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The effect of oral administration of encapsulated *Lactiplantibacillus plantarum* on the efficacy and immunogenicity of *Aeromonas hydrophila* vaccine in common carp

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Abstract

In the present study the effect of oral administration of probiotics *Lactiplantibacillus plantarum* in free form and microencapsulated with alginate/chitosan on immunogenicity and efficacy of Aeromonas *hydrophila* vaccine was evaluated in common carp. Three hundred and sixty common carps (48±5.1 gBW) were randomly divided into four equal groups in triplicates. Group 1 was vaccinated against *A. hydrophila*. Group 2 received the same vaccination and was also administered a diet supplemented with *Lactobacillus plantarum*. Group 3 was vaccinated and fed with encapsulated *L. plantarum*. Group 4, serving as the control, was fed with a basic diet without any supplementation. Biometrical measurement, blood and intestinal samples were taken on day zero, 30 and 60 of the experiment. Growth performance indices (Feed conversion ratio, specific growth rate, Protein efficacy ratio and food efficacy ratio) as well as immunological parameters (Antibody titer, serum lysozyme, complement and bactericidal activity, NBT reduction, globulin level and myeloperoxidase activity) were measured and compared among the groups. Meanwhile hematological parameters (Red Blood Cells, White Blood Cells, Hemoglobin and Hematocrit), intestinal enzyme activity (lipase, proteas, amylase and ALP). Antioxidant status (MDA level, SOD, GSH and catalase activity) and some serum biochemical indices (glucose, urea, Ca, Tg, ALP, CPK and Bilirubin) were measured and compared among the groups. On day 60 of the experiment the remained fish in each group were challenged with virulent *A. hydrophila* and cumulative mortality was recorded for 14 days. Results showed that the highest growth indices and intestinal enzyme activity were recorded in group 3 which were fed with encapsulated *L. plantarum*. Most of the immune indices evaluated in the study showed a significant increase in treatments 3 and 2 compared to the control group. The blood parameters and serum biochemical indices did not show significant differences among the groups. The mortality rate after the challenge was significantly lower in treatments 2 and 3 (30%) compared to the control group (60%). Overall, it can be concluded that not only the administration of *L. plantarum* play a role in improving the efficacy and immunogenicity of the injectable *A. hydrophila* vaccine in common carp, but also microencapsulation of this probiotic with alginate/chitosan enhances its effect on the vaccine's efficacy and immunogenicity. Therefore, the use of this microencapsulation method is recommended to improve the efficacy of the probiotic and the vaccine.

Key words: *Aeromonas hydrophila* vaccine, *Lactiplantibacillus plantarum*, Microencapsulation, Common carp, Immunogenicity

Introduction

Common carp (*Cyprinus carpio*) is one of the most widely cultivated fish species globally, with a significant role in aquaculture. Annually, global production of

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common carp exceeds 5 million tons, making it a cornerstone of freshwater fish farming, especially in Asia. The species is favored for its adaptability to various environments, fast growth rate, resistance to diseases, and its remarkability to many populations (FAO, 2023). This species is considered to be the most important species in Iraq aquaculture which dominates culture in fresh and brackish water with over 85% of the Iraqi aquaculture production (Esmaeili, 2021).

Cyprinid fish, particularly *Cyprinus carpio*, are highly susceptible to *A. hydrophila*, which causes fatal diseases like Aeromonas Hemorrhagic Septicemia. This severe infectious disease affects a wide range of aquatic animals, leading to extensive internal and external bleeding and resulting in a high mortality rate among susceptible species (Chen et al, 2020). *A. hydrophila* is one of the primary causes of the annual mortality of common carp in Iraq (Hossain and Heo, 2021).

One method of combating Aeromonas infections in aquaculture is through antibiotic therapy. However, the use of antibiotics in this setting comes with several disadvantages. A significant concern is the development of antibiotic-resistant bacteria, which can pose serious risks to both aquatic life and human health. Furthermore, the accumulation of antibiotics in the environment can disrupt ecosystems, harming beneficial microorganisms and potentially leading to bioaccumulation in fish that may be consumed by humans. Overreliance on antibiotics also tends to obscure underlying issues such as poor water quality or inadequate husbandry practices, which need to be addressed to ensure the sustainability of aquaculture. The high cost of antibiotics and the need for repeated treatments are also disadvantages of antibiotic therapy in aquatic animals (Gilani et al, 2024).

Vaccination is often considered the best and most logical method for preventing and controlling *Aeromonas* infections in fish (Schulz et al, 2020). Vaccination plays a significant role in reducing the incidence and severity of infections caused by this pathogenic bacterium; it boosts the immune response of fish, making them more resilient to *A. hydrophila* (Farias et al, 2020). With the implementation of proper vaccination protocols, the aquaculture industry can mitigate the economic losses associated with these infections, leading to sustainable fish production and improved food security (Nayak et al, 2022).

Extensive research has been conducted to prevent *A. hydrophila* infections in various aquatic organisms, including common carp by development of an extremely effective *A. hydrophila* vaccine (Abdul et al, 2022; Nayak, 2020). However, even after vaccination, fish can still exhibit symptoms of the disease when exposed to high levels of stress.

One of the problems with vaccines produced against Aeromonas infections is their relatively low efficacy and immunogenicity, which is largely related to the antigenic structure of these bacteria and the rapid changes in their surface antigens (Zhang et al, 2023). Therefore, the use of immune stimulants, such as probiotics, after fish vaccination against Aeromonas is strongly recommended to enhance immune responses (Wang et al, 2020).

Probiotics not only influence fish growth performance and health status by establishing and restoring balance in the gut bacterial flora, but they also enhance vaccine efficacy and immunogenicity by improving fish immune responses and health status (Hosainifar et al, 2020). Numerous studies have been conducted on the effect of oral probiotic administration on fish vaccine efficacy and immunogenicity. Guimarães et al, (2022) reported an improvement in the efficacy of the streptococcosis vaccine tilapia following Lactobacillus species administration.

Lactoplantibacillus plantarum is one of the most important probiotic bacteria, with its probiotic properties in fish well established. The effects of this bacterium have

been demonstrated in various fish species, including common carp, tilapia, and trout (Radkhah et al, 2024; Alishahi et al, 2022; Mohammadian et al, 2022). One challenge with administering oral probiotics in fish is the loss and degradation of these bacteria in the gastrointestinal environment, which diminishes their probiotic potency. To enhance the effects of this bacterium, it is crucial to protect the bacteria from gastrointestinal conditions. Various microencapsulation methods have been developed for this purpose. The use of biodegradable polymers, such as chitosan and alginate, for microencapsulating probiotics and shielding them from gastrointestinal conditions has recently gained increased attention from researchers (Ahmadmoradi et al, 2024; Hosseini et al, 2022). In addition to protecting probiotic bacteria in adverse gastrointestinal conditions, alginate and chitosan also act as immunostimulants, boosting the fish's immune response and overall health.

In the present study, based on our previous experience and research, alginate and chitosan were selected for the microencapsulation of the *Lactobacillus plantarum* using the emulsification method. Subsequently, the effect of the microencapsulated probiotic on the efficacy and immunogenicity of the *Aeromonas hydrophila* vaccine in common carp was evaluated.

Materials and methods

Bacterial strains

L. plantarum was selected from 30 lactic acid bacteria isolated from intestinal flora of wild and reared healthy cyprinid fish of cyprinid farms of Ahvaz, Iran, based on their in-vitro probiotic characteristics. The selected isolates were primarily identified microbiologically according to morphology of colonies, Gram staining, biochemical tests, and finally molecular identification via 16S rRNA gene sequencing (Mohammadian et al, 2016; Mohammadian et al, 2022).

