Research Article

Immune-enhancing and protective effects of lactobacillus *delbrueckii bulgaricus* on common carp vaccinated against *Aeromonas hydrophila*

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Abstract

In this study, effect of *lactobacillus delbrueckii bulgaricus (L. bulgaricus),* both in its free form and microencapsulated with alginate/chitosan, on immunity and efficacy of *Aeromonas hydrophila* vaccine was assessed in common carp. A total of 360 common carp (48±5.1 g) were randomly divided in four groups, each in tree replicates. Groups 1 to 3 were vaccinated against *A. hydrophila* and fed diets supplemented with basal diet, L. *bulgaricus*-treated feed, and encapsulated *L. bulgaricus* -treated feed, respectively, and group 4 was considered as the control. Samples were taken on days zero, 30 and 60 of the experiment. Growth performance indices (including FCR, SGR, PER, and FER) and immunological parameters (antibody titer, serum lysozyme, complement and bactericidal activity, NBT reduction, globulin levels, and myeloperoxidase activity) were

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evaluated. Additionally, hematological parameters (RBC, WBC, Hb, and Hct), intestinal enzyme activities (lipase, protease, amylase, and ALP), antioxidant status (maladeyaldehide (MDA) levels, superoxide dismutase (SOD), glutathione (GSH), and catalase activity), and serum biochemical indices (glucose, urea, calcium, triglycerides, ALP, CPK, and bilirubin) were compared among the groups. On day 60 of the experiment, the remaining fish in each group were challenged with virulent *A. hydrophila*, and cumulative mortality was recorded over a 14-day period. The results indicated that group 3 exhibited the highest growth indices and intestinal enzyme activity. Most of the immune parameters showed a significant increase in groups 3 and 2 compared to control group (p<0.05). Hematological and biochemical parameters remained consistent across all groups (p>0.05). Following the challenge, the mortality rates were lower in groups 3 (20%) and 2 (30%) compared to control group (60%). Overall, these data exhibited that administration of *L. bulgaricus* is able to enhance efficacy and immunogenicity of injectable *A. hydrophila* vaccine. Also, alginate/chitosan-microencapsulation of this probiotic further amplifies its positive effects on the vaccine's efficacy.

Keywords: *Aeromonas hydrophila* vaccine, *Lactobacillus delbrueckii bulgaricus*, Common carp, Immunogenicity

Introduction

The common carp (*Cyprinus carpio*) is among the most extensively farmed fish species worldwide and plays a crucial role in aquaculture. Its annual global production surpasses 5 million tons, establishing it as a key species in freshwater fish farming, particularly in Asia. Common carp is valuable fish species for its versatility in various environments, rapid growth, disease resistance, and broad market appeal (FAO, 2023). In Iraq, it is considered the most significant aquaculture species, accounting for over 85% of the country's aquaculture production in both fresh and brackish waters (Mutethya and Yongo, 2021; Mohammed *et al*., 2024; Rasul, 2024).

Cyprinid fish, especially *Cyprinus carpio*, are highly vulnerable to *Aeromonas hydrophila*, which causes severe diseases such as *Aeromonas* hemorrhagic septicemia. This serious infection affects a broad spectrum of aquatic species, resulting in significant internal and external bleeding and leading to high mortality rates among susceptible species (Chen *et al*., 2020). *A.*

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hydrophila is a major contributor to the annual mortality of common carp in Asia particularly in Iraq. Antibiotic therapy is one approach to managing *Aeromonas* infections in aquaculture, but it has several disadvantages. One major concern is the emergence of antibiotic-resistant bacteria, which can threaten both aquatic organisms and human health. Additionally, antibiotics can accumulate in the environment, disrupting ecosystems and harming beneficial microorganisms, which may also lead to bioaccumulation in fish that humans consume. Excessive reliance on antibiotics can also mask underlying problems such as poor water quality or inadequate management practices, which need to be addressed to ensure sustainable aquaculture. Furthermore, the high cost and necessity for repeated treatments are additional limitations of antibiotic use in aquatic settings (Gilani *et al*., 2024).

Vaccination is widely regarded as the most effective and rational approach for preventing and managing *Aeromonas* infections in fish. It plays a crucial role in reducing both the incidence and severity of infections caused by this pathogenic bacterium by enhancing fish immune response, thus increasing their resilience to *A. hydrophila* infection (Farias *et al*., 2020). By implementing effective vaccination protocols, the aquaculture industry can lessen the economic impacts associated with these infections, leading to more sustainable fish production and improved food security (Li *et al*., 2021).

Extensive research has focused on preventing *A. hydrophila* infections in various aquatic species, including common carp, through the development of highly effective vaccines (Nayak, 2020). Nevertheless, even with vaccination, fish may still display symptoms of the disease when subjected 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. 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 influence both fish growth performance and health status by establishing and restoring balance in the gut bacterial flora, and can enhance vaccine efficacy and immunogenicity by improving fish immune responses and health status. Numerous studies have been conducted on the effect of oral probiotic administration

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on fish vaccine efficacy and immunogenicity. Guimarães *et al*. (2022) and Radkhah *et al*. (2024) reported an improvement in the efficacy of the streptococcosis vaccine tilapia following *Lactobacillus* species administration.

Lactobacillus delbrueckii bulgaricus is a highly significant probiotic bacterium, with well-documented benefits for fish. Its positive effects have been observed in various fish species, including common carp, tilapia, and trout (Alishahi *et al*., 2022; Mohammadian *et al*., 2023; Radkhah *et al*., 2024). One of major challenges in administering oral probiotics to fish is the loss and degradation of these bacteria within the gastrointestinal tract, which reduces their efficacy. To overcome this issue, it is essential to protect the bacteria from harsh gastrointestinal conditions. Recent research has increasingly focused on using microencapsulation techniques to address this problem. Biodegradable polymers like chitosan and alginate have emerged as promising materials for microencapsulating probiotics, protecting them from gastrointestinal degradation (Hosseini *et al*., 2022; Ahmadmoradi *et al*., 2024). In addition, alginate and chitosan can serve as immunostimulants, enhancing fish immune responses.

In this study, building on our previous research, we chose alginate and chitosan for the microencapsulation of *Lactobacillus bulgaricus* using the emulsification method. We then evaluated the impact of the microencapsulated probiotic on the efficacy and immunogenicity of the *A. hydrophila* vaccine in common carp (Akter *et al*., 2020; Puvanasundram *et al*., 2022).

Materials and methods

Bacterial strains as probiotic

L. bulgaricus isolate utilized in this study was initially identified through a combination of colony and cell morphology, Gram staining, biochemical assays, and 16S rRNA gene sequencing (GenBank accession number EU520326) (Mohammadian *et al*., 2016). This strain was cultured for 30 hours at 37°C in MRS broth (BD Difco, Sparks, MD, USA).

