بررسی تاثیرات *لاکتوباسیلوس اسیدوفیلوس* و *لاکتوباسیلوس پلانتاروم* روی سیستم ایمنی، فاکتورهای رشد و مقاومت به باکتری بیماری‌زا در ماهی قزل‌آلای رنگین‌کمان

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چکیده

مقدمه: این مطالعه با هدف بررسی اثر پروبیوتیک‌های *لاکتوباسیلوس اسیدوفیلوس* و *لاکتوباسیلوس پلانتاروم* به‌صورت تنها و همراه با هم (سینرژیسم)، بر سیستم ایمنی، فاکتورهای رشد و درصد زنده‌مانی ماهی قزل‌آلای رنگین‌کمان انجام گرفت.

مواد و روش‌ها: در سطح اول یا in vitro عصارهٔ باکتریوسین باکتری‌های مدنظر با دستگاه خشک‌کن انجمادی، تغلیظ و در آزمون‌های ضدمیکروبی علیه عوامل بیماری‌زای ماهی *ائروموناس هیدروفیلا* با روش میکرودایلوشن استفاده شد. پس از آن با آزمون MTT اثر این عصاره بر لنفوسیت‌های خون ماهی بررسی شد. در سطح دوم یا in vivo، پس از خوراک‌دهی ماهیان هر تیمار با CFU 10^8 باکتری در مدت ۶۰ روز، شاخص‌های رشد ازجمله درصد افزایش وزن، ضریب رشد ویژه و میزان رشد روزانه محاسبه شدند. پس از آن، با خونگیری از ماهی قزل‌آلا افزایش لنفوسیت‌های خون نیز بررسی شد. درنهایت، با چالش آلوده‌سازی تیمارها با باکتری بیماری‌زای *کرایزوباکتریوم آکواتیکوم*، میزان زنده‌مانی ماهیان پس از تغذیه با پروبیوتیک‌ها بررسی شد.

نتایج: در هر دو سطح، *لاکتوباسیلوس اسیدوفیلوس* بیشترین تأثیر را بر فاکتورهای رشد و سیستم ایمنی ماهی داشته و لنفوسیت‌های خون را به‌صورت معنی‌داری افزایش داده است ($p<0.05$). همچنین، آزمون سنجش خاصیت ضدمیکروبی میکرودایلوشن برای این لاکتوباسیل توانست بیش از ۹۰ درصد تعداد کلونی‌های *آئروموناس هیدروفیلا* را کاهش دهد. تیمار تغذیه‌شده با این لاکتوباسیلوس نیز بیشترین درصد زنده‌مانی در اثر چالش آلوده‌سازی را داشت. پس از آن، تیمار هم‌افزایی و در آخر تیمار *لاکتوباسیلوس پلانتاروم* قرار داشت که هر دو نسبت به شاهد افزایش معنی‌داری در شاخص‌های بررسی‌شده ایجاد کردند.

بحث و نتیجه‌گیری: درنهایت، می‌توان نتیجه گرفت استفاده از این پروبیوتیک‌ها باعث بهبود شاخص‌های رشد و تحریک سیستم ایمنی با افزایش تعداد لنفوسیت‌ها و کاهش اثر باکتری‌های بیماری‌زا می‌شود.

واژه‌های کلیدی: تست ضدمیکروبی، ماهی قزل‌آلای رنگین‌کمان، لنفوسیت سیستم ایمنی، *لاکتوباسیلوس اسیدوفیلوس*، *لاکتوباسیلوس پلانتاروم*

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An Experimental Study of the Effects of *Lactobacillus Acidophilus* and *Lactobacillus Plantarum* Probiotic Bacteria on the Immune System, Growth Factors, and Disease Resistance in the Rainbow Trout

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Abstract

Introduction: The present study aimed to examine the effects of *Lactobacillus acidophilus* and *Lactobacillus plantarum* probiotics separately and synergistically on the immune system, growth factors, and disease resistance in the rainbow trout.