Probiotic preparation

Lyophilized *L. plantarum* was inoculated in 10 ml Man Rogosa Sharpe (MRS) broth medium incubated at 37 $^{\circ}C$ for 48 hours using anaerobic jar. Following incubation of plates, the bacteria were harvested by centrifugation (10 min in 3000 g), and cells were washed tree times with PBS (pH = 7.2). The probiotic concentration was adjusted to 3×10^{8} CFU g food $^{-1}$ through OD absorption in 620 A by spectrophotometer

Microencapsulation of L. plantarum

Microencapsulation of *L. plantarum* with chitosan/alginate (MLCA) was done according to the emulsification method (Jiang et al, 2013; Hosseini et al, 2022). Briefly, the mixture of *L.plantarum* (10^{8} CFU g^{-1}), sodium alginate, and 15% (v/v) glycerol was dropped into 0.1 M $CaCl_2$ by passing through a cannula-like syringe in the presence of nitrogen gas pressure. The sodium alginate final concentration was 2% (w/v). Formed microcapsules were incubated for 30 min and then washed with 0.85% saline to remove unreacted $CaCl_2$. The chitosan (MW 10,000) solution 0.8% (w/v) was used to coat microcapsules for 30 min followed by two times washing. The microcapsules coated with chitosan-alginate were further coated with 0.1% (w/v) sodium alginate for 10 min followed by washing. Then microcapsules were stored at 4 $^{\circ}C$ until used. The control microcapsules without bacteria were also prepared by the same procedure.

Diet preparation

In this study, the experimental diet was prepared according to the method. The experimental diets were prepared based on Van Doan et al, (2016) as follows: Diet 1 basal diet without supplementation (for group1 and group 4), Diet 2 incorporated with 10^{8} CFU/g *L. Plantarum* (group 2), and Diet 3 incorporated with 10^{8} CFU/g encapsulated *L. bulgaricus* (group 3). To prepare feed containing probiotics at a concentration of 10^{8} CFU/g, first, the concentration

of the bacteria in initial stock was determined. The appropriate amount of bacterial stock for one kilogram of feed was mixed with 50 milliliters of PBS and sprayed uniformly over the feed. Then, liquid gelatin at 5 grams per liter and (55°C) was sprayed over the feed to protect the probiotic bacteria from dispersing in water. For the control group, all steps were repeated without the probiotic bacteria. To maintain high levels of probiotics, fresh batches of the diets were prepared every two weeks (Hosseini et al., 2022).

Vaccination against *A. hydrophila*

The high-virulence *A. hydrophila* of this experiment was selected from 12 pathogenic *A. hydrophila* isolated from diseased common carp referred to Fish Health Laboratory of Veterinary Faculty of Shahid Chamran University of Ahvaz, Iran. The severity of these isolates was evaluated based on in-vitro and in-vivo virulence assays and the highest virulent isolate was selected as a vaccine seed. Selected *A. hydrophilic* was identified using 16S RNA PCR- methods and confirmed through nucleotide sequencing.

Formalin killed *A. hydrophila* was prepared according to Abdy et al., (2017) briefly, *A. hydrophila* was first cultured in TSB medium and incubated for 36 hours at 30˚C. The bacteria were adjusted to 10^{10} cfu ml^{-1}, inactivated with 0.5% formalin for one hour. Formalin Killed Cells (FKC was washed twice (6000 g; for 30 min) with phosphate-buffered saline (PBS); then, it was cultivated in TSA plates and incubated at 30°C for 24h to guarantee outright bacterial inactivation. The immunization was conducted by injection in the peritoneal area 100 microliters per fish of *A. hydrophila* bacterin with a concentration of 10^{10} bacteria per milliliter, in the first day of experiment following the booster in day 21th of experiment.

Fish and experimental design

Three hundred and sixty healthy common carp (*cyprinus carpio*) fingerlings (48±5.1 g, Mean± SD) that had no previous history of parasitic infections and no signs of disease (gross and microscopic examination of gills, skin) were obtained from Azadegan cyprinid farm, Ahvaz, Iran. The fish were transferred to the laboratory of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran acclimatized to laboratory conditions for 2 weeks in 500-L plastic quarantine tanks at 27±2 °C and fed with the control diet. The fish was randomly divided into 4 groups of 90 equal pieces, each group consisting of 3 replicates (30 fish each) stored for 60 days. The fish were divided into four equal groups with three replicates each (each replicate consisting of 30 fish). The first group was vaccinated with the injectable *A. hydrophila* vaccine. The second group was vaccinated and also fed with probiotics at a concentration of 10^8 CFU/g in the feed. The third group was vaccinated with the *A. hydrophila* and fed with microencapsulated probiotics. The fourth group served as the control group and was fed with a basal diet.

In each tank, approximately 25% of the water was exchanged daily, and 100% of the water was exchanged once a week, The fish were fed ad libitum twice daily, at 7:00 a.m. and 6:00 p.m., with the diets being hand-fed. Basic physicochemical parameters of the water were measured weekly to ensure optimal conditions. The O_2 concentration was maintained at no less than 5 mg $^{L-1}$ and pH ranged from 7.5 to 8.2 throughout the study period.

For the whole 60-day raising period, the feeding rate was ad libitum at 3% biomass and the uneaten feed was then siphoned away and dried separately in order to calculate the feed conversion ratio (FCR).

Sample collection

Sampling was performed on days zero, 30 and 60 of experiment and a total of 9 fish (3 fish from each replicate) were randomly

collected from each group for hemato-immunological and biochemical assays. After anesthetizing the fish with 2-phenoxiethanol (400 ppm), blood was collected from the caudal vein using a 1 mL syringe. Then, the blood samples were transferred to 1.5 ml microtubes with or without anticoagulant for hematological and immunological parameters respectively. Serum samples were collected by centrifuging at 3000 rpm for 10 minutes and stored at -20 ° C until used.

Growth performance

In the sampling points on days 0, 30 and 60, fish in each replica were weighed. The survival rate and growth performance of fish were calculated using the following equations:

Weight gain (WG) = final weight (g) - initial weight (g)

Specific growth rate (SGR %) = 100 - (ln final weight - ln initial weight)/Duration of experiment

Feed conversion ratio (FCR) = feed given (dried weight)/ weight gain (wet weight)

Survival rate (%) = (final fish number/ initial fish number) *100و

Food Efficacy Rate (FER) = 100 (final weight – Initial weight)/feed consumed,

Daily weight gain (DWG) = (final weight/initial weight)/time,

Protein efficiency ratio (PER) = Weight gain (g)/protein intake

Digestive enzyme activity

To analyze the digestive enzymes, the selected fish for sampling were euthanized after blood collection, and then intestine samples were taken following dissection. The intestine was dissected out using sterile technique at low temperature (on the ice-pack) and homogenized. Extracts utilized for enzyme assay were obtained after homogenization of intestine in cold 50 mM Tris–HCl buffer, pH 8.0 (1:9 v/w) followed by centrifugation (13.500 ×g; 30 min at 4 °C). The supernatant was then collected and kept at −80 °C in small portions for later determinations (Mohammadian et al., 2022). Bradford (1976) was used to assess the activity of total protein content in the gut using the diluted supernatant and bovine serum albumin as a reference. The α-amylase activity of the intestine was also measured using a soluble starch solution (Sigma– Aldrich) as the substrate, as described by Areekijseree et al. (2004). Amylase activity was measured in mol maltose generated per milligram of protein per minute. At room temperature, trypsin activity was determined using N-BenzoylL-arginine ethyl ester (BAEE) as a substrate in the presence of 0.1 mM HCl (Erlanger et al, 1961). The lipase activity was measured by quantifying the release of fatty acids from the enzymatic hydrolysis of triglycerides into glycerol in a stabilized olive oil emulsion (Fluka™) (Borlongan, 1990).

