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Assessment of Immunogenicity and Protective Efficacy of *Yersinia ruckeri* Vaccine in Rainbow Trout Fed with Microencapsulated Probiotics

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Abstract

This study investigated the effects of food supplementation with *Bacillus subtilis* and *Lactobacillus bulgaricus*, in both free and alginate/chitosan-microencapsulated forms, on the immune response and efficacy of a *Yersinia ruckeri* vaccine in rainbow trout (*Oncorhynchus mykiss*). A total of 540 rainbow trout (20 ± 5.1 g) were randomly divided into six groups (three replicates each): Group A (control) received a basal diet; Group B was vaccinated against *Y. ruckeri* and fed the basal diet; Groups C and D were vaccinated and fed diets supplemented with free or microencapsulated *L. bulgaricus*, respectively; and Groups E and F were vaccinated and fed diets containing free or microencapsulated *B. subtilis*, respectively. Sampling was conducted on days 0, 30, and 60 of study to evaluate immunological parameters (antibody titer, lysozyme activity, bactericidal activity, NBT reduction, globulin levels), and hematological parameters (RBC, WBC, Hb, Hct). On day 60 of the experiment, all groups were challenged with autogenous *Y. ruckeri*, and cumulative mortality was recorded over 14 days. According to the results groups D and F showed significantly higher growth performance than the control. Similarly, immunological parameters were significantly enhanced in microencapsulated probiotic groups, whereas hematological values did not change statistically among the groups. Post-challenge, mortality rates were markedly lower in Group D (13.3%) and Group F (23.3%) compared to the control (86.7%). In conclusion, although both probiotics, *L. bulgaricus* and *B. subtilis*, enhanced the immunogenicity and efficacy of the vaccine against *Y. ruckeri*, microencapsulation of the probiotics with alginate and chitosan significantly improved vaccine performance.

Key words: *Yersinia ruckeri vaccine, Bacillus subtilis, Lactobacillus bulgaricus*, Rainbow trout, Immunogenicity

Introduction

The increasing global population has significantly amplified the demand for aquatic animal, with aquaculture anticipated to fulfill a substantial portion of this demand in the future (FAO, 2023).

The rainbow trout is one of the most commercially valuable fish species due to its high marketability, rapid growth, domestication, and ease of breeding. It is widely cultivated in many countries, particularly in Iran. In 2022 Iran held the distinction of being the prominent global

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producer of rainbow trout with a production volume of approximately 194,000 tons. The country accounted for approximately 20.3% of the total global production of finfish (Salehi et al, 2023). However, Yersiniosis, caused by *Yersinia ruckeri*, remains one of the most significant health challenges affecting rainbow trout farming.

Yersinia ruckeri is a Gram-negative bacterium that represents a pathogen of significant concern in aquaculture, particularly affecting rainbow trout (*Oncorhynchus mykiss*) and other freshwater fish species across the globe. The disease caused by *Y. ruckeri*, known as yersiniosis, can lead to severe outbreaks characterized by high mortality rates and substantial economic losses for aquaculture operators (Murray et al, 2015). Yersiniosis leads to clinical signs such as anemia, septicemia, and lesions in various organs, thereby posing a considerable challenge for sustainable aquaculture practices. As the industry continues to grow, the frequency and impact of such diseases highlight the urgent need for effective control measures, making the development of robust vaccination strategies paramount.

Vaccination plays a crucial role in disease prevention in aquaculture. The administration of *Y. ruckeri* vaccines has demonstrated significant potential in eliciting protective immune responses, ultimately reducing the incidence of disease outbreaks (Salas-Leiton et al, 2010).

Various vaccination methods have been explored, with parenteral administration being the most commonly used approach. While effective, this method presents inherent challenges, including handling stress and the invasive nature of injections, which may compromise the fish's immune function and growth performance (Ghosh et al, 2016; Aramoon et al, 2024).

Intraperitoneal injection vaccination has gained increasing attention over the past decade due to its high efficacy, strong immunogenicity, and latency of immunity compared to other vaccination methods in aquaculture (Mondal et al, 2015). The effectiveness of vaccines in aquatic animals is generally lower than in mammals, necessitating the implementation of strategies to enhance their performance. One such approach is the use of probiotics to improve vaccine efficacy in fish (Mohammadian et al, 2022).

Probiotics as live microorganisms that confer health benefits when administered in adequate amounts have gained considerable attention for their adjuvant effects to vaccines in aquaculture. These beneficial bacteria help to maintain intestinal microbial balance, enhance nutrient absorption, and boost the immune response (Ouwehand et al, 2004). Specific probiotic strains, such as *Bacillus spp.* and Lactobacillus *spp.*, have demonstrated as immune-modulating properties, which can potentially enhance the efficacy of vaccines when delivered concurrently (Larger et al, 2012). By promoting gut health and modulating immune responses, probiotics have the potential to improve the protective efficacy of *Y. ruckeri* bacterins.

Encapsulation of probiotics is a cutting-edge technique that protects these microorganisms from environmental stressors, such as pH variations and digestive enzymes, thus enhancing their stability and viability during transit through the gastrointestinal tract (Huiyi et al, 2013). The process of encapsulation allows for a controlled release of probiotics, ensuring that they arrive at their target site in functional form, boosting the overall effectiveness of the immune response. Research indicates that encapsulated probiotics can significantly improve the survival rates of beneficial bacteria during digestion, leading to enhanced bioavailability and a more robust therapeutic effect (Arasu et al, 2021; Aakool et al, 2025).

Understanding the interactions between microencapsulated probiotics and vaccines is critical for elucidating the mechanisms underlying enhanced immune

responses in fish. Beyond immediate disease prevention, this research offers broader implications for long-term fish health, including improved resilience to multiple stressors (Shakouri, 2022). Given the growing challenges in aquaculture disease management and sustainability, a deeper understanding of these interactions is essential.

Moreover, innovative vaccination strategies align with the core goals of sustainable aquaculture, such as reducing antibiotic reliance, improving animal welfare, and enhancing productivity through integrated health management. Advancing this knowledge can lead to optimized vaccination protocols that prioritize fish health, ultimately supporting more resilient and sustainable global aquaculture systems.