Probiotic preparation

L. bulgaricus was selected from 30 strains of lactic acid bacteria isolated from the intestinal flora of both wild and farmed healthy cyprinid fish from cyprinid farms in Ahvaz, Iran, based on their in-vitro probiotic properties. The selected isolates were initially identified microbiologically by examining colony morphology, Gram staining, biochemical tests, and finally,

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through molecular identification using 16S rRNA gene sequencing (Mohammadian *et al*., 2016; Mohammadian *et al*., 2022). The lyophilized bacteria were then inoculated into 10 mL of Man Rogosa Sharpe (MRS) broth and incubated at 37°C for 48 hours in an anaerobic jar. After incubation, the bacteria were collected by centrifugation (10 minutes at 3000 g), and the cells were washed three times with phosphate-buffered saline (PBS, pH = 7.2). The probiotic concentration was adjusted to 3×10^8 CFU per gram of feed using optical density at 620 nm with a spectrophotometer.

Microencapsulation of *L. bulgaricus* via emulsification method

Microencapsulation of *L. bulgaricus* with chitosan/alginate (MLCA) was done according to the extrusion method (Dezfuly *et al*., 2020). Briefly, the mixture of L. *bulgaricus* (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 $-75\ ^{\circ}C$ for 6 h and lyophilized for 18 h. The control microcapsules without bacteria were also prepared by the same procedure. The morphologic observation and size measurement of MLAC were performed by scanning electron microscopy (SEM) (Philips XL 20, Oregon, USA).

Diet preparation

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. bulgaricus* (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 (10^{10} cfu mL^{-1}) was 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

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repeated without the probiotic bacteria. To maintain high levels of probiotics, fresh batches of the diets were prepared every two weeks.

Vaccination

The high-virulence *A. hydrophila* used as vaccine seed in this experiment was selected from 12 pathogenic isolates obtained from diseased common carp at the Fish Health Laboratory of the Veterinary Faculty at Shahid Chamran University of Ahvaz, Iran. The selected *A. hydrophila* strain was identified using 16S rRNA PCR methods and confirmed through nucleotide sequencing. Formalin-killed *A. hydrophila* was prepared following the method outlined by Abdy *et al*. (2017). Briefly, *A. hydrophila* was cultured in TSB medium and incubated for 36 hours at 30°C. The bacteria were then adjusted to 10^{10} CFU/mL and inactivated with 0.5% formalin for one hour. The formalin-killed culture (FKC) was washed twice (6000 g for 30 minutes) with phosphate-buffered saline (PBS), plated on TSA plates, and incubated at 30°C for 24 hours to ensure complete bacterial inactivation. Immunization was performed by injecting 100 µL of *A. hydrophila* bacterin at 10^{10} bacteria/mL into peritoneal cavity of each fish on the first day of the experiment. A booster injection was administered on the 21st day of the experiment.

Fish and experimental design

Apparently healthy common carp (*Cyprinus carpio*)(360 fish) weighing 48 ± 5.1 g were sourced from Azadegan Cyprinid Farm, Ahvaz, Iran. The fish were transferred to the laboratory at the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran, where they were acclimated to laboratory conditions for 2 weeks in 500-L plastic quarantine tanks maintained at 27 ± 2°C and fed a control diet. The fish were then randomly divided into four groups of 90 fish each, with each group consisting of three replicates (30 fish per replicate), and were kept for 60 days. The experimental groups were as follows: group 1 was vaccinated with the injectable *A. hydrophila* bacterin; group 2 was vaccinated and fed probiotics at10^{8} CFU/g in the feed; group 3 was vaccinated with *A. hydrophila* and fed microencapsulated probiotics; and Group 4 served as control group (basal diet). Approximately 25% of the water of each tank was exchanged daily, and 100% of water was exchanged weekly. The fish were fed ad libitum twice daily, at 7:00 a.m. and 6:00 p.m., with diets hand-fed. Weekly measurements of basic physicochemical parameters of the water

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were conducted, maintaining oxygen concentration at no less than 5 mg/L and pH levels between 7.5 and 8.2 throughout the study. During the 60-day period, the feeding rate was set at 3% of the biomass, with uneaten feed siphoned away and dried separately to calculate the feed conversion ratio (FCR).

Sample collection

Sampling was conducted on days 0, 30, and 60 of the experiment. A total of 9 fish (3 from each replicate) were randomly selected from each group for hemato-immunological and biochemical analyses. After anesthetizing the fish with 2-phenoxyethanol (400 ppm), blood was drawn from the caudal vein using a 1 mL syringe. The blood samples were then transferred to Eppendorf tubes, with or without anticoagulant, depending on whether they were for hematological or immunological analysis. Serum samples were collected and stored at -20°C until further use.

Growth performance

In the sampling points on days 0, 30 and 60, fish in each replication 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) -100.

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 assess the activity of digestive enzymes, the selected fish were euthanized after blood collection, and intestinal samples were collected through dissection. The intestines were dissected using sterile techniques at a low temperature and then homogenized. Enzyme assays were performed using extracts obtained from homogenizing the intestines in cold 50 mM Tris–HCl buffer, pH 8.0 (1:9 v/w), followed

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by centrifugation (Mohammadian *et al*., 2022).

Total protein content in the gut was assessed using the Bradford method (1976), with the diluted supernatant and bovine serum albumin as a reference. The α-amylase activity was measured using a soluble starch solution (Sigma–Aldrich) as the substrate, following the method described by Areekijseree *et al*. (2004). Amylase activity was expressed as micromoles of maltose produced per mg of protein per minute. Trypsin activity was determined at room temperature using N-Benzoyl-L-arginine ethyl ester (BAEE) as a substrate in the presence of 0.1 mM HCl (Hummel, 1959; Erlanger *et al*., 1961). Lipase activity was assessed by measuring the release of fatty acids from the enzymatic hydrolysis of triglycerides in a stabilized olive oil emulsion (Fluka TM), as described by Borlongan (1990). Total alkaline phosphatase (ALP) activity was measured at 410 nm and 37°C using P-nitrophenyl phosphate as the substrate and 2-amino-2-methyl-1-propanol buffer (0.84 mM, pH 10.3), following a modified method (Otto *et al*., 1946). Protease activity was measured using casein (Sigma–Aldrich) as the substrate, with the result subsequently reacted with Folin's reagent (Anson, 1938, with modifications). Enzyme activities were recorded as absorbance using a spectrophotometer (UV-2802S; Unico, Shanghai, China) and reported as specific activity (U mg^-1 protein min^-1) after appropriate modification.

Immunological parameters

Lysozyme activity assay

Serum lysozyme activity was measured using a turbidometric method as described by Ellis (1990). In this method, 135 µL of *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), was mixed with 15 µL of each serum sample. A reduction in absorbance of 0.001 min^{-1} was defined as one unit of lysozyme activity.