Materials and Methods: At in vitro level, bacteriocin extracts of the target bacteria were used in the antimicrobial test against the fish pathogen *Aeromonas hydrophila*. Next, their effects on the fish blood lymphocytes were evaluated. At in vivo level, after feeding the fish in each treatment with the lactobacilli at the concentration of 10^8 CFU g^{-1} for 60 days, the growth indices were calculated. Then, blood samples from the rainbow trout were used to analyze any increase in the number of lymphocytes. Furthermore, given the infectious challenge with the pathogen *Chryseobacterium aquaticum*, the survival rates of the fish were determined.

Results: *L. acidophilus* had the best effect on the growth factors and significantly increased the number of fish blood lymphocytes ($p < 0.05$). The broth microdilution assay also was shown to reduce the amount of *A. hydrophila* colonies by more than 90%. Treatment with this lactobacillus species produced the highest survival rate with this infectious challenge. Then, the synergistic and the *L. plantarum* treatments were analyzed, respectively, which each showed a significant increase in the indices under study.

Discussion and Conclusion: According to obtained results, it might be concluded that the feeding by *L. acidophilus* could likely enhance the immune responses and growth indices in fish both separately and synergistically with *L. plantarum***.**

Key words: Antimicrobial Tests, the Rainbow Trout Fish; Lymphocytes; Immune Response, *Lactobacillus Acidophilus*, *Lactobacillus Plantarum*

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Introduction

Aquaculture has rapidly become a dynamic and growing industry in recent decades. Seafood, as one of the healthiest food sources, can play a vital role in food security and community health. Nowadays, aquaculture farmers tend to increase stocking densities to use the maximum capacity of the breeding environment for higher production. With an increase in stocking densities, there would be an increase in fish stress levels and disease susceptibility; therefore, it is necessary to strengthen the fish against such factors. In addition, increasing the growth factors, enhancing the immune system of the fish, especially valuable and economic species, are the primary needs of aquaculture farmers and the most essential approach most researchers would adopt in this regard (1).

The incidence of infectious bacteria, such as *Aeromonas hydrophila* (2, 3) and *Chryseobacterium aquaticum* (4), is one of the primary reasons for a decrease in production in many aquaculture farms. However, the overuse of antibiotics and chemical drugs to treat bacterial infections may lead to severe problems such as increased production costs, environmental contamination, and consequently leading to environmental hazards for other organisms in the ecosystem, and finally, the development of antimicrobial-resistant strains in the host body (5). Nowadays, probiotics or microbial supplements are used as substitutes for antibiotics. The addition of probiotics to the diet would increase digestive enzyme activity and stimulate appetite, maintain microbial balance in the host's intestine, build beneficial compounds, including vitamins and some enzymes, stimulate and enhance immune system efficiency, and improve probiotic food development (6, 7).

The most common probiotics used in aquaculture include lactic acid bacteria, including a variety of different bacteria called Lactobacillus. Lactobacilli are gram-positive, non-motile, non-sporozoic, catalase-negative, and oxidase-negative bacilli, which convert various sugars to lactate and acetate. Most lactobacilli are safe and may be antagonists of pathogenic bacteria (8). *Lactobacillus plantarum* has a long history of safe and natural consumption in a variety of food products and is one of the most critical agents with a therapeutic potential to prevent or treat infections (9, 10).

Lactobacillus acidophilus which is one of the natural intestinal microflora of humans and animals is one of the most widely used probiotics and is known to be one of the species of Lactobacillus most resistant to acidic conditions such as gastric juice and bile salts (11). The mechanism and function of these probiotics have been investigated in many studies (9, 12). The rainbow trout (*Oncorhynchus mykiss*) is one of the most valuable economical fish and is one of the most important cold-water fish species in the aquaculture industry in many countries. The use of probiotic therapy to improve the growth factors and strengthen the immune system of this valuable fish species may greatly help the aquaculture industry and protect the environment. Recent studies have focused more on one species of lactobacilli, and few studies have ever attempted to make comparisons of several species of lactobacilli, especially their synergistic effect (13). In addition, few studies have ever attempted to conduct comprehensive research on probiotics aiming to enhance immune response and disease resistance in addition to growth rates in fish, and most studies have succeeded in addressing only one of these aspects. Moreover, to investigate the above aspects in the probiotic-fed treatments individually and synergistically, the present study also investigated the antibacterial activity of the bacteriocin production of these probiotics on the pathogenic bacteria under study and its effect on fish blood lymphocytes, which in turn was very new.