This study evaluates the protective efficacy and immunogenicity of *Y. ruckeri* vaccine following oral administration of microencapsulated probiotics. This research aims to develop an integrated vaccination strategy that improves both fish health and growth performance while offering a sustainable disease management solution for aquaculture.

Materials and methods

Identification of Probiotic Bacterial Strains

The *Lactobacillus bulgaricus* and *Bacillus subtilis* strains used in this study were identified through colony morphology, Gram staining, biochemical assays, and 16S rRNA gene sequencing (NCBI GenBank accession number EU520326, SAMN13108112) as described by Mohammadian et al. (2016). These strains were cultured in MRS broth (BD Difco, Sparks, MD, USA) at 37°C for 30 hours.

Probiotic Preparation

L. bulgaricus and *B. subtilis* were selected from 30 probiotic strains isolated from the intestinal flora of wild and farmed cyprinid fish in Ahvaz, Iran. Selection criteria were based on *in vitro* probiotic properties. Initial identification involved colony morphology, Gram staining, and biochemical tests, followed by molecular confirmation through 16S rRNA gene sequencing (Mohammadian et al, 2016; Mohammadian et al, 2022).

Lyophilized *L. bulgaricus* cultures were inoculated into 10 mL of MRS broth and incubated anaerobically at 37°C for 48 hours. Meanwhile *B. subtilis* were inoculated into 10 mL of TSB and incubated at 37°C for 48 hours. Cells were then harvested via centrifugation (3000×g, 10 minutes), washed three times with phosphate-buffered saline (PBS, pH 7.2), and adjusted to a final concentration of 3×10^8 CFU/g optical density measurements at 620 nm with a spectrophotometer.

Microencapsulation of Probiotics via Emulsification

Microencapsulation of *L. bulgaricus* and *B. subtilis* with chitosan/alginate (MLCA) was carried out using the extrusion method as described by Tulaby Dezfuly et al. (2020). Briefly 10^8 CFU/ml of each bacteria *L. bulgaricus* and *B. subtilis* were suspended in 1–2% (v/v) sodium alginate and emulsified in olive oil containing 0.5–1% Span 80 under continuous stirring (800 rpm). Gelation was induced by adding 0.3 M $CaCl_2$, forming alginate microspheres, which were collected via centrifugation (4000 g, 10 min) and washed two times with PBS (pH 7.2). For enhanced stability, microspheres were coated with 0.7% (w/v) chitosan (dissolved in 1% acetic acid, pH adjusted to 5) for 30–60 min. Encapsulated probiotics were stored at 4°C in PBS for subsequently use. The encapsulation efficiency (EE%) was assessed via plate counting after capsules dissolution in 0.1 M sodium citrate (pH 6.5).

Diet Preparation

Experimental diets were formulated based on the methodology of Van Doan et

al, (2016): To prepare probiotic-supplemented diets, bacterial stock solutions (10^{10} CFU/mL) were sprayed onto the feed to achieve a final concentration of 10^8 CFU/g. To enhance probiotic stability, liquid gelatin (5 g/L, at 55°C) was sprayed onto the feed. Control diets were prepared using the same method without probiotics. To ensure probiotic efficacy, the diets were restocked every two weeks.

Vaccine Preparation and Administration

The *Y. ruckeri* strain used in this study was isolated from diseased rainbow trout at the Fish Health Laboratory, Veterinary Faculty, Shahid Chamran University of Ahvaz, Iran. Strain identification was conducted using 16S rRNA sequencing.

Formalin-killed *Y. ruckeri* (FKC) was prepared following the method described by Abdy et al. (2017). Bacteria cultured in TSB medium were incubated at 37°C for 36 hours, adjusted to 10^{10} CFU/mL, and inactivated with 0.5% formalin for 1 hour. The inactivated culture was washed twice (6000×g, 30 minutes) with PBS, plated on TSA, and incubated at 30°C for 24 hours to confirm complete inactivation. The fish were immunized intraperitoneally with 100 µL of FKC containing 10^{10} bacteria/mL on Day 1, followed by a booster dose on Day 21.

Fish and Experimental Design

A total of 540 apparently healthy rainbow trout (*Oncorhynchus mykiss*), weighing 20±2.1 g, were obtained from a commercial aquaculture farm in Khoramabad, Iran. The fish were transferred to the Fish Health Laboratory at the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. They were acclimated to laboratory conditions for two weeks in 500-L plastic quarantine tanks maintained at 27 ± 2°C and fed a basal control diet.

After acclimation, the fish were randomly divided into four experimental groups, each including three replicates with 30 fish per each replicate. The experimental period lasted 60 days, with treatments as described earlier.

The tank management included partial daily water exchange (approximately 25%) and complete water exchange once a week. The fish were hand-fed experimental diets as satiation twice daily, at 7:00 a.m. and 6:00 p.m. The water quality parameters, including dissolved oxygen (≥5 mg/L) and pH (7.5–8.2), were monitored weekly. The feeding rate was maintained at 3% of total biomass, and uneaten feed was siphoned out, dried, and weighed to calculate the feed conversion ratio (FCR).

Table 1: The experimental treatment names and grouping

Code	Treatment Name
A	Treatment 1: Control
B	Treatment 2: Vaccinated with *y. ruckeri* vaccine
C	Treatment 3: Vaccinated with *y. ruckeri* vaccine + *B. subtilis*
D	Treatment 4: Vaccinated with *y. ruckeri* vaccine + encapsulated *B. subtilis*
E	Treatment 5: Vaccinated with *y. ruckeri* vaccine + *L. bulgaricus*
F	Treatment 6: Vaccinated with *y. ruckeri* vaccine + encapsulated *L. bulgaricus*

Sample Collection

Sampling was performed on days 0, 30, and 60 of the experiment. From each group, nine fish (three per replicate) were randomly selected for hematological and immunological analyses. Fish were anesthetized using 2-phenoxyethanol (400 ppm) before sampling. Blood samples were collected from the caudal vein using 1 mL syringes and transferred into Eppendorf tubes, either with heparin or without anticoagulant, depending on the type of analysis. The serum samples were taken

with centrifugation (3000 g, 10 minutes) and the serum was stored at −20°C until further analysis.