According to a modified approach (Otto et al., 1946), total ALP activity in homogenized tissue was measured at 410 nm and 37 °C using Pnitrophenyl phosphate as substrate and 2- amino-2-methyl-1-propanol buffer (0.84 mM, pH= 10.3). Casein (Sigma–Aldrich) was used as a substrate for measuring protease activity, and the result was subsequently reacted with Folin's reagent (Anson, 1938, with modifications). The absorbencies of each individual sample were determined using a spectrophotometer (UV-2802S; Unico, Shanghai, China), and the enzyme activities that were recorded as absorbance were modified and then reported as specific activity (U mg-1 protein min-1) (Erlanger et al, 1961).

Immunological parameters

Lysozyme activity assay

Serum lysozyme activity was determined turbidometrically according to the method described by Ellis. 1990. One hundred and thirty five µl of the *Micrococcus 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) were mixed with 15 µl of each sample. Reduction of absorbance of 0.001 min^{-1} of samples was defined as one unit of lysozyme activity.

Alternative complement pathway activity

Alternative Complement Activity Pathway (ACP) of serum samples was measured and calculated based on Yano (Boshra et al., 2006) method using rabbit red blood cells (RaRBC).

Briefly, Veronal buffer used for the serum samples was diluted (5 times); then, 1% Rabbit RBC was gently poured into each well. Following 24 h incubation at 4 °C, the samples were centrifuged (five minutes in 3500 g). Afterward, 150 µl of supernatants were transferred to the wells of the microplate; then, the OD of each well was measured at 540 nm by an ELISA reader (Accu Reader, Taiwan).

Respiratory burst activity

The respiratory burst activity of leukocytes was evaluated using Nitro Blue Tetrazolium (NBT) according to the method suggested by Alishahi et al (2019) with minor modifications. In brief, 100 µl of blood samples were mixed with 100 µl of NBT (0.2 % in distilled water). The plate was well shaken and incubated for 30 min at 25 ˚C. Afterward, 2000 µl dimethylformamide was gently added to 100 µl of the prepared mixture; then, the final substance was centrifuged (at 3000 rpm for 10 minutes). Finally, the optical density of the supernatant was measured by a spectrophotometer (Shimadzu, Japan) at 620 nm.

Serum Bactericidal activity

The serum bactericidal activity was measured according to Yin et al (Dezfuly et al. 2020), with some modifications. The bacteria culture *(A.hydrophilla*) was pelleted (3000 g, 10 min) and washed 3 times with sterile PBS. A volume of 25 µL bacterial suspension (adjusted to 4×10^9 cells/ml) was added to 25 µL serum of fish in sterile Eppendorf tubes. Then, the tube was incubated at room temperature for 1 h. After that, plating the mixtures on TSA containing 1.5% NaCl was used to determine colony forming units (CFU)/ml.

Anti *A. hydrophila* antibody titer

A. hydrophila antibody levels in plasma were measured by ELISA with some modifications (Skov *et al*., 2018). Concisely, Microplate (Nunc, Denmark) was coated with 50 µL well ^{1} of formalin-killed and sonically disrupted *A.hydrophila* (100 µg/mL) antigen at a 1:15 dilution in bicarbonate coating buffer (pH=9.6) for 18 h at 4°C. After washing the plate; Common carp plasma samples (100 µL) were then, added at a 1:20 and 1:1 dilution respectively in PBS+0.05% Tween-20 (PBS-T) containing 0.1% skim milk. After 90 min incubation at 25°C, 100 µL of mouse anti common carp monoclonal immunoglobulin at a 1:4000 dilution in PBS-T containing 0.1% skim milk was added to all wells and then shaken for 60 min. After washing, 50 µL of goat anti-mouse IgG HRP conjugate (Sigma-Aldrich) at a 1:2500 dilution in PBS-T containing 0.1% skim milk was added and incubated for 60 min. Plates were washed as above and 50 µL TMB (3,3', 5,5; -tetramethylbenzidine - H2O2) chromogenic solution was added to each well for 10 min at 25°C. The reaction was stopped with 50 µL 2 N H_2SO_4. Lastly, serum and mucus antibody levels were read spectrophotometrically at 450 nm by an ELISA reader (Accu Reader, Taiwan).

Hematology and biochemical indices

RBC and WBC counts were determined using an improved Neubauer hemocytometer. Hemoglobin (Hb) concentration (g dl^{-1}) was estimated by cyano methemoglobin method using Drabkin's reagent. Hematocrit (Hct) was determined using microhematocrit capillaries filled with blood and centrifuged at 10000×g for 5 min and expressed as percentage of total blood volume (Thrall, 2004).

Serum biochemical indices, including levels of urea, calcium, glucose, triglycerides, alkaline phosphatase, creatine phosphokinase, and bilirubin, were measured using an autoanalyzer and commercial laboratory kits.

Antioxidant Status

Liver samples were obtained from each fish after euthanasia. After blood sampling and dissection of the fish, liver samples were collected, weighed, and then homogenized in ratio of 1–9 (w/v) of cold potassium phosphate buffer (0.1 M, pH =7.4, 4°C) at 10,000x g for 60 s. The homogenate was centrifuged (9,000x g, 30 min, 4°C); the supernatant was removed and aliquoted, then kept at −80°C. Catalase (CAT) (E.C. 1.11.1.6), superoxide dismutase (SOD) (E.C. 1.15.1.1) (McCord and Fridovich, 1969), and GSH level were determined according to the standard methods.

Intestinal bacterial flora

Samples of intestine were analyzed to quantify the total and Lactobacilli counts. Nine samples from each group were taken in each sampling point after blood sample collection and dissection. One gram of the samples was then homogenized by 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH=7.0) and stirred into 1 min in the stomacher (Heidolph instruments, Germany). Serial dilutions of each were then prepared under the sterile condition and spread on MRS and TSA plates. Following the 48 h incubation at 30 ℃, the number of colony on each plate was counted and reported as colony-forming units (CFU) per gram of sample.

Determination of LD 50

Before performing the challenge, the following steps were taken to calculate the median rate of lethality of the *A. hydrophila* for common carp (Alishahi et al., 2024). Briefly *A. hydrophila* was cultured in TSB culture medium for 48 hours at 37˚ C. The bacteria were adjusted to 10^8 cfu ml^{-1} after centrifugation (4000 rpm, 10 min). The 10-fold serial dilutions (10^5 to 10^8 CFU ml^{-1}) of the *A. hydrophila* were prepared in PBS and 0.1 ml of each concentration of *A. hydrophila* was injected intraperitoneally to 10 fish (each replicate) in a separate aquarium. The dead fish were netted and recorded daily for 10 days. The rate of mortality was analyzed and the LD50 was determined by Probit software using version 22 of SPSS. The LD50 of *A. hydrophila* in common carp calculated as 1.2×10^6. At the end of the study, the fish were challenged with this concentration of bacteria (Aramon et al, 2024).

Bacterial challenge

The remained fish in each group (at least 30 fish,10 fish from each replicate) were injected with live *A. hydrophila* via intraperitoneal route at day 60 of experiment. Firstly, the sedation of fish was done by 2-phenoxyethanol (300 mg l^{-1}) and 100 microliter of 1.2×10^6 cfu ml^{-1} of *A. hydrophila* (LD_{50} concentration) was injected intraperitoneally. After injection of the bacteria the fish were put to 100 L aquaria. The dead fish of each treatment were netted and checked two times daily for 10 days and the number of dead fish was recorded. The cumulative mortality rate (CMR) was calculated after mortality recording for10 days (Alishahi et al., 2018 and 2024). For confirmation of the cause of death re-isolation of *A. hydrophila* was done from the kidney and liver of the dead fish.