Materials and Methods

Bacteriocin Preparation: *L. plantarum* (ATCC13643) and *L. acidophilus* (ATCC314) were purchased as a freeze-dried powder from the Pasteur Institute of Iran. After activation of the bacteria under study, they were incubated in an MRS broth medium at pH 6.5 for 48 h at 37 ° C. After incubation, centrifugation (6000 rpm for 10 min) was performed for each falcon, and the supernatant was passed through a 0.2 μm pore-size bacterial filter. Finally, a freeze dryer was used to concentrate the extracts. Each initial treatment consisted of 5 ml of protein solution, which, after being concentrated, reached a volume of 0.15 ml, about 30 times as concentrated. Then, 0.25- and 0.5-ml solutions were obtained by dilution, which was 20 and 10 times more concentrated than in the initial solutions, respectively (11). For *L. plantarum* treatment, after 48h, the optical absorption at 280nm was 0.140 and for *L. acidophilus* treatment, it was 0.151.

Antimicrobial Activity: The pathogenic bacterium *Aeromonas hydrophila* (ATCC7965) was purchased as a powder from the Pasteur Institute of Iran. After activation and culturing in a Tryptic Soy Broth medium to reach a 0.5 McFarland concentration, it was ready to be used for antimicrobial test. The microdilution method is one of the most basic methods of testing antimicrobial susceptibility, as described elsewhere (11). In this method, for this experiment, a 0.5 McFarland solution was prepared by the probiotics under study in an MRS medium and the pathogenic *A. hydrophila* in a TSB medium. *L. acidophilus, A. hydrophila, L. plantarum*, and *hydrophila* were simultaneously cultured in BHI broth mediums until reaching a 0.5 McFarland concentration. Then, a dilution serial was prepared for each row in a 96-well plate, up to the twelfth well. After completion of the last three wells in each row, the lowest concentrations were cultured by sterile swabs on the plates containing solid mediums and were incubated at 37 ° C for 48 h to grow the colonies. Subsequently, the antagonistic effect and the ability to inhibit bacterial growth at the lowest concentrations were evaluated by counting the colonies (14).

The Effect of Bacteriocin on Fish Lymphocyte: First, blood was drawn from the tail vein of the rainbow trout, and after lymphocyte cells were isolated, the MTT assay was performed for bacteriocin concentrations with three replications (15). One well contained a PBS solution instead of a bacteriocin, which was considered as a control. Finally, the OD of the wells was read at 492 to 630 nm by the ELISA reader.

Experimental Set-up and Fish Management: In this study, 120 healthy rainbow trout without any symptoms of infection and with an average weight of 25 ± 2 g were obtained from one of the local fish farms and transferred to Isfahan University Laboratory. The fish were randomly distributed in plastic tanks with half their capacity filled with water (15 in each container). After distribution, they were allowed to adapt to the new conditions for ten days. During this time, they were fed with standard fish food twice daily at 1.5% of their body weight each time. Throughout the period, water quality was maintained by at least 30% daily renewal. An average water temperature of 14 ± 2° C and a pH of 7.8 were maintained (16). Then oxygen was supplied by water pumps. After the adaptation period, the fish were divided into four groups: Treatment 1 (control), Treatment 2 (*l.acidophilus*), Treatment 3 (*l. plantarum*), and Treatment 4 (containing both probiotics to investigate the synergistic effect), and each treatment had a replicate. The fish were fed two meals a day at 1.5% of their body weight for eight weeks. The amount of feed distributed in each group was changed weekly by measuring fish weight.

Preparation of Probiotic-Supplemented Diet: The lactobacilli under study were suspended in a 10 ml sterile physiological serum and sprayed with 5 ml of sunflower oil on the commercial food purchased for the rainbow trout. The food was then dried for 2 hours at room temperature and stored in the refrigerator for later use. In the control group, only the serum and the liquid oil were sprayed (17). The feasible bacterial count in the fish feed was then determined using the pour plate count method, and the final bacterial count was determined to be 10^8 CFU g^{-1}. The commercial diet for the rainbow trout contained 40% crude protein, 16% crude fat, 3.5% fiber, 10% ash, and 1.1% phosphorous (9).