Immunological parameters

Anti *Y. ruckeri* Ab titer

Anti *Y. ruckeri* antibody titer in plasma was measured by ELISA method with some modifications (Skov et al., 2018). Concisely, Microplate (Nunc, Denmark) was coated with 50 µL well 1 of formalin-killed and sonically disrupted *Y. ruckeri* (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 trout 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. The 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 H2SO4. Lastly, serum and mucus antibody levels were read spectrophotometrically at 450 nm by an ELISA reader (Accu Reader, Taiwan).

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 (w/v) in 0.02 M sodium phosphate buffer (SPB), pH 5.8 (Sigma-Aldrich), was mixed with 15 µL of each serum sample. The absorbance of the suspension was recorded after 60 and 180 seconds. A reduction in absorbance of 0.001 min^{-1} was defined as one unit of lysozyme activity in sample.

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 resulted 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

In order to measure the bactericidal activity of samples, the suggested method by Ellis (1990) has been used with some slight modifications. In short, each serum sample was mixed with 25 µl of sterile PBS, then gently added to 50 µl of 10^6 cfu/ml of prepared *A. hydrophila*. 50 µl MTT (dimethylthiazol-diphenyl tetrazolium bromid) (Sigma, M5655) has been added to the suspension following 6 h incubation at 25 ˚C. Finally, the concentration of *A. hydrophila* (Formazan positive cells) was measured spectrophotometrically at 600 nm by an ELISA reader (Accu Reader, Taiwan).

Serum protein and globulin

Total serum protein was determined using the biuret method (Pars Azmun Kit, Tehran, Iran) following the manufacturer's protocol. Briefly, 10 µL of serum was mixed with 1 mL of biuret reagent, incubated for 10 minutes at 37°C, and absorbance was measured at 546 nm. Albumin levels were measured using the bromocresol green (BCG) method Globulin concentration was calculated by subtracting albumin from

total protein values. All measurements were performed in triplicate. (Alishahi et al, 2018).

Myeloperoxidase (MPO) Activity Assay

Briefly, 50 µL of serum was mixed with 100 µL o-dianisidine solution (0.167 mg/mL in 50 mM phosphate buffer, pH 6.0) and 50 µL of 0.0005% H_2O_2. After 10 min incubation at room temperature, the reaction was stopped by adding 50 µL of 1% NaN_3, and absorbance was measured at 450 nm. Enzyme activity was calculated using the extinction coefficient of 11,300 $M^{-1}cm^{-1}$. The quality control included blank samples (buffer instead of serum) and negative controls (without H_2O_2) in each assay run (Katzenback et al, 2014).

Hematological parameters

Red blood cell (RBC) and white blood cell (WBC) counts were determined using an improved Neubauer hemocytometer. Hemoglobin (Hb) concentration (g/dL) was estimated using the cyanomethemoglobin method with Drabkin's reagent. Hematocrit (Hct) was determined by filling microhematocrit capillaries with blood, followed by centrifugation at 10,000×g for 5 minutes. The hematocrit value was expressed as the percentage of total blood volume (Thrall, 2004).

Determination of LD50

The median lethal dose (LD_{50}) of *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*) was determined according to the method described by Alishahi et al. (2024). The bacterial strain was cultured in tryptic soy broth (TSB) at 37°C for 48 hours. Following centrifugation, the bacterial concentration was adjusted to 10^8 CFU/mL in phosphate-buffered saline (PBS). Serial ten-fold dilutions (10^5-10^8 CFU/mL) were prepared, and 0.1 mL of each dilution was administered via intraperitoneal injection to groups of 10 fish maintained in separate aquaria. Mortality was recorded daily for 10 days post-injection.

The LD_{50} value (1.2×10^6 CFU/mL) was calculated using Probit analysis (SPSS v.22). This concentration was subsequently used for the final challenge study in surviving fish.

Bacterial Challenge

On day 60 of the study, ten fish from each replicate group were challenged with *Yersinia ruckeri* via intraperitoneal injection. Prior to injection, The fish were anesthetized using 2-phenoxyethanol (300 mg/L). Each fish received 100 µL of bacterial suspension containing 1.2×10^6 CFU/mL (previously determined LD_{50} dose). Following injection, The fish were maintained in 100-L observation aquaria under standard conditions. Mortality was monitored twice daily for 14 days post-challenge. All dead fish were immediately collected and subjected to necropsy. To confirm Y. *ruckeri* infection as the cause of mortality, bacterial re-isolation was performed from kidney tissue using standard microbiological techniques. Cumulative mortality rates were calculated based on 10-day mortality data (Alishahi et al, 2018).

Statistical Analysis

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

Results

Immunological parameters

Immunological data for the experimental groups are presented in Table 2, Figures 1 and 2. The results revealed statistically significant increases ($P<0.05$) in key

immune parameters, including nitroblue tetrazolium (NBT) reduction, total protein, globulin concentrations, and myeloperoxidase (MPO) activity, in probiotic-treated groups compared to the control, particularly in those receiving microencapsulated formulations in both days 30 and 60 of the experiment. In contrast, lysozyme activity and albumin levels showed no significant differences among groups ($P>0.05$). The most pronounced immune responses were seen in Group D (vaccinated + microencapsulated *Lactobacillus bulgaricus*) and Group F (vaccinated + microencapsulated *Bacillus subtilis*).

Most evaluated immune parameters showed significant enhancement in vaccinated fish fed probiotic-supplemented diets compared to those receiving non-probiotic controls. Notably, groups receiving microencapsulated probiotics demonstrated superior immunostimulatory effects, with significantly higher immune indices ($P<0.05$) than those fed non-encapsulated probiotics (except for serum lysozyme activities), which remained statistically similar ($P>0.05$). Although temporal fluctuations were observed between days 30 and 60 post-vaccination, no significant differences ($P>0.05$) were found between the two probiotic strains (*L. bulgaricus* and *B. subtilis*), indicating comparable immunomodulatory efficacy.