Statistical analysis

Before statistical analysis of data, their normality was determined using the Kolmogorov-Smirnov test. One-way ANOVA with Multiple Comparisons Test was used to compare the different groups, followed by Tukey's test ($P<0.05$) and then, the quantitative data were presented as mean ± standard deviation. All statistical analyses were performed using SPSS software (Version 24).

Results

Growth indices

The results of the growth indices comparison between the experimental goups are presented in Table 1. As shown in the table, nearly all growth indices including feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), daily weight gain (DWG), and food efficiency ratio (FER) improved significantly in the

probiotic-fed treatments compared to the control and the vaccinated group (without probiotics) at both sampling times (P<0.05). The group vaccinated with *A. hydrophila* and fed with *Lactobacillus plantarum* microencapsulated with alginate and chitosan demonstrated the highest growth indices at both sampling stages compared to the other treatments (P<0.05). Survival rates were 100% across all the experimental treatments, with no mortality observed during the study.

Table 1: Growth performance indices of the experimental groups at days 30 and 60 of experiment

	Groups	SGR	FCR	PER	DWG	FER
Day 30	A	0.56±0.03 ^{ab}	2.08±0.13 ^{b}	1.51±0.10 ^{b}	0.34±0.04 ^{b}	48.24±3.16 ^{b}
	B	0.65±0.15 ^{a}	1.86±0.44 ^{ab}	1.49±0.31 ^{b}	0.40±0.08 ^{b}	47.66±9.96 ^{b}
	C	0.74±0.09 ^{a}	1.45±0.19 ^{a}	2.13±0.37 ^{a}	0.50±0.08 ^{a}	78.01±7.88 ^{a}
	D	0.47±0.14^{b}	2.23±0.52 ^{b}	1.45±0.30 ^{b}	0.31±0.07 ^{b}	46.37±9.73 ^{b}
Day 60	A	1.09±0.19^{ab}	2.31±0.07	1.35±0.14	0.91±0.23 ^{b}	39.96±4.6 ^{b}
	B	1.16±0.03^{ab}	2.06±0.15 ^{ab}	1.22±0.07 ^{b}	0.95±0.02	39.17±2.32 ^{b}
	C	1.24±0.05^{a}	1.87±0.45 ^{a}	1.31±0.19 ^{a}	1.02±0.21 ^{a}	43.42±2.22 ^{a}
	D	0.98±0.02^{b}	2.24±0.19 ^{b}	1.26±0.09 ^{b}	0.96±0.09 ^{b}	36.91±4.77 ^{b}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Digestive enzyme activity

The results of the comparison of digestive enzyme activities in the experimental groups at three sampling points are presented in Table 2. As shown in table 2, ALP and amylase showed a significant increase in both probiotic-treated groups (with and without microencapsulation) on days zero, 30 and 60 of the study, compared to the control treatment (P<0.05). However, the activities of protease, lipase, and trypsin were significantly increased only in the vaccinated treatment fed with microencapsulated probiotics compared to the control group in days 30 and 60 of experiment (P<0.05).

Table 2: The activity of intestinal digestive enzymes of the experimental groups at days zero, 30 and 60 of experiment

	Groups	ALP	Amylase	protease	Lipase	Tripsine
Day 0	A	14.54±3.07 ^{a}	47.48±11.30 ^{a}	0.12±0.02 ^{a}	7.59±2.84^{a}	65.41±18.5^{a}
	B	13.04±2.04 ^{a}	42.29±24.65 ^{a}	0.14±0.02 ^{a}	8.22±2.31^{a}	47.43±0.19^{a}
	C	13.79±2.55 ^{a}	44.88±17.97 ^{a}	0.13±0.02 ^{a}	7.91±2.58^{a}	56.42±19.7^{a}
	D	13.41±2.29 ^{a}	43.59±21.31 ^{a}	0.13±0.01^{a}	8.06±2.45 ^{a}	51.92±17.4^{a}
Day 30	A	12.20±4.18 ^{b}	43.70±21.9 ^{b}	0.12±0.03 ^{b}	8.11±1.93 ^{b}	45.87±16.3 ^{b}
	B	17.67±3.45 ^{a}	43.89±18.67 ^{b}	0.11±0.02 ^{b}	7.99±2.87 ^{b}	53.73±18.9 ^{b}
	C	16.71±4.04 ^{a}	64.83±20.33 ^{a}	0.16±0.01^{a}	11.8±3.65 ^{a}	92.49±26.8 ^{a}
	D	12.54±3.07 ^{b}	47.48±11.5 ^{b}	0.12±0.02 ^{b}	7.59±2.84 ^{b}	65.41±27.3 ^{b}
Day 60	A	14.41±4.28 ^{b}	47.62±11.23 ^{b}	0.13±0.02 ^{b}	8.13±2.26 ^{a}	52.55±19.2 ^{b}
	B	15.50±8.99 ^{a}	73.29±11.33 ^{a}	0.16±0.03 ^{ab}	9.79±1.83 ^{a}	63.50±12.6^{ab}
	C	17.79±3.74 ^{a}	80.55±24.49 ^{a}	0.17±0.03 ^{a}	9.54±2.74 ^{a}	87.81±30.5 ^{a}
	D	13.04±2.04 ^{b}	42.29±24.65 ^{b}	0.14±0.02 ^{b}	8.22±2.31 ^{a}	47.43±15.2^{b}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Immunological parameters

The data related to the serum immune response are summarized in Table 3 and figures 1 to 3. The comparison of immune indices between the experimental treatments at the three sampling stages showed that most immune indices, including anti-*A. hydrophila* antibody titer, lysozyme activity, NBT reduction, protein and globulin levels, and antitrypsin and myeloperoxidase activities, exhibited significant increases ($P<0.05$) in the probiotic-treated groups, particularly those treated with microencapsulated probiotics, compared to the control group at days 30 and 60 of experiment. However, some immunological indices, such as alternative complement activity, albumin levels, and serum bactericidal activity, did not show significant differences between the experimental groups ($P>0.05$).

Table 3: The immunological indices of the experimental groups at days zero, 30 and 60 of experiment

	Groups	complement	Bactericidal	Protein	Albumin	Globulin	Antitrypsin	mylopor
Day 0	A	5.77±0.46 ^{a}	0.27±0.09 ^{a}	3.81±0.27 ^{a}	0.96±0.11 ^{a}	2.85±0.32 ^{a}	0.91±0.17 ^{a}	0.31±0.09 ^{a}
	B	5.97±0.50 ^{a}	0.25±0.11 ^{a}	3.87±0.20 ^{a}	0.95±0.09 ^{a}	2.92±0.18 ^{a}	0.84±0.28 ^{a}	0.37±0.14 ^{a}
	C	5.87±0.48 ^{a}	0.25±0.12 ^{a}	3.84±0.13 ^{a}	0.96±0.10 ^{a}	2.88±0.25 ^{a}	0.88±0.23 ^{a}	0.34±0.11 ^{a}
	D	5.92±0.49 ^{a}	0.21±0.08 ^{a}	3.86±0.16 ^{a}	0.95±0.10 ^{a}	2.90±0.21 ^{a}	0.86±0.26 ^{a}	0.35±0.12 ^{a}
Day 30	A	6.27±0.46 ^{a}	0.24±0.09 ^{a}	4.01±0.47 ^{ab}	0.93±0.05 ^{a}	3.07±0.46 ^{ab}	0.78±0.26 ^{b}	0.40±0.12 ^{b}
	B	6.53±0.64 ^{a}	0.31±0.06 ^{a}	4.27±0.76 ^{a}	0.95±0.11 ^{a}	3.33±0.77 ^{a}	1.20±0.17 ^{ab}	0.49±0.11 ^{a}
	C	6.23±0.67 ^{a}	0.29±0.09 ^{a}	4.38±0.43 ^{a}	0.92±0.13 ^{a}	3.46±0.25 ^{a}	1.56±0.23 ^{a}	0.43±0.11 ^{ab}
	D	5.97±0.4 ^{a}	0.28±0.11 ^{a}	3.87±0.27 ^{b}	0.95±0.14 ^{a}	2.92±0.67 ^{b}	0.84±0.23 ^{b}	0.37±0.13 ^{b}
Day 60	A	6.00±0.87 ^{a}	0.26±0.13 ^{a}	4.01±0.44 ^{a}	0.94±0.12 ^{a}	3.06±0.44 ^{ab}	0.89±0.26 ^{b}	0.32±0.12 ^{a}
	B	5.67±1.05 ^{a}	0.21±0.14 ^{a}	4.14±0.32 ^{a}	0.93±0.11 ^{a}	3.22±0.28 ^{a}	1.08±0.34 ^{ab}	0.33±0.11 ^{a}
	C	6.20±0.54 ^{a}	0.24±0.09 ^{a}	4.15±0.65 ^{a}	0.93±0.08 ^{a}	3.22±0.83^{a}	1.31±0.22 ^{a}	0.33±0.10 ^{a}
	D	5.77±0.50 ^{a}	0.29±0.12 ^{a}	3.81±0.13 ^{a}	0.96±0.09 ^{a}	2.85±0.18 ^{b}	0.91±0.28	0.31±0.11 ^{a}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences ($P<0.05$) within each sampling time).