Measurement of Growth Factors in Probiotic-treated Fish: For the biometric analysis of the rainbow trout, sampling was performed at time zero and the end of the sixtieth day. For this purpose, ten fish from each treatment were randomly selected and anesthetized by 200mg/ml of clove powder. Then, growth percentages, specific growth coefficients, and daily growth rates were calculated for each treatment (18).

Cell Viability of Probiotic-treated Fish Lymphocyte: After feeding the trout with the probiotics for eight weeks, three fish were randomly selected from each treatment. After taking 2.5 ml of blood from the tail vein of the rainbow trout and adding 0.5 ml of heparin, 2 ml of lymphodex was poured under the sterile hood into the falcon, and the blood was added to it slowly. The falcons were then centrifuged (1800 rpm for 15 min) to create three detectable phases. The middle white layer was the lymphocytes separated by the sampler and was poured into a new falcon. Finally, the DMEM medium and the sterile physiological serum were added (9). The immunocompetent cell population, consisting of lymphocytes, was measured using the MTT assay (9). For this purpose, the culture medium containing rainbow trout blood lymphocytes was added to each well of 96 well plates, and three replicates were considered for each one. Plates were incubated for 24 h, then 20 µL of MTT was added to each well and incubated again for 16 h. Finally, the OD of the wells was read at 492 to 630 nm by the ELISA device.

The Effect of Probiotics on Fish Infected with Pathogenic Bacteria: The pathogenic bacterium *Chrysobacterium aquaticum* (KCTC12483) was purchased as a lyophilized powder from the Pasteur Institute of Iran. After two weeks of feeding the fish, each trout received an injection of 20 µL of the 24-hour culture medium from 10^7 CFU g^{-1} dilution of *Chryseobacterium aquaticum*. For up to two weeks, casualties were reported daily (13).

Statistical Analysis: The results of the MTT test were presented in bar charts in Excel, and samples with significant differences were compared with the negative control at a high confidence level ($p < 0.05$) in SPSS ANOVA v.21.

Results

Antimicrobial Activity: The broth microdilution method was used to evaluate the antibacterial effects of the extract produced by the Lactobacilli under study. This method is one of the most basic methods of antimicrobial susceptibility testing, succeeded in dramatically reducing the number of pathogenic colonies compared to the control, indicating the antibacterial properties of the bacteriocins. In the broth microdilution method, comparisons of single-culture *A. hydrophila* colonies and *A. hydrophila* and probiotic co-cultures in three different dilutions revealed a significant decrease in the growth of this bacterium and a high inhibitory effect of the probiotics under study. Tetracycline was used as a positive control, and no bacteria were detected in the presence of this antibiotic.

The Effect of Bacteriocin on Fish Lymphocyte: The results of this section are illustrated in Figure 1. In general, *L. acidophilus* had the most growth stimulating effect. With increasing concentrations from 0.5 to 2 mg/ml, all bacteria showed an increasing trend in their impact on lymphocyte cells. *L. acidophilus* at concentrations of 0.5, 1, and 2 mg/ml increased the growth of blood lymphocyte cells dose-dependently. The greatest effect of this bacterium was observed at 2 mg/ml concentration, which was 2.25 times higher than that of the negative control, causing the proliferation of blood lymphocyte cells. *L. plantarum* was also able to increase the growth of blood lymphocyte cells by increasing the concentration and stimulating the growth of 2.15 times more than in the negative control. There was a significant difference between both of these bacteria and the control in all three concentrations.

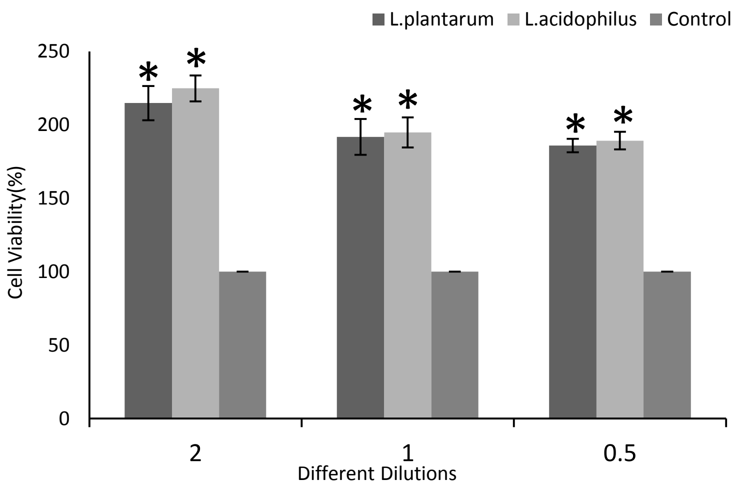

Fig. 1- The Cytotoxicity test (MTT) results for rainbow trout blood lymphocytes inoculated with bacteriocin of two lactobacillus under study.
* Samples with a significant difference with negative control