Alternative complement pathway activity

The activity of the alternative complement pathway (ACP) in serum samples was assessed according to the method by Boshra *et al*. (2006), using rabbit red blood cells (RaRBC). Briefly, serum samples were diluted five-fold with Veronal buffer, and 1% Rabbit RBC was gently added to each well. After 24 hours of incubation at 4°C, the samples were centrifuged at 3,500 g for five minutes. Subsequently, 150 µL of the supernatant from each sample was

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transferred to a microplate well, and the optical density (OD) of each well was measured at 540 nm using an ELISA reader (Accu Reader, Taiwan).

Respiratory burst activity

The respiratory burst activity of leukocytes was assessed using the nitro blue tetrazolium (NBT) test, following the method proposed by Alishahi *et al*. (2019) with slight modifications. In brief, 100 µL of blood was mixed with 100 µL of NBT solution (0.2% in distilled water). The mixture was thoroughly shaken and incubated for 30 minutes at 25°C. After incubation, 2000 µL of dimethylformamide was gently added to 100 µL of the mixture, and the resulting solution was centrifuged at 3000 rpm for 10 minutes. The optical density of the supernatant was then measured at 620 nm using a spectrophotometer (Shimadzu, Japan).

Serum bactericidal activity

The respiratory burst activity of leukocytes was assessed using the Nitro Blue Tetrazolium (NBT) test, following the method proposed by Alishahi *et al*. (2019) with slight modifications. In brief, 100 µL of blood was mixed with 100 µL of NBT solution (0.2% in distilled water). The mixture was thoroughly shaken and incubated for 30 minutes at 25°C. After incubation, 2000 µL of dimethylformamide was gently added to 100 µL of the mixture, and the resulting solution was centrifuged at 3000 rpm for 10 minutes. The optical density of the supernatant was then measured at 620 nm using a spectrophotometer (Shimadzu, Japan).

Anti *A. hydrophila* antibody titer

The serum *A. hydrophila* antibody titer was assessed using an ELISA method (Skov *et al*., 2018). Briefly, microplates were coated with 50 µL per well of a *sonicated A. hydrophila* antigen (100 µg/mL) at a 1:15 dilution and incubated for 18 hours at 4°C. After washing, samples were added at 1:20 dilutions in PBS with 0.05% Tween-20 (PBS-T) and 0.1% skim milk. The samples were incubated for 90 minutes at 25°C. Then, 100 µL of mouse anti-common carp monoclonal immunoglobulin, diluted 1:4000 in PBS-T with 0.1% skim milk, was added. Following another wash, 50 µL of goat anti-mouse IgG HRP conjugate (diluted 1:2500 in PBS-T with 0.1% skim milk) was added and incubated for 60 minutes. After washing, 50 µL of TMB chromogenic substrate was applied for 10 minutes at 25°C, and the reaction was stopped with 50 µL of 2 N H2SO4. Antibody levels in the serum and mucus

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were then measured spectrophotometrically at 450 nm using an ELISA reader.

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

After collecting blood samples and dissecting the fish, liver samples were taken, weighed, and homogenized in cold potassium phosphate buffer (0.1 M, pH 7.4, 4°C) at a ratio of 1:9 (w/v) using a homogenizer set at 10,000x g for 60 seconds. The homogenate was then centrifuged at 9,000x g for 30 minutes at 4°C. The supernatant was carefully removed, aliquoted, and stored at −80°C for further analysis. The activities of catalase (CAT) (E.C. 1.11.1.6) (Aebi, 1974), superoxide dismutase (SOD) (E.C. 1.15.1.1) (McCord and Fridovich, 1969), and the levels of glutathione (GSH) (Beutler *et al*., 1963) were measured according to standard protocols.

Intestinal flora

Intestinal samples were analyzed to quantify total bacterial and Lactobacilli counts. At each sampling point, following blood collection and dissection, nine samples were taken from each group. One gram of each sample was homogenized in 9 ml of sterilized phosphate-buffered saline (PBS, 0.1 M, pH 7.0) and mixed for 1 minute using a stomacher (Heidolph Instruments, Germany). Serial dilutions were then prepared under sterile conditions and spread on MRS and TSA plates. After 48 hours of incubation at 30°C, the number of colonies on each plate was counted and reported as colony-forming units (CFU) per gram of sample.

Determination of LD 50

LD_{50} of *A. hydrophila* in common carp was calculated based on method proposed by Alishahi *et al*. (2024). Briefly *A. hydrophila* was cultured in TSB culture medium for 48 hours at 37˚ C. The bacteria were adjusted

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to 10^8 cfu mL^{-1} after centrifugation. 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 it was injected intraperitoneally to 10 fish in a separate aquarium. The dead fish was netted and recorded daily for 10 days. The rate of mortality was analyzed and the LD_{50} was determined by Probit software using version 22 of SPSS. The LD_{50} of *A. hydrophila* in common carp calculated as 1.2×10^6 . At the end of the study, the fish were challenged with this bacterial concentration.

Bacterial challenge

The remaining fish in each group were injected intraperitoneally with live *A. hydrophila* on day 60 of the experiment. Fish were first sedated using 2-phenoxyethanol (300 mg L^{-1}), and 100 µm of *A. hydrophila* at 1.2×10^6 CFU ml^{-1} (LD_{50}) was injected intraperitoneally. Following the bacterial injection, the fish were placed in 100-liter aquaria. Dead fish from each treatment were collected and checked twice daily for 10 days, with the number of dead fish recorded. The cumulative mortality rate was calculated after recording mortality over the 10-day period (Alishahi *et al*., 2014 and 2018). To confirm the cause of death, *A. hydrophila* was re-isolated 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 quantitative data were presented as Mean ± Standard deviation. All statistical analyses were performed using SPSS software (Version 24).

Results

Growth indices

The comparison of growth indices among the experimental groups is summarized in Table 1. The data reveal that nearly all growth metrics, including feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), daily weight gain (DWG), and food efficiency ratio (FER), were significantly enhanced in the groups fed with probiotics compared to the control and the vaccinated group without probiotics at both sampling points ($p<0.05$). Notably, the group vaccinated with *A. hydrophila* and fed with *L. bulgaricus* microencapsulated with alginate and chitosan exhibited the highest growth indices at both sampling stages,

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(p<0.05). Moreover, all experimental treatments achieved a 100% survival rate, with no mortality recorded throughout the study.

Table 1. Growth indices of the experimental groups at days 30 and 60 of experiment. A: vaccinated group, B: vaccinated and L.bulgaricus treated group, C: vaccinated and encapsulated L.bulgaricus treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (p<0.05) within each sampling time).