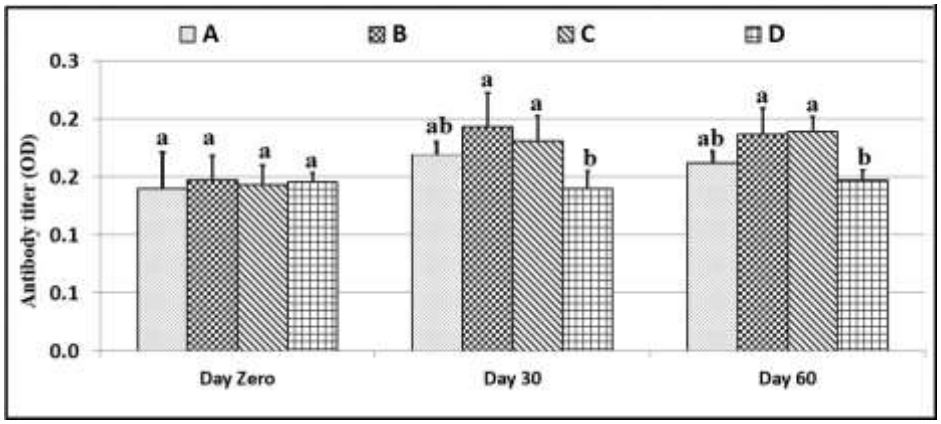

Figure 1: Anti *A.hydrophila* antibody titer of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

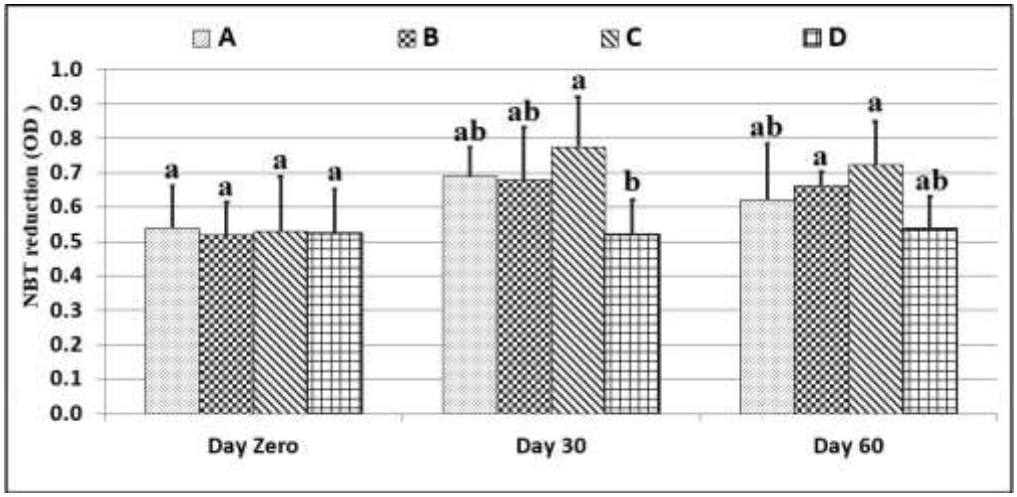

Figure 2: Nitro Blue Tetrazolium (NBT) reduction of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

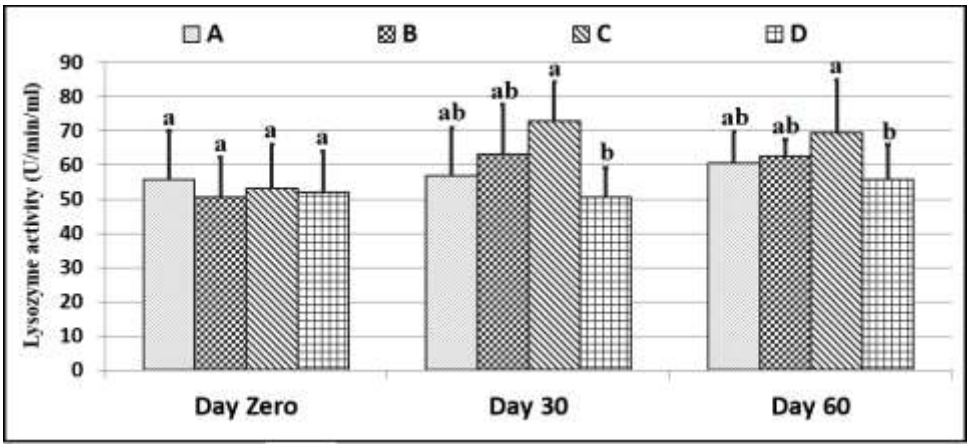

Figure 3: Lysozyme activity of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

Hematological and biochemical parameters

The data related to the serum Hematological parameters are summarized in Table 4. A comparison of the hematological parameters among the experimental groups at different sampling stages showed that the red globular-related indices (including red RBC, hemoglobin, and hematocrit) were not affected by vaccination or probiotic administration (P>0.05). However, the white blood cell counts significantly increased in the probiotic-fed treatments, particularly in those fed with microencapsulated probiotics, on days 30 and 60 of the study compared to the control group (P<0.05).

Table 4: The hematological parameters of the experimental groups at days zero, 30 and 60 of study

	Groups	Hb	PCV	RBC	WBC
Day 0	A	9.14±2.1[a]	35.20±3.03[a]	1.34±0.16[a]	21.2±6.2[a]
	B	8.74±1.91[a]	35.61±6.47[a]	1.42±0.16[a]	18.35±5.23[a]
	C	8.9±4.87[a]	34.46±6.43[a]	1.39±0.16[a]	20.26±7.2[a]
	D	8.86±1.34[a]	34.00±5.49[a]	1.39±0.17[a]	18.5±5.48[a]
Day 30	A	9.01±1.95[a]	35.40±4.51[a]	1.33±0.17[a]	20.5±4.86[b]
	B	8.65±2.71[a]	37.83±5.7[a]	1.35±0.21[a]	27.64±4.34[a]
	C	9.19±2.73[a]	37.20±3.84[a]	1.38±0.31[a]	31.76±4.24[a]
	D	9.14±1.94[a]	35.20±5.13[a]	1.34±0.19[a]	20.5±4.92[b]
Day 60	A	8.7±3.12[a]	35.60±4.86[a]	1.31±0.18[a]	22.1±4.81[b]
	B	8.71±2.94[a]	34.75±5.2[a]	1.39±0.21[a]	25±4.86[a]
	C	8.72±2.34[a]	33.00±4.65[a]	1.34±0.17[a]	28±4.34[a]
	D	8.94±2.67[a]	33.60±6.34[a]	1.41±0.19[a]	18.5±5.24[b]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

The results of the comparison of serum biochemical indices between the experimental groups at different sampling points are presented in Table 5. The serum biochemical indices examined, including urea, calcium, glucose, triglycerides, ALP, Total and direct Bilirubin, and Creatine Phosphokinase were not affected by probiotic (with or without microencapsulation) administration (P>0.05).