Response to Feed Intake and Growth Performance in the Rainbow Trout: The results of the growth factors are illustrated in figures 2 and 3. Regarding weight gain percentages (%WG), the treatment containing *L. acidophilus* with a 188% WG over 60 days showed the greatest effect on fish growth. Then, the synergistic treatment was the second highest with 144% WG, and finally, *L.plantarum* with 116% WG. All the treatments were able to show WG above 100%, whereas the control treatment showed only 56% WG over the two-month period. The same was correct for daily growth rate (DGR) and specific growth rate (SGR), in that the *L. acidophilus* treatment had the highest DGR and SGR, which were 0.78 and 1.76, respectively. Then, the synergistic treatment showed the second-highest DGR and SGR, 0.6 and 1.48, respectively. Finally, the *L. plantarum* treatment with DGR and SGR of 1.28 and 0.48, respectively, showed the least effect on growth. However, compared to the control with the DGR and the SGR of 0.23 and 0.74, respectively, it had a favorable impact on the fish growth factors.

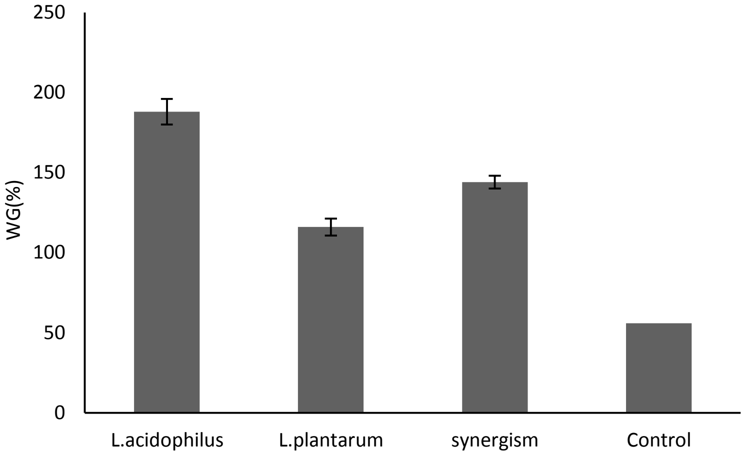

Fig. 2- Weight gain percentages (%WG) in the rainbow trout treated with *lactobacillus acidophilus*, *lactobacillus plantarum,* and synergism from day 0 to 60, with a significant difference with negative control.

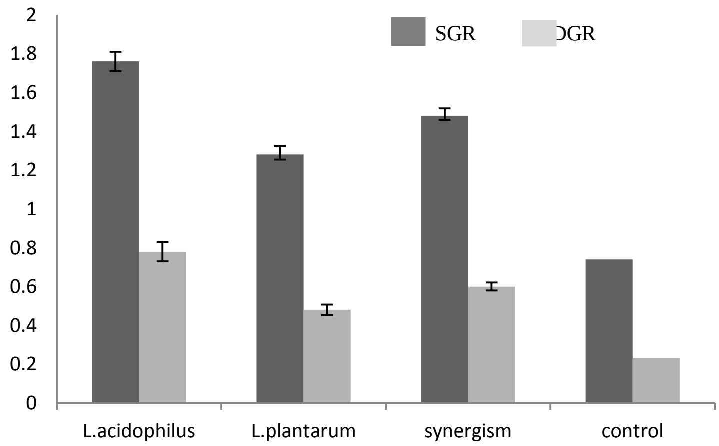

Fig. 3- Daily growth rate (DGR) and specific growth rate (SGR), over 60 days in the rainbow trout treated with *lactobacillus acidophilus*, *lactobacillus plantarum,* and synergism, with a significant difference with negative control

Cell Viability Assay of Probiotic-treated Fish Lymphocyte: The *L. acidophilus*-fed treatment showed the highest growth rate in the blood lymphocyte cells and showed the highest survival rate (%SR) of 2.0185 times more than in the control treatment (Figure 4). Then, the *L. plantarum* treatment had an SR of 1.788 times, which was very close to that of the synergistic treatment, an increase in the SR of 1.766 times. The control treatment showed only 100% survival. Compared with the control of the *L. acidophilus* treatment, it was able to increase the viability of blood lymphocytes by approximately twice.