Table 2: Immunological indices of experimental groups at days 0, 30, and 60

time	group	NBT OD	Lysozyme U/mg/min	Protein g/dl	Albumin g/dl	Globulin g/dl	Bactericidal	Myloproxidase
Day zero	Control	0.75±0.1^{a}	175.04±58.46^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.24^{a}
	Vaccinated	0.75±0.1^{a}	175.04±58.46^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.24^{a}
	Vac+L.bul	0.75±0.1^{a}	175.04±58.46^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.24^{a}
	Vac+L.b+En	0.75±0.1^{a}	175.04±58.46^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.24^{a}
	Vac+B.s	0.75±0.1^{a}	175.04±58.46^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.24^{a}
	Va+B.s+En	0.75±0.1	175.04±58.4^{a}	4.08±0.61^{a}	0.92±0.25^{a}	3.16±0.51^{a}	0.17±0.03^{a}	0.65±0.2^{a}
Day 30	Control	0.75±0.1^{b}	180.0±44.7^{a}	3.61±0.67^{b}	0.9±0.26^{a}	2.72±0.68^{b}	0.14±0.05^{b}	0.63±0.2^{c}
	Vaccinated	0.79±0.08^{b}	186.66±18.2^{a}	4.82±1.1^{ab}	0.87±0.23^{a}	3.95±1.28^{ab}	0.16±0.07^{ab}	0.81±0.4^{b}
	Vac+L.bul	0.84±0.1^{ab}	166.67±66.6^{a}	5.24±0.67^{a}	0.86±0.24^{a}	4.38±0.88^{a}	0.17±0.04^{a}	1.06±0.2^{a}
	Vac+L.b+En	0.90±0.08^{a}	158.33±87.6^{a}	5.26±1.06^{a}	0.89±0.25^{a}	4.36±0.94^{a}	0.17±0.02^{a}	1.25±0.3^{a}
	Vac+B.s	0.86±0.08^{ab}	160.0±59.6^{a}	4.44±0.93^{ab}	0.83±0.26^{a}	3.81±0.80^{ab}	0.15±0.03^{ab}	0.98±0.4^{ab}
	Va+B.s+En	0.88±0.1^{a}	173.33±64.1^{a}	4.48±0.9^{ab}	0.86±0.11^{a}	4.01±0.41^{a}	0.17±0.01^{a}	1.2±0.3^{a}
Day 60	Control	0.77±0.08^{b}	158.33±50^{a}	4.17±0.9^{b}	0.84±0.36^{a}	3.34±0.62^{b}	0.18±0.017^{a}	0.7±0.28^{c}
	Vaccinated	0.85±0.10^{ab}	162.49±58.3^{a}	4.61±0.3^{ab}	0.81±0.15^{a}	3.8±0.46^{ab}	0.17±0.025^{a}	0.94±0.10^{b}
	Vac+L.bul	0.88±0.05^{ab}	150.0±69.3^{a}	4.71±0.8^{ab}	0.87±0.22^{a}	4.04±0.76^{a}	0.17±0.02^{a}	0.95±0.04^{b}
	Vac+L.b+En	0.91±0.07^{a}	162.5±82^{a}	4.92±0.26^{a}	0.84±0.16^{a}	4.12±0.41^{a}	0.16±0.03^{a}	1.24±0.46^{a}
	Vac+B.s	0.87±0.07^{ab}	152.49±44.5^{a}	4.61±0.38^{ab}	0.89±0.08^{a}	3.73±0.37^{ab}	0.18±0.036^{a}	1.07±0.28^{ab}
	Va+B.s+En	0.91±0.09^{a}	166.75±81.6^{a}	5.09±0.54^{a}	0.88±0.37^{a}	4.21±0.86^{a}	0.17±0.057^{a}	1.35±0.34^{a}

A (control, basal diet); B (vaccinated, basal diet); C (vaccinated + *L. bulgaricus*); D (vaccinated + microencapsulated *L. bulgaricus*); E (vaccinated+*B. subtilis*); F (vaccinated+microencapsulated *B. subtilis*). Different lowercase superscript letters (a, b, c) indicate significant differences among groups at each sampling time ($P<0.05$; one-way ANOVA).

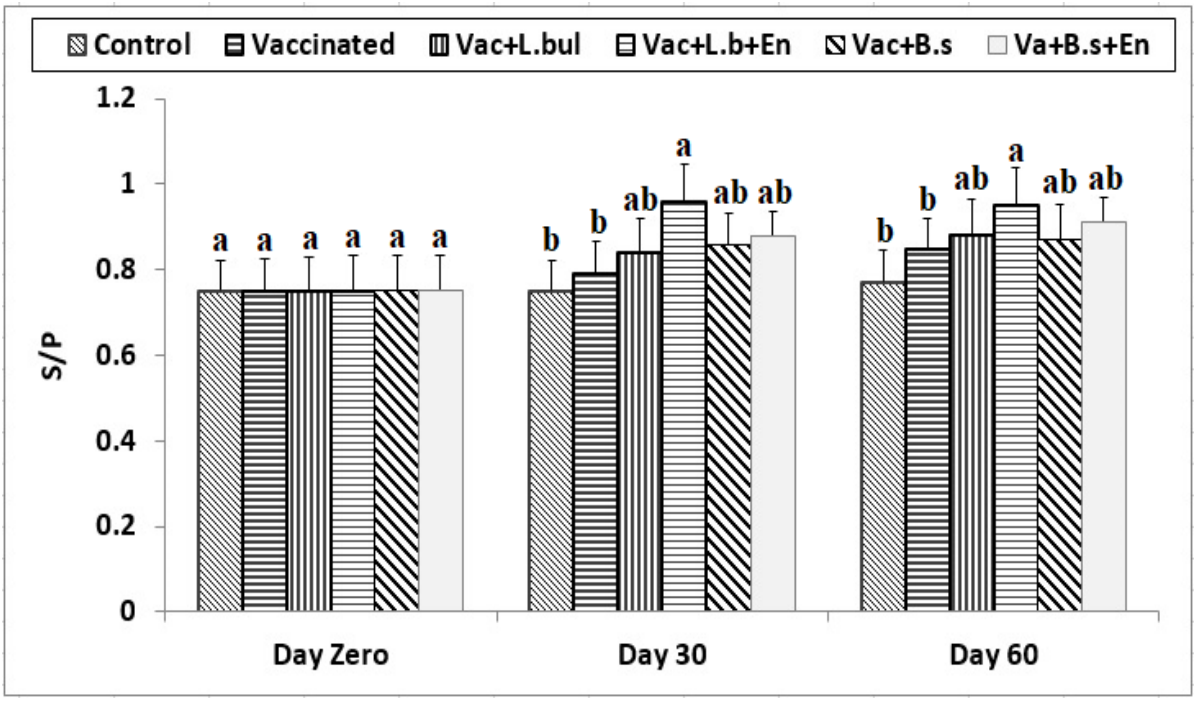

Figure 1: Serum Anti *Y.ruckeri* Ab titer at days 0, 30, and 60. A (control, basal diet); B (vaccinated, basal diet); C (vaccinated + *L. bulgaricus*); D (vaccinated + microencapsulated *L. bulgaricus*); E (vaccinated + *B. subtilis*); F (vaccinated + microencapsulated *B. subtilis*). Different lowercase superscript letters (a, b, c) indicate significant differences among groups at each sampling time (P<0.05; one-way ANOVA)