Table 5: The serum biochemical parameters of the experimental groups at days zero, 30 and 60 of study

		UREA	CA	GLU	TG	ALP	CPK	OT-B	PT-B
Day 0	A	10.97±2.52[a]	8.97±1.02[a]	157.3±40.77[a]	270±70.5[a]	336±75.8[a]	81.83±25.7[a]	269.6±44.81[a]	1.97±0.18[a]
	B	13.33±2.34[a]	8.53±1.02[a]	146.6±40.77[a]	213.6±72.21[a]	331.3±75.8[a]	82.33±25.9[a]	228.3±41.8[a]	1.85±0.17[a]
	C	12.15±2.05[a]	8.75±0.29[a]	152±35.3[a]	241.8±11.1[a]	333.6±29.72[a]	82.08±7.65[a]	249±46.61[a]	1.91±0.32[a]
	D	12.74±2.28[a]	8.64±0.66[a]	149.3±38.06[a]	227.7±40.82[a]	332.5±18.45[a]	82.21±16.71[a]	238±46.16[a]	1.88±0.24[a]
Day 30	A	11.83±2.17[a]	8.90±0.47[a]	144.6±36.70[a]	221.6±56.81[a]	314.3±52.76[a]	91.00±12.18[a]	266±45.71[a]	1.91±0.27[a]
	B	12.00±0.76[a]	9.10±0.20[a]	120.3±40.02[a]	240±25.98[a]	371±41.24[a]	83.67±19.31[a]	226.6±33.41[a]	2.1± 0.17[a]
	C	12.67±1.73[a]	9.07±0.10[a]	122.3±17.47[a]	257.6±21.73[a]	342.6±25.72[a]	83.67±9.87[a]	212.6±49.66[a]	1.9±0.35[a]
	D	10.97±1.53[a]	8.97±0.5[a]	157.3±26.27[a]	270.5±26.7[a]	336±29.72[a]	81.83±17.6[a]	269.6±46.9[a]	1.9±0.30[a]
Day 60	A	12.33±2.05[a]	9.07±0.29[a]	139±35.35[a]	234.4±51.7[a]	359±42.98[a]	91.50±7.65[a]	222.3±41.04[a]	1.94±0.32[a]
	B	12.67±2.31[a]	8.47±0.55[a]	124±20.66[a]	250.3±79.32[a]	332.3±45.3[a]	92.33±35.8[a]	237.3±38.8[a]	1.9±0.36[a]
	C	13.33±2.08[a]	8.97±0.93[a]	129±33.78[a]	220.6±11.14[a]	378.6±84.24[a]	88.33±18.01[a]	255.6±41.5[a]	1.7±0.39[a]
	D	13.33±0.58[a]	8.53±0.40[a]	146.6±22.61[a]	213.6±93.26[a]	331.3±30.37[a]	82.33±22.59[a]	228.3±56.2[a]	1.8±0.33[a]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Antioxidant status

The antioxidant status in the liver of the fish from the experimental treatments is presented in Table 6. The level of MDA (malondialdehyde) in the probiotic treated groups, especially in the microencapsulated probiotic group, was significantly lower than in the control group (P<0.05). In contrast, the activities of superoxide dismutase

(SOD) and GSH (glutathione) in the probiotic treated groups showed a significant increase compared to the control group on days 30 and 60 of the study. The level of catalase enzyme did not show significant differences among the groups across the three sampling stages.

Table 6: The antioxidant status of the experimental groups at days zero, 30 and 60 of study

	Mean	MDA	SOD	GSH	catlase
Day 0	A	94.81±46.29 [a]	1.97±0.21 [a]	0.28±0.05 [a]	0.04±0.012 [a]
	B	105.48±20.92 [a]	2.58±0.45 [a]	0.29±0.04 [a]	0.03±0.011 [a]
	C	100.14±33.61 [a]	2.27±0.33 [a]	0.29±0.04 [a]	0.04±0.013 [a]
	D	102.81±27.26 [a]	2.42±0.39 [a]	0.29±0.04 [a]	0.03±0.012 [a]
Day 30	A	89.85±11.38 [b]	2.11±0.66 [b]	0.31±0.12 [a]	0.05±0.014 [a]
	B	79.21±15.97 [b]	3.63±0.54 [a]	0.37±0.13 [a]	0.05±0.011 [a]
	C	74.85±23.92 [b]	4.87±0.9 [a]	0.43±0.07 [a]	0.06±0.02 [a]
	D	106.81±46.29 [a]	1.97±0.21 [b]	0.28±0.05 [b]	0.04±0.012 [a]
Day 60	A	94.67±22.03 [ab]	2.73±0.60 [b]	0.31±0.04 [b]	0.03±0.014 [a]
	B	85.13±12.19 [b]	2.87±0.36 [ab]	0.38±0.08 [a]	0.04±0.013 [a]
	C	79.89±9.19 [b]	3.27±1.09 [a]	0.39±0.08 [a]	0.05±0.014 [a]
	D	117.48±20.92 [a]	2.58±0.45 [b]	0.29±0.04 [b]	0.03±0.011 [a]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Intestinal Bacterial flora

The total bacterial count and the count of lactic acid bacteria in the intestines of experimental groups were compared in sampling points (Table 7). The results indicated that, although the total bacterial count did not differ significantly (P>0.05) between the treatments, the number of lactic acid bacteria was significantly higher in the treatments fed with probiotic-containing diets compared to the other groups (P<0.05).

Table 7: The bacterial flora of intestineof the experimental groups at days zero, 30 and 60 of study

	Groups	Heterotroph bacteria	Lactic Acid bacteria
Day 0	A	165±26.06 [a]	122.33±33.15 [b]
	B	169±25.16 [a]	131.00±9.07 [b]
	C	167±25.61 [a]	126.67±21.11 [b]
	D	168±25.38 [a]	128.83±15.09 [b]
Day 30	A	171.3±55.9 [a]	141.00±43.71 [b]
	B	180.6±44.81 [a]	170.00±42.3 [ab]
	C	175.3±46.32 [a]	180.33±44.66 [a]
	D	169±26.06 [a]	131.00±33.1 [b]
Day 60	A	173.3±45.35 [a]	144.00±56.29 [ab]
	B	162.3±53.72 [a]	156.67±48.01 [a]
	C	171.3±30.44 [a]	156.67±31.56 [a]
	D	165±25.16 [a]	122.33±9.07 [b]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Challenge

The results of the challenge with *A. hydrophila* of treated fish showed that the highest mortality rate (60%) occurred in the control group, while the lowest mortality rate (20%) was observed in the both vaccinated groups fed with encapsulated probiotic-containing diets (Figure 4).