	Mean	SGR	FCR	PER	DWG	FER
Day 30	A	0.56±0.03 b	2.08±0.13 b	1.51±0.1 b	0.34±0.02 b	48.24±3.16 b
	B	0.74±0.19 a	1.46±0.16 a	2.06±0.21 a	0.48±0.11 a	62.14±7.67 a
	C	0.78±0.24 a	1.67±0.25 a	2.13±0.73 a	0.52±0.17 a	64.80±32.3 a
	D	0.47±0.14 b	2.23±0.09 b	1.45±0.3 b	0.31±0.07 b	46.37±9.73 b
Day 60	A	1.09±0.19 b	2.38±0.17 b	1.25±0.21 b	0.91±0.23 b	38.96±4.6 b
	B	1.34±0.1 a	2.31±0.19ab	1.30±0.03 ab	1.14±0.06 a	39.45±2.92 b
	C	1.35±0.08 a	2.06±0.054 a	1.50±0.18 a	1.13±0.18 a	47.99±11.3 a
	D	0.98±0.05 b	2.49±0.19 b	1.26±0.06 b	0.96±0.06 b	36.91±4.77 b

Digestive enzyme activity

The comparison of digestive enzyme activities in the experimental groups at three different sampling points is summarized in Table 2. According to the data, alkaline phosphatase (ALP) and amylase activities significantly increased in both groups 3 and 2 (probiotic-treated groups, with and without microencapsulation) on days 30 and 60, compared to the control group (p<0.05). In contrast, the activities of protease, lipase, and trypsin showed significant enhancement only in the group vaccinated and fed with microencapsulated probiotics on days 30 and 60, compared to the control (p<0.05). Trypsin and lipase activity were increased just in group 3 at days 30 and 60 of experiment (p<0.05).

Immunological parameters

The immunological data in experimental groups are displayed in Table 3 and Figures 1 to 3. A results showed that most of evaluated immune parameters, including anti-*A. hydrophila* antibody titer, lysozyme activity, NBT reduction, protein and globulin levels, along with antitrypsin and myeloperoxidase activities, showed significant increases (p<0.05) in the probiotic-treated groups, particularly those receiving microencapsulated probiotics, compared to the control group on days 30 and 60 of the experiment. However, some

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immune indices, such as alternative complement activity, serum bactericidal activity and albumin levels, did not show significant differences among the experimental groups (p>0.05). The highest immunological parameters were seen in group 3 which vaccinated against A. hydrophila and fed with encapsulated *L. plantarum*.

Table 2. The activity of intestinal digestive enzymes of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L.bulgaricus* treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (p<0.05) within each sampling time).

	Mean	ALP	Amylase	protease	Lipase	trypsin
Day 0	A	14.32±2.12^{a}	46.58±11.30^{a}	0.13±0.03^{a}	7.99±3.14^{a}	61.51±16.7^{a}
	B	13.65±2.44^{a}	43.67±21.70^{a}	0.14±0.02^{a}	8.22±2.31^{a}	48.43±15.2^{a}
	C	13.19±3.2.45^{a}	42.98±27.7^{a}	0.13±0.03^{a}	7.91±2.58^{a}	54.52±17.5^{a}
	D	13.41±2.29^{a}	41.9±20.31^{a}	0.13±0.02^{a}	8.06±2.45^{a}	51.82±16.3^{a}
Day 30	A	12.20±5.18^{b}	43.70±17.93^{b}	0.12±0.02^{b}	8.11±1.93^{b}	39.87±18.9^{b}
	B	14.84±3.70^{ab}	53.77±14.12^{ab}	0.17±0.04^{a}	13.72±3.71^{a}	83.37±36.3^{a}
	C	15.53±2.13^{a}	73.37±20.11^{a}	0.16±0.03^{b}	12.60±1.85^{a}	98.14±24.8^{a}
	D	14.54±3.07^{b}	47.48±11.3^{b}	0.12±0.02^{b}	7.59±2.84^{b}	65.41±19.7^{b}
Day 60	A	14.41±4.28^{ab}	47.62±11.31^{b}	0.13±0.03^{b}	8.13±2.26^{b}	52.55±12.7^{b}
	B	16.83±3.56^{a}	73.99±17.21^{a}	0.18±0.05^{a}	10.12±1.6^{ab}	70.4±25.2^{ab}
	C	16.43±7.25^{a}	73.20±20.52^{a}	0.20±0.02^{a}	11.16±1.43^{a}	86.35±31.6^{a}
	D	13.04±2.04^{b}	42.29±24.65^{b}	0.14±0.03^{b}	8.22±2.31^{b}	47.43±15.2^{b}

Table 3. The immunological indices of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and L.bulgaricus treated group, C: vaccinated and encapsulated L.bulgaricus treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (p<0.05) within each sampling time).

	Mean	complement	Bactericidal	Protein	Albumin	Globulin	Antitrypsin	mylopor
Day 0	A	5.78±0.26^{a}	0.28±0.08^{a}	3.96±0.27^{a}	0.98±0.12^{a}	2.94±0.35^{a}	0.87±0.17^{a}	0.38±0.09^{a}
	B	5.62±0.50^{a}	0.26±0.11^{a}	3.88±0.13^{a}	0.97±0.09^{a}	2.94±0.18^{a}	0.97±0.13^{a}	0.40±0.15^{a}
	C	5.37±0.68^{a}	0.23±0.12^{a}	3.69±0.22^{a}	0.99±0.12^{a}	2.78±0.25^{a}	0.86±0.27^{a}	0.37±0.13^{a}
	D	5.82±0.49^{a}	0.21±0.09^{a}	3.82±0.16^{a}	0.96±0.12^{a}	2.87±0.21^{a}	0.86±0.23^{a}	0.32±0.12^{a}
Day 30	A	6.27±0.46^{a}	0.24±0.09^{a}	4.01±0.47^{b}	0.93±0.13^{a}	3.07±0.46^{b}	0.78±0.27^{b}	0.40±0.24^{b}
	B	6.53±0.47^{a}	0.30±0.06^{a}	4.17±0.53^{ab}	0.94±0.13^{a}	3.23±0.50^{ab}	1.33±0.29	0.38±0.14^{b}
	C	6.43±0.57^{a}	0.29±0.08^{a}	4.43±0.31^{a}	0.93±0.14^{a}	3.50±0.39^{a}	1.42±0.12	0.49±0.06^{a}
	D	5.97±0.46^{a}	0.28±0.11^{a}	3.87±0.43^{b}	0.95±0.1^{a}	2.92±0.28^{b}	0.84±0.17^{b}	0.34±0.09^{b}

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	Mean	complement	Bactericidal	Protein	Albumin	Globulin	Antitrypsin	mylopor
Day 60	A	6.00±0.87 [a]	0.26±0.13 [a]	4.01±0.27 [b]	0.94±0.11 [a]	3.11±0.09 [ab]	0.89±0.23 [b]	0.32±0.11 [a]
	B	6.17±0.76 [a]	0.23±0.14 [a]	4.19±0.44 [a]	0.93±0.09 [a]	3.26±0.06 [a]	1.14±0.29 [ab]	0.33±0.13 [a]
	C	5.67±0.67 [a]	0.24±0.09 [a]	4.22±0.33 [a]	0.96±0.12 [a]	3.26±0.11 [a]	1.30±0.23 [a]	0.30±0.19 [a]
	D	5.77±0.50 [a]	0.27±0.11 [a]	3.81±0.38 [b]	0.96±0.3 [a]	2.85±0.05 [b]	0.91±0.28 [b]	0.31±0.14 [a]