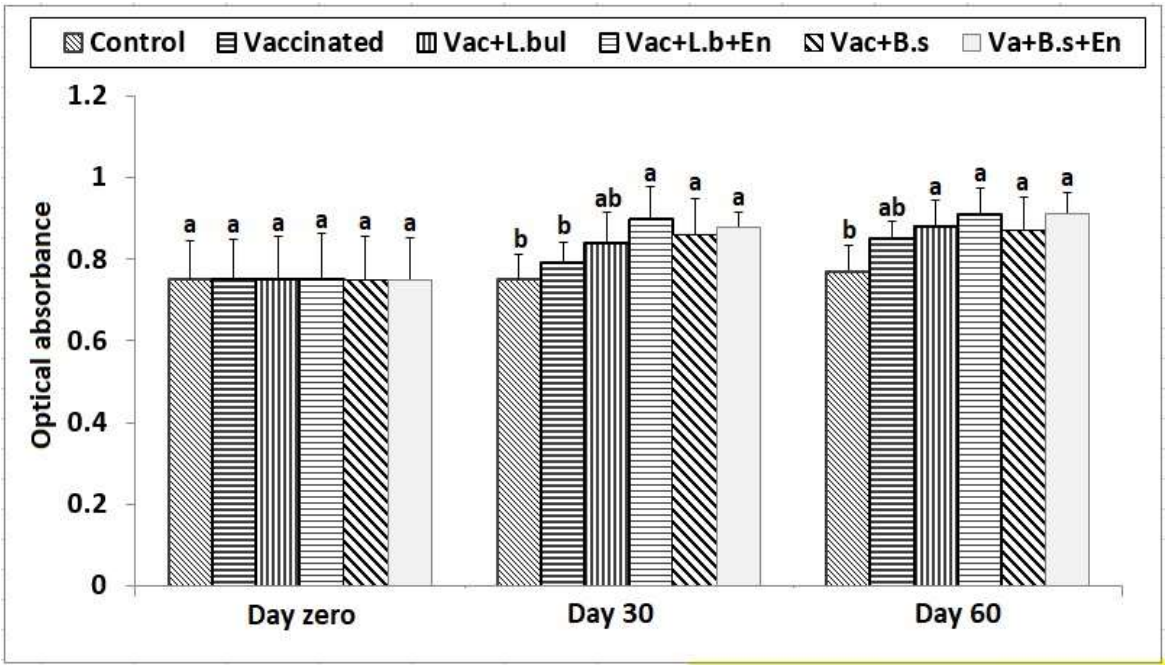

Figure 2: Serum complement activity of experimental groups at days 0, 30, and 60. A (control, basal diet); B (vaccinated, basal diet); C (vaccinated + *L. bulgaricus*); D (vaccinated + microencapsulated *L. bulgaricus*); E (vaccinated + *B. subtilis*); F (vaccinated + microencapsulated *B. subtilis*). Different lowercase superscript letters (a, b, c) indicate significant differences among groups at each sampling time (P<0.05; one-way ANOVA)

The increase in anti-*Y ruckeri* antibody titer was exclusively observed in the vaccinated group fed with a diet containing alginate- and chitosan-microencapsulated *L. bulgaricus* on days 30 and 60 of the experiment (P<0.05). A slight increase in antibody titer was observed in the vaccinated groups fed with probiotics, but this increase was not statistically significant (P>0.05).

Serum Complement activity was significantly higher (P<0.05) in all vaccinated groups receiving probiotic-supplemented diets, both microencapsulated and non-encapsulated, compared to the control group (P>0.05). The highest Complement activity on day 30 was observed in Group D (fed with a microencapsulated *B. subtilis*-supplemented diet), while Group F (fed with a microencapsulated *L. bulgaricus*-supplemented diet) exhibited the highest Complement activity by day 60 post-vaccination

Hematological parameters

The hematological parameters are summarized in Table 3. Comparative analysis of these parameters across the experimental groups at various sampling intervals revealed that red blood cell-related indices including RBC count, hemoglobin concentration, and hematocrit levels were not significantly influenced by either vaccination or probiotic supplementation (P>0.05). In contrast, total white blood cell counts demonstrated a significant elevation in the groups administered probiotics, particularly in those receiving microencapsulated formulations. This increase was most pronounced on days 30 and 60 post-treatment when compared to the control group (P<0.05).

Table 3: Hematological parameters of experimental groups at days 0, 30, and 60

Day	Group	Hb ()	PVC (%)	RBC $\times 10^6$	WBC $\times 10^4$
Day Zero		6.11±1.4[a]	24.50±2.72[a]	1.54±0.17[a]	19.00±9.9[b]
Day 30	Control	6.05±1.19[a]	23.20±2.86[a]	1.51±0.25[a]	18.00±8.3[b]
	Vaccinated	5.98±1.41[a]	25.60±2.41[a]	1.54±0.26[a]	20.00±7.07[b]
	Vac+L.bul	5.96±1.35[a]	25.80±3.11[a]	1.50±0.2[a]	22.00±13.04[b]
	Vac+L.b+En	6.03±0.5[a]	26.20±2.05[a]	1.57±0.28[a]	26.00±13.4[a]
	Vac+B.s	5.98±1.04[a]	26.40±4.9[a]	1.55±0.23[a]	22.00±8.3[b]
	Va+B.s+En	5.85±1.33[a]	25.00±3.8[a]	1.51±0.21[a]	26.00±8.9[a]
Day 60	Control	5.98±0.5[a]	24.20±3.1[a]	1.51±0.19[a]	18.00±8.3[b]
	Vaccinated	5.84±1.5[a]	25.40±1.3[a]	1.52±0.22[a]	18.00±8.3[b]
	Vac+L.bul	6.07±1.2[a]	26.20±2.17[a]	1.53±0.25[a]	20.00±12.2[b]
	Vac+L.b+En	5.96±0.4[a]	26.80±2.6[a]	1.55±0.15[a]	24.00±11.4[a]
	Vac+B.s	6.23±2.1[a]	26.00±5.1[a]	1.53±0.18[a]	18.00±8.3[b]
	Va+B.s+En	5.91±1.4[a]	25.00±3.8[a]	1.51±0.12[a]	24.00±11.4[a]