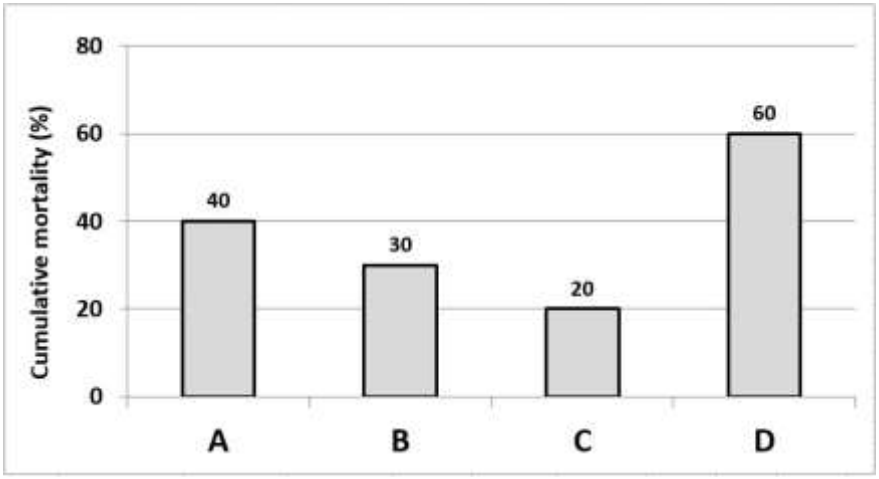

Figure 4: Mortality rate after challenge with *A.hydrophila* in the experimental groups at days60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group.

Discussion

Improving growth indices is one of the primary goals in aquaculture. The results of the current study showed that the highest growth indices were observed in the fish vaccinated with *A. hydrophila* and fed with *L. plantarum* microencapsulated with alginate/chitosan. The microencapsulation of the probiotic likely enhanced the probiotic effects of probiotic, leading to improved growth in this treatment. The ability to improve growth performance of encapsulated probiotics has previously been demonstrated in other aquatic animals such as Oriental Bream Fry (*Abramis brama orientalis*) (Asadi et al, 2016), green terror (Neissi et al, 2013), sea bass (Ashouri et al., 2018), basa fish *(Pangasius bocourti*) (Van et al, 2014) and Nile tilapia (Van et al, 2017).

It is well established that the use of dietary probiotics can positively affect the growth of fish through stimulation of appetite, elevation of digestive enzymes activity, regultion of the population of the gut microflora, modification of the intestinal morphology, promotion of feed utilization and provision of micronutrients (Pinpimai et al, 2015; Mohammadian et al, 2022). In this study, protecting probiotics through microencapsulation techniques enhances their efficacy. Similarly, significant improvement of growth parameters has been reported in Nile tilapia (*Oreochromis niloticus*) and rainbow trout fed with diets containing free or encapsulated *Saccharomyces cerevisiae* and *L. rhamnosus*, respectively (Pinpimai et al, 2015; Hooshyar et al, 2020). The improvement of growth indices in probionts supplemented groups could likely be owing to the increase in digestive enzyme activities, induced by probiotics (Jang et al, 2019). The increase in digestive enzyme activities and therefore, improved feed utilization through the use of probiotics has also been reported in *O. mykiss* as the results of other bacterial strains, like *L. casei* and *L. plantarum* or even in other fish species, like *Sparus aurata*, fed with *Lactobacillus* sp. (Assan et al, 2022). The obtained results suggested that higher amylase, trypsin, ALP and lipase activities, in the *L. plantarum*-

treated fish, might be responsible for improved growth performance. The higher intestine ALP activity indicates the intensity of nutrient absorption in the enterocytes of fish, which it can be responsible for more carbohydrate and lipid uptake (Gawlicka et al, 2000). The previous studies explained how probiotics (especially *L. plantarum*) are able to stimulate this enzyme activity within the brush border of fish enterocyte (Mohammadian et al, 2017). The improved intestine protease activity was in line with the increased PER in our study. According to the results, fish vaccinated with *A. hydrophila* and fed with encapsulated probiotic had significantly higher activities of intestinal trypsin, α-amylase, lipase and ALP. Therefore, the enhancement of digestive enzymes activity was apparently one of the main reasons for growth-stimulatory effects of the probiotic used. The elevated activities of digestive enzymes have been reported to enhance the digestion of macromolecules and therefore facilitate the absorption of nutrients within the gut lumen (Assan et al, 2022).

In this study, the vaccinated fish demonstrated improved antioxidant defense after the administration of microencapsulated probiotics at both sampling stages. The higher activities of serum SOD, CAT and GSH and the lower levels of MDA were observed in the probiotic treated groups, in particular in the microencapsulated group. In agreement with our results, the protective effects of encapsulated or non-encapsulated Lactobacillus strains have been reported previously against oxidative stress caused by stressors in aquatic animal models (Giri et al, 2018).The previous studies have revealed that there are insufficient levels of endogenous antioxidants in cultured fish to cope with external stressors, and improvement of antioxidant defense capacities of fish is highly important (Ghanei-Motlagh et al, 2020). On the other hand, *Lactobacillus plantarum* possess direct scavenging activities against active oxidants by production of enzymes or metabolites with potent antioxidant abilities such as SOD, GSH, and butyrate (Wang et al, 2020). Likewise, Administration of Lactobacillus spp. can positively alter the antioxidant defense system of fish through regulation of antioxidant-dependent signaling pathways (Hoseinifar et al, 2020). SOD and CAT are involved in the disproportionation of superoxide anion radical, and the degradation of hydrogen peroxide, respectively (Yousefi et al, 2019; Ghanei-Motlaghet al, 2021). GSH is a tripeptide non-enzymatic antioxidant which plays an important role in the balance of intracellular redox reactions (Haddad and Harb, 2005). MDA is a secondary product of lipid peroxidation reflecting the cell membrane injury mediated by free radicals.

The results of the current study showed that common carp vaccinated with *A. hydrophila* and fed with probiotics (with or without microencapsulation) did not exhibit significant differences in blood indices and serum biochemical parameters with control group ($P>0.05$). Hemato-biochemical tests are important tools to assess the health status of fish (Fazio, 2019). Irianto and Austin reported no change in the number of RBC in *O. mykiss* fed with probiotics for 14 days. Similarly, The *Micrococcus luteus* administrated fish showed no increase in the number of hematological parameters in *O. niloticus* (El-Rhman et al, 2009). Contrary to our finding, Firouzbakhsh et al, (2011) reported the improved hematological indices in *Astronotus ocellatus* treated by probiotic mixed diet. They suggest that the increase in hematological parameters may be due to the higher growth rates, which lead to increased hematopoiesis and oxygen-carrying capacity. However, this conclusion is limited as changes in hematological indices due to nutrient manipulation often reflect ion regulatory or respiratory issues, indicating higher energy demands to maintain homeostasis rather than supporting growth.

Out results showed that vaccination against *A. hydrophila* and the administra-

tion of probiotics (with or without encapsulation) had no significant effect on serum biochemical indices. The lack of impact on blood and serum biochemical parameters suggests that these treatments do not adversely affect the health of the fish. However, it is possible that a longer administration period or a higher concentration of probiotics could improve blood and serum biochemical indices. Nonetheless, under the conditions used in the current study, vaccination and probiotic administration did not affect these parameters.

Groups 3 and 2, vaccinated and fed with *L. plantarum* with and without microcapsulation, exhibited a significant increase in WBC at days 30 and 60 of trial. In similar works, elevated WBC demonstrated in *O. mykiss* received dietary probiotics. (Mohammadian et al, 2017). The increase in WBC count in the probiotic fed fish seems to be the result of induced activities in the anterior part of the head kidney.

In this study, most immune indices, including anti-*A. hydrophila* antibody levels, serum lysozyme activity, NBT reduction, serum globulin and protein levels, anti-trypsin, and myeloperoxidase, showed a significant increase in group 3 (vaccinated fish with *A. hydrophila* and fed with diet containing *L. plantarum* microencapsulated with chitosan/alginate) compared to the control group ($P<0.05$). However, some indices, such as serum complement activity and serum antimicrobial power, did not show significant differences among the treatments ($P>0.05$). Elevated lysozyme activity in groups 2 and 3 suggests that these applied probiotics can likely provoke the immunity system of common cap. In agreement with our finding, higher level of serum lysozyme in *O. mykiss* fed with *L. casei, L. plantarum*, and *C. divergens* was reported previously (Mohammadian et al, 2019).