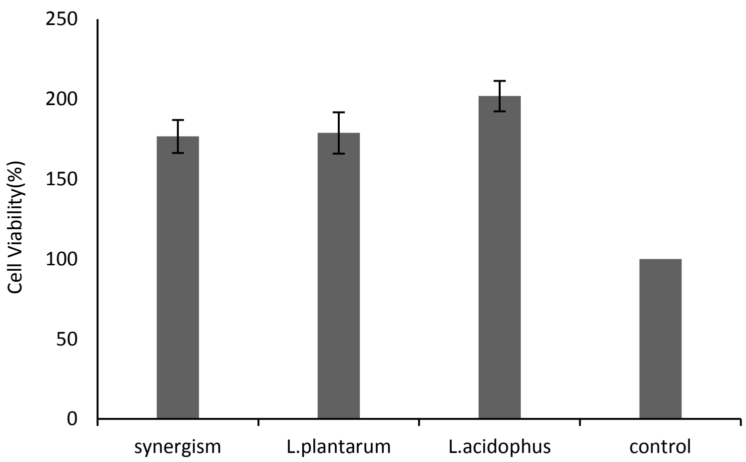

Fig. 4- Cell viability of blood lymphocytes of the rainbow trout treated with *lactobacillus acidophilus*, *lactobacillus plantarum*, and synergism within 60 days, with a significant difference with negative control

The Effect of Probiotics on Fish Infected with Pathogenic Bacteria: After inoculation with *Chrysobacterium aquaticum* for 14 days, the lowest mortality was related to *l.acidophilus* treatment, which was only 26%. Thereafter, the two synergistic and *l. plantarum* treatments had the same mortality rate of 33%, which was reasonable compared to the control treatment with 53% mortality. Figure 5 presents the mortality results of each treatment, along with their percentages.

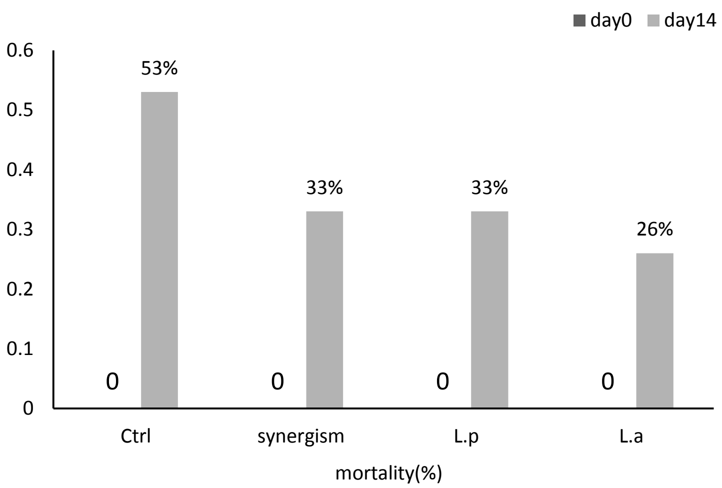

Fig. 5- The Mortality Rate of each Treatment after Inoculation with *Chrysobacterium Aquaticum* during 14 Days

Discussion and Conclusion

The purpose of this study was to evaluate the antimicrobial activity of bacteriocins produced by *L. acidophilus* and *L. plantarum* against the pathogenic fish bacteria in the in vitro part and to evaluate the overall nutritional effect of these two probiotics in the improvement of the immune response, resistance to pathogenic bacteria, and increase in growth factors in the in vivo part of the study. However, few studies have ever been conducted on several species of *Lactobacillus probiotics* on such a large scale, investigating the use of both bacteriocins and the direct feeding of probiotics both individually and synergistically to the principal species of commercial fish (19).