A (control, basal diet); B (vaccinated, basal diet); C (vaccinated + *L. bulgaricus*); D (vaccinated + microencapsulated *L. bulgaricus*); E (vaccinated + *B. subtilis*); F (vaccinated + microencapsulated *B. subtilis*). Different lowercase superscript letters (a, b, c) indicate significant differences among groups at each sampling time (P<0.05; one-way ANOVA).

Challenge

Following challenge with *Y. ruckeri*, mortality rates varied significantly among treatment groups (Figure 1). The control group showed the highest mortality (93.4%), while immunized fish fed with probiotic-supplemented diets, regardless of encapsulation status, demonstrated markedly improved survival, with the

lowest mortality observed in group D (10%) which was fed with encapsulated L. bulgaricus. Notably, no significant difference (P>0.05) was detected between the two probiotic strains (*Lactobacillus bulgaricus* and *Bacillus subtilis*) in their enhancement of vaccine efficacy.

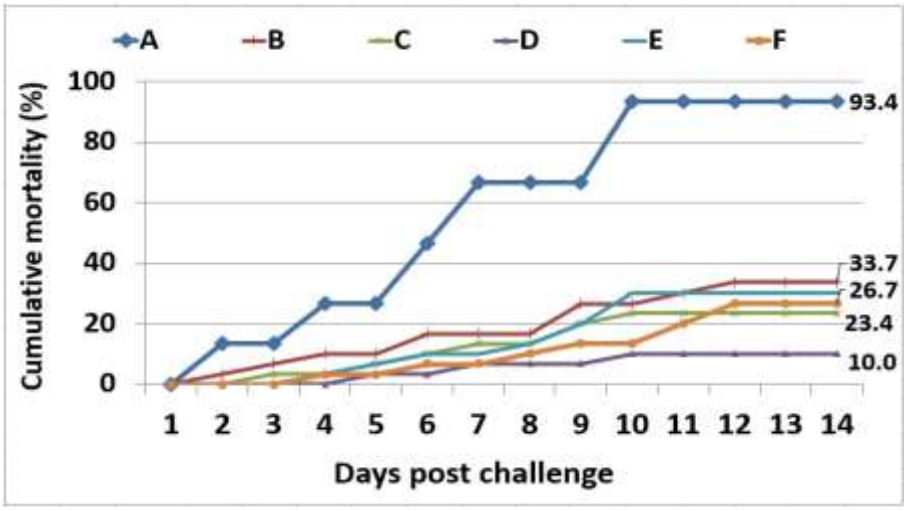

Figure 3: Mortality rate after challenge with *Y. Ruckeri* in the experimental groups at days zero, 30 and 60 of experiment. A: Control group, B: vaccinated group, C: vaccinated and *L.bulgaricus* treated group, D: vaccinated and encapsulated *L.bulgaricus* group, E: vaccinated and *B. subtilis* treated group, F: vaccinated and encapsulated *B. subtilis* group.

Discussion

Although preventive approaches such as vaccines, probiotics, and antimicrobial peptides are currently employed in aquaculture, their efficacy is often limited by antigenic variability among pathogen strains and poor immune response in fishes, thereby necessitating continued dependence on antibiotics (Mohammad and Rahimi, 2023). This highlights the pressing need for more effective infection control strategies and improved pathogen detection methodologies (Cain, 2022). Increasing the effectiveness of vaccines fosters greater confidence among fish farmers and encourages the adoption of vaccination as an alternative to antibiotic treatment (El-Sayed & Khattab, 2023). Accordingly, the present study not only utilized two probiotic strains (*L. bulgaricus* and *B. subtilis*) but also explored the potential of microencapsulation to enhance their immunomodulatory efficacy.

In the present study, dietary supplementation with microencapsulated *L. bulgaricus* and *B. subtilis* significantly enhanced the immunological responses of vaccinated rainbow trout. This enhancement was reflected in elevated serum bactericidal and nitroblue tetrazolium (NBT) activity, and higher globulin concentrations. Although a significant increase in anti-*Y.* ruckeri antibody titer was observed in the vaccinated group fed with microencapsulated *L. bulgaricus*, no statistically significant elevation was detected in the other treatment groups. This finding likely provides evidence for the probiotic strain-specific role in stimulating a specific immune response in fish.

These findings align with those of Purwandari et al, (2024), who reported improved immune function in tilapia following supplementation with encapsulated *Lactobacillus* strains.

The elevated NBT activity observed in Groups D and F of this study indicates an enhanced phagocytic activity of leukocytes. These findings are consistent with the previous research (Balcázar et al, 2007;

Mohammadian et al, 2019), which reported increased NBT levels in rainbow trout fed *Lactobacillus* species. In contrast, Mozanzadeh et al, (2023) reported variable outcomes, potentially attributable to differences in probiotic strain specificity or experimental condition.

Although the effects of probiotics on respiratory burst activity may vary depending on the strain used various evidences support their immunostimulatory potential. Recent studies by Purwandari et al, (2024) and Guimarães et al, (2024) corroborate the immunoenhancing efficacy of encapsulated probiotics, aligning closely with the findings of the current study. The increase in NBT activity provides compelling evidence for the role of microencapsulated probiotics in potentiating innate immune responses through the activation of phagocytic mechanisms.

The use of alginate and chitosan as encapsulating agents has been shown to significantly enhance the stability and functional efficacy of probiotics. These biodegradable polymer-based matrices not only protect probiotics during gastrointestinal transit but also cause controlled release, thereby maximizing their immunological and physiological impact in aquaculture systems (Villegas-Navarro and Medina-Vera, 2023).