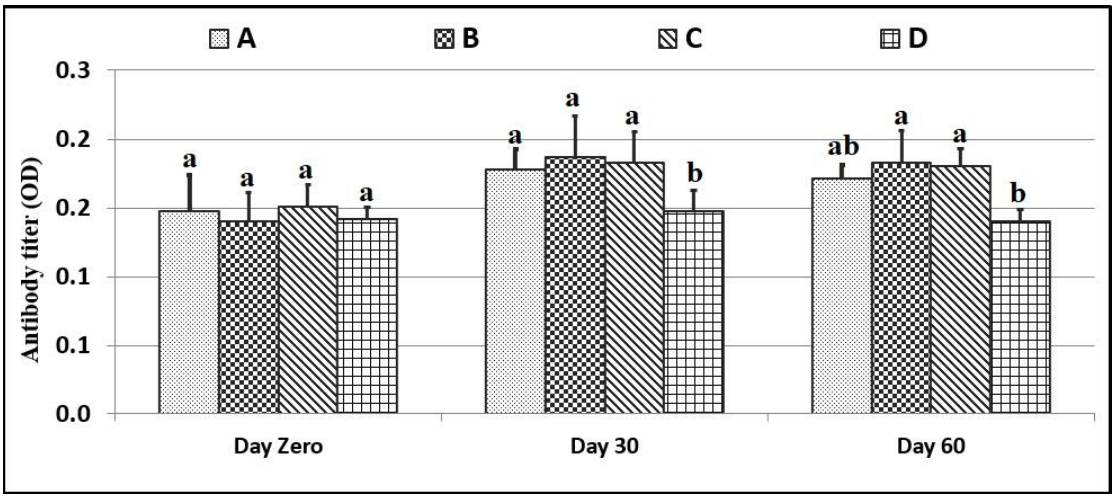

Figure 1. Anti A. hydrophila antibody titer of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and L. bulgaricus treated group, C: vaccinated and encapsulated L. bulgaricus treated group, D: control group. (different lowercase letters on the standard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

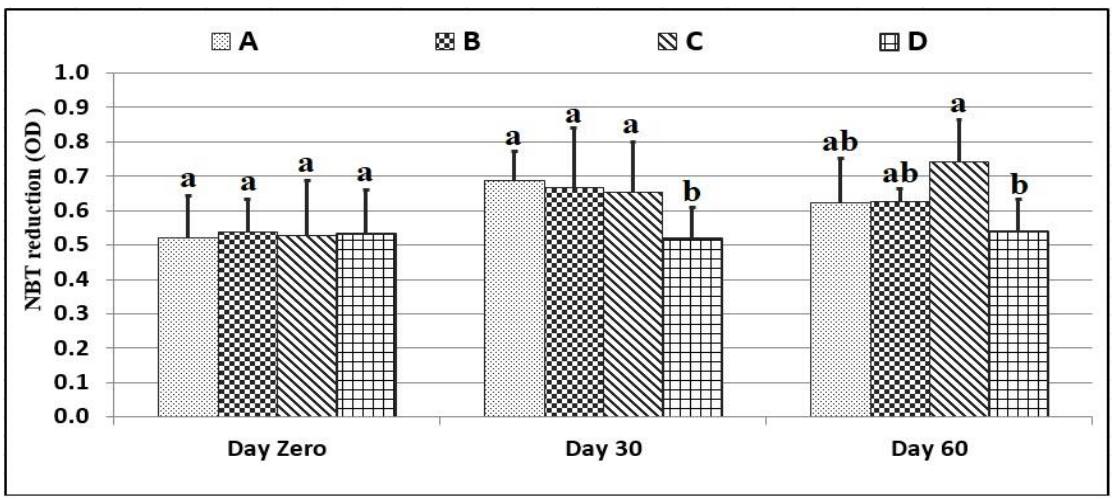

Figure 2. NBT reduction of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L. bulgaricus* treated group, D: control group. (different lowercase letters on the standard deviation bar indicate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

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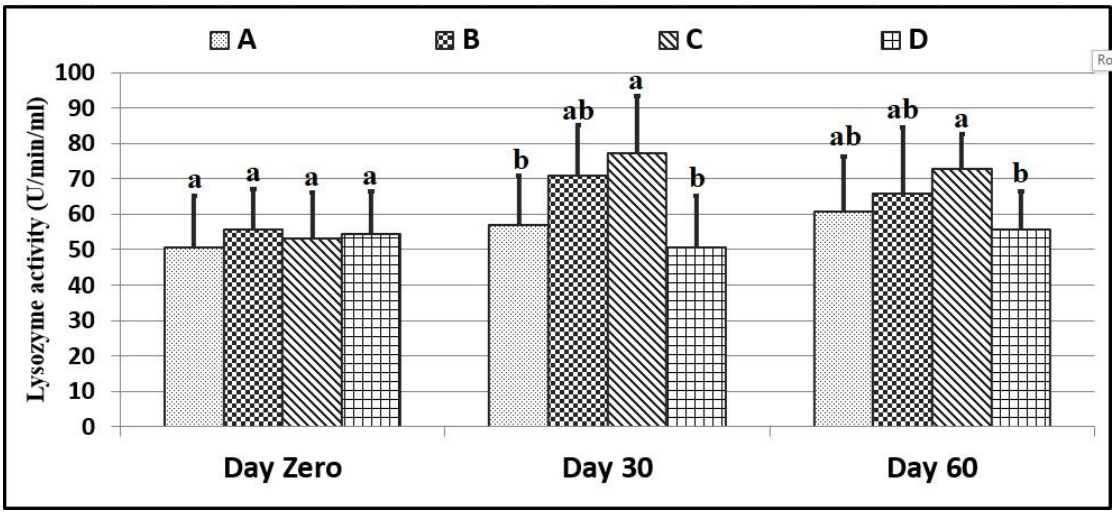

Figure 3. Lysozyme activity of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L. bulgaricus* treated group, D: control group. (different lowercase letters on the standard deviation bar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

Hematological and biochemical

The data concerning hematological parameters are showed in Table 4. A comparison of these parameters across the experimental groups at various sampling stages revealed that indices related to red blood cells, such as RBC, hemoglobin, and hematocrit, were not influenced by either vaccination or probiotic administration (p>0.05). In contrast, white blood cell counts showed a significant increase in the groups fed with probiotics, especially those receiving microencapsulated probiotics, on days 30 and 60 of the study compared to the control group (p<0.05). 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).

Antioxidant status

The antioxidant status in the liver of fish from the experimental groups is detailed in Table 6. The level of malondialdehyde (MDA) in the probiotic-treated groups, particularly in the group receiving microencapsulated probiotics, was significantly lower than in the control group (p<0.05).