According to the results of this study, the addition of *L. acidophilus* and *L. plantarum* probiotics separately and synergistically to the rainbow trout diet led to a significant difference between the experimental and control treatments. Regarding the white blood cell count, the immune system in the experimental treatments was stimulated by the probiotic use, and the highest lymphocyte levels were related to the *L. acidophilus* treatment and the synergism treatment, respectively. Based on some studies, probiotic mixtures appear to outperform single strains, such as the case of the *L. plantarum* treatment, which was weaker than its synergism with *L. acidophilus* (20). However, this was not the case for the *L. acidophilus* treatment.

Some studies have examined the effect of a single probiotic on the immune system in fish, such as the study by Kamgar and colleagues, who investigated the effect of *Bacillus subtilis* as a probiotic on the hematological and biochemical factors of the rainbow trout. The number of white blood cells, lymphocytes in the treatment group was significantly higher than in the control group ($p < 0.05$). Also, a study by El Dohail and colleagues showed that the use of *L. acidophilus* at a rate of 3×10^7 CFU / g of food in the African catfish for 12 weeks improved blood parameters, which was consistent with the results of the present study (21).

One of the beneficial effects of probiotics on living organisms, according to the literature, is to improve host nutrition through the production of digestive enzymes and growth supplements, thereby enhancing survival, nutritional efficiency, preventing intestinal disorders, and digesting the nutrients contained in food ingredients (22-28). Probiotics can also improve nutrition by harmful detoxifying compounds in the diet by enzymatic hydrolysis and producing vitamins such as biotin and vitamin B12. Overall, probiotics appear to increase digestibility and absorption of food, thereby enhancing nutritional efficiency by enhancing digestive enzyme activity and secretion of these enzymes into the fish digestive tract. Also, they increase food intake even under stressful conditions and increase growth indices by balancing the digestive microflora (6, 23).

The results of the present study revealed that in the case of all the growth indices, including weight gain, specific growth rate, and daily growth rate, *L. acidophilus* and synergism treatment had the highest effect on the rainbow trout diet. Additionally, all the treatments on day 60 had higher growth factors than in the control treatment. Increased growth indices following oral administration of various probiotics in other fish species have been reported in various studies (10, 23, 29-36). Probiotics are used for two reasons: a) improving the aquatic environment, b) introducing beneficial microflora into the digestive tract. Today, the presence of probiotics can be seen in all areas of agricultural production, aquaculture, and the environment.

In the aquaculture industry, probiotics have recently been widely used in almost every area. Increased foodborne illnesses and poisoning, along with economic and social problems, have led to the growth of studies on healthy food production and the application of new antimicrobial compounds (37). The ability of lactic acid bacteria to produce acids and antimicrobials has long been used for food storage (38). Food fermentation by lactic acid bacteria reduces the amount of carbohydrates available and results in the production of a variety of low molecular mass molecules with antimicrobial properties (37, 39). The mechanism of action of these lactobacilli has been shown in various studies (12, 40-42).

In antimicrobial assays in this study, the lactobacilli’s bacteriocins, which were antimicrobial peptides, were isolated by centrifugation and concentrated by freeze-drying. Of the experimental methods, only the microdilution method was able to demonstrate the antimicrobial properties of this probiotic because it was more accurate than other methods, while the results of in vivo experiments revealed that both probiotics, whether individually or synergistically, enhanced the survival rate of the rainbow trout, which was in line with the results of some other studies on probiotic effects in different animals (43). The *L. acidophilus* and *L. plantarum* probiotics under study inhibited the growth of the pathogens in the fish gastrointestinal tract by increasing the number of beneficial bacteria in their intestine by producing lactic acid and decreasing the intestinal pH. They significantly increased their resistance to bacteria and viruses and other stressors by stimulating their immune system. It can also be concluded that the probiotics under study contributed to higher survival rates of the rainbow trout under study.

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Conflict of Interest

It is to specifically state that there is “No Conflict of Interest” with other people or organizations that could inappropriately influence or bias the content of the paper.

Ethical Approval

The research meets all applicable standards with regard to the ethics of experimentation and research integrity, and the following is being certified true.

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