One of the properties of probiotics is enhancing fish resistance against pathogenic agents in the gastrointestinal mucosa. Encapsulated probiotics have demonstrated superior efficacy compared to their non-encapsulated counterparts. For instance, Guimarães et al, (2024) reported that *Bacillus subtilis* strains, when administered in encapsulated form, exhibited strong mucosal adherence and effective competitive exclusion of *Yersinia ruckeri*. These findings confirm the hypothesis that encapsulated probiotics can substantially improve disease resistance in aquaculture species.

The present study infancies that probiotic microencapsulation using alginate and chitosan not only preserves the viability of probiotic strains but also enhances their immunostimulatory effects. Encapsulated probiotics have been shown to modulate gut microbiota composition, support intestinal health, and elicit stronger immune responses compared to free-cell formulations (Hoseinifar et al, 2020; Kuebutornye et al, 2019). Additionally, a recent investigation by Vinh et al, (2023) in rainbow trout and Alishahi et al, 2024 in common carp demonstrated that an oral vaccine formulated with alginate-chitosan conferred effective protection against *Streptococcus iniae* and *Aeromonas hydrophila* respectively.

Myeloperoxidase as an immunological indices, showed a significant increase in groups D and F compared to the control group. Probiotic administration in fish has been shown to enhance the activity of myeloperoxidase (Sahu et al, 2013). These effects are attributed to the stimulation of the immune system, which improves the fish's ability to respond to pathogens. Myeloperoxidase is involved in the production of reactive oxygen species during the immune response, both contributing to increased disease resistance in fish (Hoseinifar et al, 2020). In a similar study, administration of microencapsulated *Lactobacillus plantarum* significantly enhanced myeloperoxidase (MPO) activity in rainbow trout (*Oncorhynchus mykiss*). This observed increase can be attributed to the improvement of non-specific immune responses following microencapsulated probiotic supplementation.

Lysozyme activity remained unchanged across all vaccinated groups relative to the control, suggesting that not all innate immune markers respond uniformly to probiotic supplementation. The absence of elevated serum lysozyme activity, despite improvements in other non-specific immune parameters, could be due to the type of vaccine, probiotic, encapsulation

material, or experimental condition. The immune stimulation may have been inadequate to enhance lysozyme activity in treated fish. This is in line with findings from Niu et al, (2019), who reported synergistic effects on multiple immune markers—including lysozyme, myeloperoxidase, and glutathione peroxidase—when *Bacillus spp.*, *Lactobacillus spp.*, and *Saccharomyces cerevisiae* were co-administered in Japanese flounder (*Paralichthys olivaceus*).

This study found that vaccination against *Y. ruckeri*, whether accompanied by encapsulated or non-encapsulated probiotic supplementation, did not result in statistically significant changes in red blood cell (RBC) indices compared to unvaccinated controls ($P>0.05$). These results indicate that probiotic administration did not negatively affect general hematological health. While hematological parameters are widely accepted as reliable indicators of physiological status in fish (Fazio, 2019), The previous studies have reported inconsistent outcomes regarding the effects of probiotics on RBC profiles, likely due to the strain-specific responses and variations in experimental conditions (Irianto and Austin, 2002; El-Rhman et al, 2009; Firouzbakhsh et al, 2011). The current findings are consistent with those of El-Rhman et al, (2009), who observed no significant RBC changes in *Oreochromis niloticus* fed with *Micrococcus luteus*-supplemented diets. In contrast, Firouzbakhsh et al, (2011) reported enhanced erythropoiesis in *Astronotus ocellatus*, potentially due to increased metabolic demands associated with accelerated growth.

Notably, Groups D and F, which received encapsulated *L. bulgaricus* and *B. subtilis* alongside vaccination, showed significantly elevated white blood cell (WBC) counts at both 30 and 60 days post-vaccination. Significant increases in WBC levels have been observed in *Oncorhynchus mykiss* following dietary probiotic supplementation, likely linked to the stimulation of hematopoietic tissues such as the anterior kidney (Mohammadian et al, 2022). These findings highlight the immunostimulatory capabilities of encapsulated probiotics, particularly their role in augmenting innate immune responses. Further investigation is warranted to determine optimal probiotic formulations, dosages, and treatment durations to consistently enhance hematological and immunological health in aquaculture species.

The fish that were administered alginate/probiotic combinations exhibited significantly higher survival rates when exposed to *Yersinia ruckeri*. This enhanced disease resistance has been linked to increased immune response parameters, such as elevated antibody levels and improved phagocytic activity (Khosravi and Safari, 2023).

The findings of this study demonstrated that rainbow trout (*Oncorhynchus mykiss*) vaccinated against *Y. ruckeri* and fed with diets containing encapsulated *Lactobacillus bulgaricus* and *Bacillus subtilis* exhibited the lowest mortality rates (10% and 93.4%, respectively) compared to the control group, which suffered an 87% mortality rate. Additionally, fish vaccinated and fed non-encapsulated forms of these probiotics experienced slightly higher mortality rates (30% and 36.7%, respectively). These results underscore the synergistic effect of vaccination and encapsulated probiotic supplementation in enhancing the survival of fish under pathogenic challenge conditions.

Encapsulation of probiotics using alginate and chitosan likely played a critical role in improving their efficacy. Microencapsulation protects probiotics from the harsh gastric environment, ensuring higher viability as they transit through the digestive tract and enabling targeted release in the intestine, where they can exert their immunostimulatory effects (Mondal et al, 2015). Encapsulation also

prolongs the shelf life of probiotics and enhances their stability during storage and administration (Sultana et al, 2000). This improved delivery mechanism could explain the enhanced immune responses observed in encapsulated groups compared to non-encapsulated ones.

The use of probiotics, especially in encapsulated forms, has been widely reported to enhance the immune responses of the fish, as evidenced by significant reductions in mortality under pathogenic challenges. Similar studies have shown that dietary supplementation with encapsulated probiotics can boost the production of immune-related enzymes such as lysozyme, myeloperoxidase, and alkaline phosphatase, as well as cytokines like IL-1β and TNF-α (Niu et al, 2019; Mukherjee et al, 2019). These responses play a pivotal role in strengthening the fish's innate and adaptive immune systems, enabling them to resist infections more effectively.