The alternative complement activity is accounted as another indicator of innate immune response in the case of infectious disease (Bavia et al, 2022). In the present study, NBT reduction was elevated in group 3. Consistent to our finding, Andani et al, (2012), and (Mohammadian et al, 2019) showed that administration of Lactobacillus bacteria increases the serum complement and NBT activity in *O. mykiss*. On the other hand, contradictory findings were also reported (Mozanzadeh et al, 2023), attributing the possible difference in experimental procedure and even bacterial strains. The obtained results showed that when fish recieved *L. plantarum*, the NBT reduction was higher than the control group. NBT reduction is an indicator for respiratory burst activity of immune-related cells in fish (Zhu & Su, 2022). The findings of respiratory burst activity following the probiotics treatment in fish are often contradictory, while some studies indicated that probiotics did not have any significant impact on this nonspecific defense mechanism of fish (Mozanzadeh et al, 2023). Several *in vitro* and *in vivo* studies showed a significant increase in respiratory burst activity by various probiotics in many aquatic animals including fish (Zhu and Su, 2022). This study further confirmed that the probiotics might be responsible for degrading free radicals production by host phagocytic cells.

Myeloperoxidase and anti-trypsin, as two immunological indices, showed a significant increase in groups 2 and 3 compared to the control group. The probiotic administration in fish has been shown to enhance the activity of anti-trypsin and myeloperoxidase (Sahu et al, 2013). These effects are attributed to the stimulation of the immune system, which improves the ability to respond to pathogens of the fish. Anti-trypsin activity is linked to the regulation of protease enzymes, while myeloperoxidase is involved in the production of reactive oxygen species during the immune response, both contributing to increased disease resistance in the fish (Hoseinifar et al, 2016).

The intestinal bacterial count and flora of fish in the different treatment groups were influenced by the treatments. Although the number of heterotrophic bacteria did not show significant differences among the

groups (P>0.05), the number of lactic acid bacteria significantly increased in group 3 (vaccinated fish fed with microencapsulated probiotics) and group 2 (vaccinated fish treated with probiotic) compared to the control group (P<0.05). In similar studies, the effect of probiotic administration on altering the intestinal bacterial flora and increasing lactic acid bacteria in the gut has been reported. Mohammadian et al, (2019) reported an increase in the proportion of lactic acid bacteria in the intestines of rainbow trout fed with a diet containing *Lactobacillus bulgaricus.* It is likely that the probiotic bacteria established in the gut, after proliferating, led to changes in the intestinal bacterial flora, replacing other bacteria, particularly Gram-negative bacteria, with beneficial lactic acid bacteria. A healthy gut microbiota can prevent colonization by pathogenic bacteria, reduce inflammation, and improve nutrient absorption, all of which contribute to a more effective immune response.

Overall, it can be concluded that the administering *L. plantarum* probiotic to the fish immunized with the *A. hydrophila* vaccine enhanced the efficacy of the vaccine and immunogenicity, as well as improved growth and health indices in common carp. Additionally, microencapsulation of this probiotic with alginate and chitosan microparticles significantly improved its positive effects on vaccine efficacy, growth indices, and fish health status. Therefore, this microencapsulation method is recommended for improving probiotic and vaccine efficacy and fish health. Further studies are suggested to refine the probiotic microencapsulation technique and its effect on the efficacy of highly demanded vaccines in aquaculture industry.

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Conflict of Interest

The authors declare no conflict of interest.

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اثر تجویز خوراکی *لاکتی پلانتی باسیلوس پلانتاروم* ریزپوشانی شده بر کارایی و ایمنی‌زایی واکسن *آئروموناس هیدروفیلا* در کپور معمولی

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چکیده

در مطالعه حاضر، تأثیر تجویز خوراکی پروبیوتیک *لاکتی پلانتی باسیلوس پلانتاروم* به صورت آزاد و کپسوله شده با آلژینات/کیتوسان بر ایمنی‌زایی و کارایی واکسن *آئروموناس هیدروفیلا* در ماهی کپور معمول ارزیابی شد. تعداد ۳۶۰ قطعه ماهی کپور معمولی (با وزن ۵/۱±۴۸ گرم) به طور تصادفی به چهار گروه در سه تکرار تقسیم شدند. گروه اول، دوم و سوم در برابر باکتری *آئروموناس هیدروفیلا* واکسینه شده و گروه اول با خوراک پایه، گروه دوم با پروبیوتیک ساده و گروه سوم با پروبیوتیک ریزپوشانی شده تغذیه شدند. گروه چهارم یا گروه شاهد با جیره پایه بدون مکمل تغذیه شد. زیست سنجی و نمونه‌گیری خون و روده در روزهای صفر، ۳۰ و ۶۰ آزمایش انجام شد. شاخص‌های عملکرد رشد (ضریب تبدیل غذایی، نرخ رشد ویژه، نسبت کارایی پروتئین و نسبت کارایی غذا) و همچنین شاخص‌های ایمنی (تیتر آنتی‌بادی، فعالیت لیزوزیم، کمپلمان و باکتری‌کشی، احیای NBT، میزان گلوبولین و فعالیت میلوپراکسیداز) اندازه‌گیری و بین گروه‌ها مقایسه شد. همچنین پارامترهای خونی (RBC، WBC، Hb و Hct)، فعالیت آنزیم‌های روده‌ای (لیپاز، پروتئاز، آمیلاز، ALP) و وضعیت آنتی‌اکسیدانی (سطح MDA، SOD، GSH و فعالیت کاتالاز) و برخی شاخص‌های بیوشیمیایی سرم (گلوکز، اوره، کلسیم، تری‌گلیسرید، ALP، CPK و بیلی‌روبین) اندازه‌گیری و بین گروه‌ها مقایسه شدند. در روز ۶۰ آزمایش، ماهیان باقی‌مانده در هر گروه با سویه بیماری‌زای *آئروموناس هیدروفیلا* مورد چالش قرار گرفتند و مرگ و میر تجمعی به مدت ۱۴ روز ثبت شد. نتایج نشان داد که بالاترین شاخص‌های رشد و فعالیت آنزیم‌های روده‌ای در گروه ۳ که واکسینه شده و با پروبیوتیک میکروکپسوله تغذیه شده بودند مشاهده شد. شاخص‌های ایمنی در تیمارهای ۲ و ۳ به طور معنی‌داری نسبت به گروه کنترل افزایش داشتند. پارامترهای خونی و شاخص‌های بیوشیمیایی سرم بین تیمارها تفاوت معنی‌داری نشان ندادند. تلفات پس از چالش در تیمارهای ۳ (۲۰ درصد) و ۲ (۲۰ درصد) به طور معنی‌داری کم‌تر از گروه کنترل (۶۰ درصد) بود. به طور کلی، می‌توان نتیجه‌گیری کرد که نه تنها تجویز این پروبیوتیک نقش مهمی در بهبود کارایی و ایمنی‌زایی واکسن تزریقی آئروموناس در ماهی کپور معمولی دارد، بلکه میکروکپسوله کردن این پروبیوتیک با آلژینات/کیتوزان اثر آن را بر کارایی و ایمنی‌زایی واکسن افزایش می‌دهد. بنابراین، استفاده از این روش میکروکپسوله‌سازی برای بهبود کارایی پروبیوتیک و واکسن توصیه می‌شود.

کلمات کلیدی: واکسن *ائروموناس هیدروفیلا*، *لاکتی پلانتی باسیلوس پلانتاروم*، ریزپوشانی، ماهی کپور، ایمنی‌زایی

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