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Table 4. The hematological parameters of the experimental groups at days zero, 30 and 60 of study. A: vaccinated group, B: vaccinated and L.bulgaricus treated group, C: vaccinated and encapsulated L. bulgaricus treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences ($p<0.05$) within each sampling time).

	STD	Hb	PCV	RBC	WBC
Day 0	A	9.23±1.81[a]	36.29±4.43[a]	1.36±0.19[a]	23±5.2[a]
	B	8.54±2.61[a]	36.67±6.47[a]	1.21±0.18[a]	19±4.68[a]
	C	9.39±4.7[a]	35.43±5.79[a]	1.41±0.16[a]	19±6.2[a]
	D	8.97±1.12[a]	35.00±5.9[a]	1.39±0.17[a]	18.85±5.78[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	8.75±3.1[a]	38.80±5.67[a]	1.37±0.21[a]	26±5.45[a]
	C	9.17±2.63[a]	37.20±4.94[a]	1.36±0.31[a]	30±4.16[a]
	D	9.14±1.94[a]	35.20±5.13[a]	1.34±0.19[a]	20±4.92[b]
Day 60	A	8.7±3.12[a]	35.60±4.86[a]	1.31±0.18[a]	22±4.81[b]
	B	8.74±2.94[a]	33.75±5.2[a]	1.39±0.21[a]	24±5.36[a]
	C	8.72±2.89[a]	33.00±4.65[a]	1.34±0.17[a]	26±4.12[a]
	D	8.94±2.67[a]	33.60±6.34[a]	1.41±0.19[a]	18.5±5.24[b]

Table 5. The serum biochemical parameters of the experimental groups at days zero, 30 and 60 of study. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L.bulgaricus* treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences ($p<0.05$) within each sampling time).

	Groups	UREA	CA	GLU	TG	ALP	CPK	OT-B	PT-B
Day 0	A	14.2±2.52[a]	8.43±1.02[a]	153.2±40.77[a]	224.7±72.52[a]	346.3±45.8[a]	86.3±28.7[a]	238.3±49.8[a]	1.81±0.19[a]
	B	11.7±2.05[a]	9.17±0.89[a]	157.3±57.65[a]	273.0±39.5[a]	366.0±11.14[a]	83.8±27.2[a]	269.7±46.6[a]	1.77±0.27[a]
	C	13.4±3.28[a]	8.95±0.96[a]	159.0±28.06[a]	251.2±46.54[a]	387.7±47.8[a]	86.8±16.1[a]	249±47.8[a]	1.91±0.24[a]
	D	11.5±2.47[a]	9.06±1.11[a]	154.7±46.3[a]	255.9±45.43[a]	324.8±52.76[a]	81.96±12.8[a]	239.3±46.1[a]	1.87±0.32[a]
Day 30	A	11.83±0.76[a]	8.96±0.82[a]	147.7±40.3[a]	221.7±21.73[a]	319.3±28.72[a]	91.3±19.3[a]	266±73.4[a]	1.91±0.59[a]
	B	11.80±0.72[a]	8.89±0.75[a]	123.0±15.10[a]	243.7±45.32[a]	366.3±14.50[a]	87.4±8.89[a]	237.3±65.3[a]	1.85±0.23[a]
	C	13.00±2.3[a]	8.93±0.84[a]	123.3±10[a]	238.0±35.09[a]	349.3±18.77[a]	86.5±27.72[a]	230.3±72.51[a]	1.99±0.12[a]
	D	10.97±2.05[a]	8.97±0.27[a]	157.3±35.35[a]	270.0±37.98[a]	336.0±45.6[a]	81.8±7.65[a]	269.7±46.61[a]	1.97±0.3[a]
Day 60	A	12.33±2.13[a]	9.07±0.46[a]	139.0±20.66[a]	234.0±35.22[a]	359.0±29.72[a]	91.5±35.8[a]	222.3±53.93[a]	1.94±0.06[a]
	B	12.67±1.84[a]	8.90±0.52[a]	126.7±34.93[a]	223.7±76.4[a]	313.3±45.03[a]	93.3±25.77[a]	340.3±89.63[a]	1.90±0.12[a]
	C	13.00±2.1[a]	8.41±0.7[a]	129.3±38.70[a]	221.3±24.4[a]	331.0±91.2[a]	92±8.72[a]	255.3±64.83[a]	1.90±0.29[a]
	D	13.33±1.65[a]	8.53±0.73[a]	146.7±40.77[a]	213.7±70[a]	331.3±75.80[a]	82.3±25.7[a]	228.3±44.81[a]	1.85±0.18[a]

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Table 6. The antioxidant status of the experimental groups at days zero, 30 and 60 of study. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L.bulgaricus* treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (p<0.05) within each sampling time).

	Mean	MDA	SOD	GSH	catlase
Day 0	A	93.31±41.2 [a]	1.87±0.23 [a]	0.29±0.04 [a]	0.046±0.01 [a]
	B	101.22±34.2 [a]	2.51±0.42 [a]	0.28±0.03 [a]	0.037±0.01 [a]
	C	100.4±39.6 [a]	2.31±0.21 [a]	0.29±0.03 [a]	0.043±0.01 [a]
	D	98.83±27.23 [a]	2.44±0.33 [a]	0.29±0.04 [a]	0.032±0.01 [a]
Day 30	A	89.85±11.38 [ab]	2.11±0.66 [b]	0.31±0.12 [b]	0.051±0.02 [b]
	B	77.38±18.77 [b]	3.80±1.25	0.38±0.11 [ab]	0.059±0.02 [ab]
	C	72.78±29.19 [b]	3.73±0.85	0.42±0.13	0.068±0.01[a]
	D	106.81±36.29 [a]	1.97±0.2 [b]	0.28±0.05 [b]	0.041±0.01 [b]
Day 60	A	94.67±22.03 [ab]	2.73±0.60 [b]	0.31±0.04 [b]	0.032±0.02 [b]
	B	80.25±18.18 [b]	3.54±1.19 [ab]	0.37±0.09 [b]	0.046±0.01 [ab]
	C	76.86±14.37 [b]	4.99±1.38 [a]	0.56±0.05 [a]	0.059±0.01[a]
	D	117.48±20.92 [a]	2.58±0.45	0.29±0.04 [b]	0.034±0.01 [b]

In contrast, the activities of superoxide dismutase (SOD) and glutathione (GSH) were significantly higher in the probiotic-treated groups compared to the control group on days 30 and 60 of the study. However, the level of catalase enzyme did not show significant differences among the treatments across the three sampling stages.

Intestinal bacterial flora

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

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 (30%) was observed in the both vaccinated groups fed with probiotic-containing diets (microencapsulated and non-microencapsulated) (Fig. 4).

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Table 7. The bacterial flora of intestine of the experimental groups on days zero, 30 and 60 of study. A: vaccinated group, B: vaccinated and *L.plantarom* 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).

