and thus increases the growth of aquatic animals (Zang *et al*., 2019). The possible mechanisms involved in this phenomenon can be the role of probiotics in detoxifying nutrients, the breakdown of indigestible dietary compounds by hydrolyzing enzymes such as amylase and protease, and the production of vitamins such as biotin and vitamin B12, promoting the growth and development of hosts (Suzer *et al*., 2008; Balcázar et. al., 2006). Also, Qin *et al*. (2018) and Avella *et al*. (2012) studied the mechanisms through which probiotics would affect the growth of fish. Qin *et al*. (2018) (2018) used *L. casei*; Avella *et al*. (2012) applied *L. rhamnosus*, and both studies revealed that feeding with the probiotics increased the levels of insulin-like growth factor (IGF-1) and IGF-2 in zebrafish. So, this could also be a possible mechanism through which *L. casei* improved zebrafish growth parameters in the present study. Future studies are recommended to focus on the cellular pathways involved in the positive effects of these probiotics, which will also be investigated by the present research team.

Regarding intestinal morphological analyses in the present study, we observed an increase in the number of goblet cells and increased intestinal villous length in the groups fed with *L. casei* probiotic. This highlights one of the positive effects of probiotics on the structure and function of the gastrointestinal tract in fish. In this regard, several studies have shown that probiotics improved intestinal structure in terms of the homogeneity, density, and/or length of intestinal villous (Sáenz de Rodrigáñez *et al*. 2009; Standen *et al*. 2016).

Similar to the results of the present study, feeding with lactobacilli was reported to have positive impacts on intestinal morphology in zebrafish in studies by Qin *et al*. (2018) and Falcinelli *et al*. (2015). *Lactobacillus rhamnosus* and *L. lactis*, as lactic acid bacteria, have previously been shown to increase intestinal villous length and improve the microscopic morphology of the intestine in tilapia fish (Xia *et al*., 2018). In addition, in the study of Pirarat *et al*. (2011), a significant increase in the length of intestinal villous was observed in the tilapia fish fed with probiotic supplements as compared to the group receiving the basic diet. This was in accordance with the results of our study in zebrafish.

Other studies investigating the effects of feeding with probiotics on increasing the length of intestinal villous in fish are González-Félix *et al*. (2018) in a study on *Totoaba macdonaldi* and Merrifield *et al*. (2010) in their study on the effects of *Pediococcus acidilactici* on the proximal part of the intestine of rainbow trout. On the other hand, in contrast to our results, Jang *et al*.

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(2019) stated that feeding Olive flounder (*Paralichthys olivaceus*) with probiotics did not have a significant effect on the length of intestinal villous compared to the control group. the possible mechanisms that may be involved in increasing the length of intestinal villous, as observed in the present study, includes the production of short-chain fatty acids from sugars in the gastrointestinal tract by the act of probiotic bacteria. This is important as short-chain fatty acids, especially butyric acid, are the principal energy sources of intestinal epithelial cells, so this activity, as mentioned by Pelicano *et al*. (2005), can play an important role in increasing the length of intestinal villous.

Other histological changes in intestinal tissue sections was increase the number of intestinal goblet cells. Several reports, including Reda and Selim (2015) and Standen *et al*. (2016) have reported an elevation in the number of goblet cells after feeding with probiotics. In this regard, according to Lazado and Caipang (2014), increase goblet cell count can subsequently lead to increase mucus production, therefore hindering pathogens from binding to receptors on intestinal epithelium.

Overall, preserving and strengthening the intestinal bacterial flora can maintain and enhance the structure and function of intestinal epithelium in fish partly by inhibiting the attachment of potential pathogens, and thus repressing the production of their virulence factors such as extracellular enzymes and toxins. As a result, mucosal damage is reduced, and the disruption of the intestinal epithelium integrity is prevented (Ringø *et al*., 2010). Another noteworthy point is that the increased length of intestinal villous, as observed in this study, can increase the absorption of nutrients from the intestine, as expressed by Standen *et al*. (2016) and Pirarat *et al*. (2011). Considering the remarkable increase in intestinal villous length, which was observed in the present study, extending the duration of feeding with probiotics would have significantly improved the growth parameters compared to the control group. In this regard, due to the importance of the growth rate of fish in the aquaculture industry, the probiotic of *L. casei* can be used as a dietary supplement to achieve this goal.

Conflict of interest

Authors have no conflict of interest on this work.

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