For instance, Aly et al, (2008) reported significantly higher survival rates in *Oreochromis niloticus* challenged with *Aeromonas hydrophila* when fed with a diet containing a mixture of *Bacillus subtilis* and *Lactobacillus acidophilus*.

Probiotics enhance disease resistance through multiple mechanisms, including competitive exclusion of pathogens, production of antimicrobial substances, modulation of gut microbiota, and stimulation of host immune responses (Ringø et al, 2010). Encapsulated probiotics, in particular, exhibit superior efficacy due to their targeted release and prolonged activity in the intestine, as shown by Mondal et al, (2022). Furthermore, the use of alginate and chitosan for encapsulation offers additional benefits, such as biocompatibility and biodegradability, while also acting as immunomodulatory agents themselves (Sultana et al, 2000; Adorian et al, 2019).

In this study, the significant reduction in mortality among the fish fed with encapsulated probiotics suggests that these supplements not only enhanced the immune response but also acted as a protective barrier against the virulent pathogen *Y. ruckeri*. Similar findings were reported by Van Hai (2015), who noted that probiotics could enhance phagocytic activity and respiratory burst in fish, leading to more efficient pathogen clearance. Additionally, Adorian et al, (2019) highlighted the dose-dependent efficacy of probiotics, with optimal concentrations yielding maximum immunostimulatory effects.

Hai et al, (2008) also demonstrated enhanced resistance in tilapia against *A. hydrophila*, *Pseudomonas fluorescens*, and *S. iniae* following dietary administration of a probiotic blend containing *B. subtilis* and *Lactobacillus acidophilus*, outperforming treatments with single strains. Likewise, Beck et al, (2015) reported that a 1:1 mixture of *Lactococcus lactis* (BFE920) and *Lactobacillus plantarum* (FGL0001) at 10^7 CFU/g provided immunoprotective benefits in Japanese flounder (*Paralichthys olivaceus*) against *S. iniae* challenge. Moreover, Mukherjee et al, (2019) evaluated the dietary application of *Bacillus methylotrophicus*, *B. amyloliquefaciens*, and *B. licheniformis* in *Labeo rohita* fingerlings, reporting improvements in growth, hematological profiles, immune parameters, and disease resistance.

The use of *L. bulgaricus* and *B. subtilis* as probiotic supplements enhanced both the efficacy and immunogenicity of the *Y. ruckeri* vaccine. Moreover, microencapsulation of these probiotic strains with alginate and chitosan further improved vaccine efficacy in rainbow trout. No statistically significant differences were observed between the two probiotic strains in terms of their immunomodulatory effects, suggesting comparable efficacy. These findings warrant further investigation to explore their potential application in the large-scale aquaculture vaccination programs.

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Conflict of Interest

The authors declare no competing interests.

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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)

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ارزیابی ایمنی‌زایی و اثربخشی حفاظتی واکسن یرسینیا راکری در ماهی قزل‌آلای رنگین‌کمان تغذیه‌شده با پروبیوتیک‌های ریزپوشانی‌شده

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چکیده

در این مطالعه اثرات مکمل غذایی با *باسیلوس سوبتیلیس* و *لاکتوباسیلوس بولگاریکوس*، به هر دو شکل آزاد و ریزپوشانی شده با آلژینات/کیتوزان، بر پاسخ ایمنی و اثربخشی واکسن *یرسینیا راکری* در ماهی قزل‌آلای رنگین‌کمان (*Oncorhynchus mykiss*) مورد بررسی قرار گرفت. در مجموع ۵۴۰ ماهی قزل‌آلای رنگین‌کمان (۵/۱ ± ۲۰ گرم) به طور تصادفی به شش گروه (هر کدام با سه تکرار) تقسیم شدند: گروه A (کنترل) یک رژیم غذایی پایه دریافت کرد؛ گروه B علیه *یرسینیا راکری* واکسینه شد و با رژیم غذایی پایه تغذیه شد؛ گروه‌های C و D به ترتیب واکسینه شدند و با رژیم‌های غذایی حاوی *لاکتوباسیلوس بولگاریکوس* آزاد یا ریزپوشانی شده تغذیه شدند؛ و گروه‌های E و F به ترتیب با رژیم‌های غذایی حاوی *باسیلوس سوبتیلیس* آزاد یا ریزپوشانی شده تغذیه شدند. نمونه‌برداری در روزهای ۰، ۳۰ و ۶۰ مطالعه برای ارزیابی پارامترهای ایمنی‌شناسی (تیتر آنتی‌بادی، فعالیت لیزوزیم، فعالیت باکتری‌کشی، NBT، سطح گلوبولین) و پارامترهای خونی (RBC، WBC، Hb، Hct) انجام شد. در روز ۶۰ آزمایش، همه گروه‌ها با *یرسینیا راکری* اتوژن مورد آزمایش قرار گرفتند و مرگ و میر تجمعی طی ۱۴ روز ثبت شد. نتایج نشان داد که گروه‌های D و F (پروبیوتیک‌های ریزپوشانی شده) عملکرد رشدشان به طور قابل توجهی نسبت به گروه کنترل بالاتر بود. به طور مشابه، پارامترهای ایمنی‌شناسی در گروه‌های پروبیوتیک ریزپوشانی شده به طور قابل توجهی افزایش یافتند، در حالی که مقادیر خون‌شناسی از نظر آماری بین گروه‌ها تغییر نکرد. پس از چالش، میزان مرگ و میر در گروه D (۱۳،۳ درصد) و گروه F (۲۳/۳ درصد) در مقایسه با گروه کنترل (۸۶/۷ درصد) به طور قابل توجهی کمتر بود. در نتیجه، اگر چه هر دو پروبیوتیک، *لاکتوباسیلوس بولگاریکوس* و *باسیلوس سوبتیلیس*، ایمنی‌زایی و اثربخشی واکسن علیه *یرسینیا راکری* را افزایش دادند، ریزپوشانی پروبیوتیک‌ها با آلژینات و کیتوزان به طور قابل توجهی عملکرد واکسن را بهبود بخشید.

کلمات کلیدی: واکسن *یرسینیا راکری*، *باسیلوس سوبتیلیس*، *لاکتوباسیلوس بولگاریکوس*، قزل‌آلای رنگین‌کمان، ایمنی‌زایی

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