	Mean	Heterotroph bacteria	Lactic Ac bacteria
Day 0	A	144.37±34.06 [a]	131.4±3.15 [a]
	B	165.64±29.16 [a]	122.3±9.07 [a]
	C	154.73±45.63 [a]	126.6±21.12 [a]
	D	166.7±25.38 [a]	124.50±15.09[a]
Day 30	A	151.33±55.90 [a]	141.3±43.71[b]
	B	172.50±37.48 [a]	160.6±50.86[ab]
	C	165.67±40.22 [a]	183.3±36.3[a]
	D	159±26.06 [a]	131±33.15[b]
Day 60	A	152.33±45.35 [a]	141.03±56.29[b]
	B	161.67±33.31 [a]	149.3±54.24[ab]
	C	164.33±53.50 [a]	169.3±45.54 [a]
	D	154±25.16 [a]	128.3±9.07 [b]

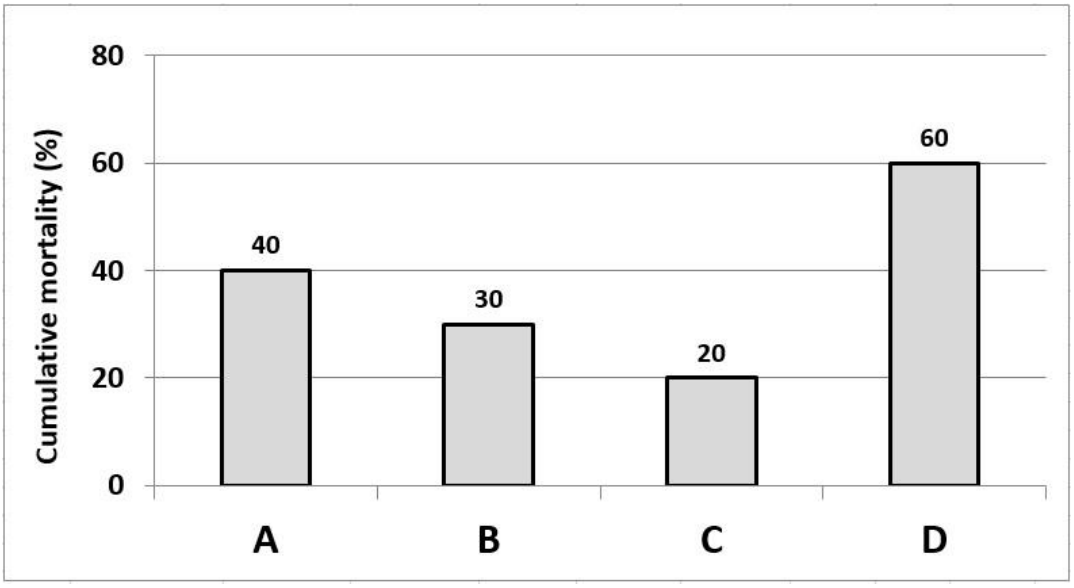

Figure 4. Mortality rate after challenge with *A.hydrophila* in the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.bulgaricus* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group.

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Discussion

Immunological and resistance against bacterial challenge

Various immunophysiological variables including anti-*A. hydrophila* antibody levels, serum lysozyme activity, NBT reduction, serum globulin and protein levels, anti-trypsin, and myeloperoxidase were higher in vaccinated-fish fed diet containing *L. bulgaricus* microencapsulated with chitosan/alginate than control fish (p<0.05). An increase in lysozyme activity observed in groups 2 and 3 suggests that the administered probiotics may boost fish immune response as showed in other species such as *O. mykiss* fed *L. casei*, *L. plantarum*, and *C. divergens* (Son *et al*., 2009; Mohammadian *et al*., 2029).

NBT reduction was significantly higher in group 3. Andani *et al.* (2012), Balcazar *et al.* (2007), and Mohammadian *et al*. (2019), also reported an increase in complement and NBT in rainbow trout fed *Lactobacillus*, while Mozanzadeh *et al*. (2023) reported a contradictory result, possibly due to differences in experimental protocols or bacterial strains used. Our results indicated that NBT reduction was higher in the group receiving *L. bulgaricus* compared to the control group. NBT reduction serves as an indicator of respiratory burst activity in immune cells of fish (Zhu and Su, 2022). While the impact of probiotics on respiratory burst activity has been mixed, with some studies showing no significant effects (Mozanzadeh *et al*., 2023), many in vitro and in vivo studies have demonstrated significant increases in respiratory burst activity with various probiotics in aquatic animals, including fish (Zhu and Su, 2022). This study further supports the idea that probiotics may enhance the production of reactive oxygen species by host phagocytic cells.

Both myeloperoxidase and anti-trypsin activities were significantly elevated in groups 3 compared to the control group. Probiotic supplementation in fish has been shown to improve the activities of anti-trypsin and myeloperoxidase (Pandiyan *et al*., 2013). These effects are attributed to the stimulation of the immune system, which enhances the fish's ability to combat pathogens. Anti-trypsin activity helps regulate protease enzymes, while myeloperoxidase is involved in generating reactive oxygen species during the immune response, contributing to increased disease resistance in fish (Hoseinifar *et al*., 2016).

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Growth indices and intestinal enzyme activity

The results of this study indicated that the best growth performance was achieved in vaccinated fish fed diet containing *L. bulgaricus* microencapsulated with alginate/chitosan. This microencapsulation likely amplified the probiotic effects, leading to improved growth in this treatment. Previous research has also demonstrated that microencapsulated probiotics can enhance growth performance in various aquatic species, including oriental bream fry (*Abramis brama orientalis*) (Asadi *et al*., 2016), green terror (Neissi *et al*., 2013), sea bass (Shouri *et al*., 2018), basa fish (*Pangasius bocourti*) (Van *et al*., 2014), and Nile tilapia (Van *et al*., 2017). It is well-established that dietary probiotics can positively impact fish growth by stimulating appetite, increasing digestive enzyme activity, regulating gut microflora, altering intestinal morphology, enhancing feed utilization, and providing essential micronutrients (Pinpimai *et al*., 2015; Mohammadian *et al*., 2022). In this study, the efficacy of probiotics was enhanced through microencapsulation techniques. Similarly, significant growth improvements have been reported in Nile tilapia (*Oreochromis niloticus*) and rainbow trout when fish were fed diets containing either free or encapsulated *Saccharomyces cerevisiae* and *L. rhamnosus*, respectively (Pinpimai *et al*., 2015; Hooshyar *et al*., 2020). The observed growth benefits in probiotic-supplemented groups are due to several functions such as secretion of digestive enzymes induced by probiotics (Yanbo *et al*., 2006; Suzer *et al*., 2008) as also was seen in *O. mykiss* administred *L. casei* and *L. bulgaricus*, and in other fish species, such as *Sparus aurata* fed with *Lactobacillus* spp. (Uzer *et al*., 2008). Thus, with an increase in digestive enzymes a better digestibility and absorption of feed can occurred resulting in a higher growth (Gawlicka *et al*., 2000). The increased protease activity observed aligns with the higher Protein Efficiency Ratio (PER) noted in this study. Fish vaccinated against *A. hydrophila* and fed with encapsulated probiotics exhibited significantly higher levels of intestinal trypsin, α-amylase, lipase, and ALP. Thus, the enhancement of digestive enzyme activity appears to be a key factor in the growth-promoting effects of the probiotic used.

Antioxidant status

In this study, vaccinated fish showed an enhanced antioxidant defense following the administration of microencapsulated

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probiotics at both sampling stages. The probiotic-treated groups, particularly those receiving microencapsulated probiotics, exhibited significantly higher activities of serum superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), along with a lower level of malondialdehyde (MDA). These findings are consistent with previous reports demonstrating the protective effects of both encapsulated and non-encapsulated *Lactobacillus* strains against oxidative stress in aquatic models (Giri *et al*., 2018; Muhammad *et al*., 2018). Prior research has highlighted that cultured fish often have inadequate levels of endogenous antioxidants to handle external stressors, underscoring the importance of enhancing their antioxidant defenses (Ghanei-Motlagh *et al*., 2020). Additionally, *Lactobacillus bulgaricus* exhibits direct scavenging activities against reactive oxidants by producing enzymes or metabolites with strong antioxidant properties, such as SOD, GSH, and butyrate (Wang *et al*., 2017). The administration of *Lactobacillus* spp. can positively influence the antioxidant defense system of fish by modulating antioxidant-dependent signaling pathways (Hoseinifar *et al*., 2020). SOD and CAT play crucial roles in the conversion of superoxide anion radicals and the breakdown of hydrogen peroxide, respectively (Yousefi *et al*., 2019; Ghanei-Motlagh *et al*., 2021). GSH is a non-enzymatic tripeptide antioxidant that is essential for maintaining intracellular redox balance (Haddad and Harb, 2005). MDA, a byproduct of lipid peroxidation, serves as an indicator of cell membrane damage caused by free radicals (Grotto *et al*., 2009).

Hemato-biochemical parameters

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 compared to control group (p>0.05). Hemato-biochemical tests are important tools to assess the health status of fish (Fazio, 2019). Different findings on effect of probiotic bacteria on fish hematology have been reported by different researchers (e.g., Irianto and Austin, 2002; *El*-Rhman *et al*., 2009; Firouzbakhsh *et al*., 2011), thus, further research works are required to assess the precise effect of probiotics on fish homeostasis. Out results showed that vaccination against *A. hydrophila* infection and the administration of probiotics (with or without encapsulation) had no significant effect on serum biochemical indices. The lack of impact on blood and serum

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biochemical parameters suggests that these treatments do not adversely affect the health status of 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 which were vaccinated and fed with *L. bulgaricus* with and without microcapsulation exhibited a significant increase in WBC at day 30 and 60 of trial. In similar works elevated WBC demonstrated in *O. mykiss* received dietary probiotics. (Mohammadian *et al*., 2017, 2022). 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.

The results of this study indicated that common carp vaccinated against *A. hydrophila* and fed probiotics, whether microencapsulated or not, did not show significant differences in blood indices or serum biochemical parameters compared to the control group (p>0.05). This lack of effect on blood and serum biochemical parameters suggests that these treatments do not negatively influence fish health. Nonetheless, it is possible that extending the treatment period or increasing the probiotic concentration could potentially enhance these indices. Under the conditions of this study, however, neither vaccination nor probiotic administration significantly affected these parameters. Hematological and biochemical parameters are crucial factor for evaluating the health status of fish (Fazio, 2019). Consistent with our findings, Irianto and Austin observed no changes in the red blood cell count of *O. mykiss* fed probiotics for 14 days. Similarly, *O. niloticus* administered *Micrococcus luteus* did not exhibit increased hematological parameters (El-Rhman *et al*., 2009). In contrast, Firouzbakhsh *et al*. (2011) reported improved hematological indices in *Astronotus ocellatus* fed a probiotic-enriched diet, suggesting that increased growth rates led to enhanced hematopoiesis and oxygen-carrying capacity. However, this interpretation is limited because alterations in hematological indices due to dietary changes often reflect issues related to ion regulation or respiration, implying a higher energy demand for maintaining homeostasis rather than solely supporting growth.

Groups 2 and 3, which were vaccinated and fed *L. bulgaricus* (both with and without microencapsulation), showed a significant increase in white blood cell (WBC) counts

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on days 30 and 60 of the trial. Similar findings have been reported where elevated WBC counts were observed in *O. mykiss* fed dietary probiotics (Mohammadian *et al*., 2017; 2022). The observed increase in WBC count in probiotic-fed fish is likely due to induced activities in the anterior part of the head kidney.

Intestinal bacterial flora

The intestinal bacterial populations in fish were affected by the different treatments. While the total count of intestinal heterotrophic bacteria did not differ significantly among the groups ($P > 0.05$), there was a notable increase in lactic acid bacteria in Group 3 (vaccinated fish receiving microencapsulated probiotics) and Group 2 (vaccinated fish treated with probiotics) compared to the control group ($p < 0.05$). Similar studies have demonstrated that probiotic administration can modify intestinal bacterial communities and increase the presence of lactic acid bacteria in the gut. For example, Mohammadian *et al*. (2019) observed an increase in lactic acid bacteria in the intestines of rainbow trout fed a diet supplemented with *Lactobacillus bulgaricus*. It is likely that the probiotics, once established and proliferated in the gut, contributed to shifts in the intestinal microbiota by replacing other bacterial populations, particularly Gram-negative bacteria, with beneficial lactic acid bacteria. A balanced gut microbiota can prevent the colonization of pathogenic bacteria, diminish inflammation, and enhance nutrient absorption, all of which support a more robust immune response.

Conclusion

In summary, the administration of *Lactobacillus bulgaricus* probiotics to carp immunized with *A. hydrophila* vaccine significantly enhanced both the vaccine effectiveness and immunogenicity, as well as improved growth and health metrics of fish. Furthermore, microencapsulation of this probiotic using alginate and chitosan microparticles notably increased its beneficial impacts on vaccine efficacy, growth indices, and overall fish health. Consequently, this microencapsulation approach is highly recommended for boosting the effectiveness of probiotics and vaccines as well as for enhancing fish health. Further studies are suggested to refine the probiotic microencapsulation technique and its effect on the efficacy of vaccines in aquaculture industry.

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Data availability

The datasets generated during and/or analyzed during the current study are available in below Google drive link: https://docs.google.com/spreadsheets/d/1nmPQNxWHFcWXviswgfSztweJLPxGToQPEcFzqijKYUg/edit?usp=drive_link

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Declarations

Ethics approval In vivo phase of this experiment has been conducted as the guidelines of the Institutional Animal Ethics Committee, Faculty of Veterinary, Shahid Chamran University, Iran (Approved NO: EE/1401.2.24.78971/SCU.ac.ir)

Competing interests: The authors declare no competing interests.

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