

## Original Article

# Evaluation of using the hyperosmotic environment on the double-streptococcus (*Streptococcus iniae*/*Lactococcus garvieae*) vaccine efficacy by bath method in juvenile great sturgeon, *Huso huso*

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**Abstract:** In this study, the effectiveness of the double-streptococcus (*Streptococcus iniae*/*Lactococcus garvieae*) vaccine was evaluated in great sturgeon, *Huso huso*. The fish were divided into six experimental groups which included a group of fish vaccinated by injection method (Treatment 1), two-step vaccination in a hyperosmotic environment followed by a vaccine by immersion method (Treatment 2), two-step vaccination by immersion method (Treatment 3), vaccination in a hyper-osmotic environment followed by a one-step vaccine by immersion method (Treatment 4), one-step vaccinated fish group by immersion method (Treatment 5) and a control group. A total of 120 juvenile great sturgeon with an average weight of  $27.4 \pm 1.7$  g were divided into 12 tanks (two replicates for each group) and were reared and tested for two months. The fish in treatments 2 and 3 received the second step of the vaccine 21 days after the first vaccination. Based on the results, the serum total protein and immunoglobulin levels and white blood cell counts (WBC), as well as serum antibody titer against *L. garvieae* and *S. iniae* in vaccinated fish by the injection method, were recorded as higher in comparison to other treatments ( $P \geq 0.05$ ). On the other hand, the mentioned parameters for Treatments 2 and 3 were higher than those of the control group and Treatments 4 and 5. After the bacterial challenge, the control group had 80% mortality, while the lowest mortality rate belonged to the fish vaccinated by the injection method (with only 20% deaths). The fish that received two stages of vaccine by the immersion method had fewer casualties than the one-step vaccinated fish. According to the study's results, using a hyperosmotic solution (15 ppt NaCl) did not have a satisfactory effect on the vaccine efficacy in great sturgeon. Also, one-step vaccination by the bath method does not provide acceptable immunity for the mentioned bacteria in this fish. In conclusion, vaccination by the injection method is suggested as the best method of vaccination for juvenile great sturgeon.

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## Introduction

Aquaculture, especially in intensive and semi-intensive farming, can be stressful for farmed fish. This stress can weaken the fish's immune system, making them more susceptible to bacterial diseases (Elnoby et al., 2021). Streptococcosis is one of the most common bacterial diseases affecting cold and warm-water fish. This disease can result in significant annual losses to the aquaculture industry. In 2008, it was estimated that the US aquaculture industry suffered a loss of approximately \$ 250 million due to this disease (Van Doan et al., 2021). According to the

reports, multiple species were responsible for causing the disease. In addition to *Streptococcus* spp., other genera of cocci bacteria, such as *Lactococcus* spp., *Vagococcus* spp., and *Enterococcus* spp., can cause similar diseases. All diseases caused by these bacteria are collectively referred to as streptococcosis (Van Doan et al., 2022). A wide range of clinical symptoms have been reported for this disease, including exophthalmia, lethargy, opacity in the eye, anorexia, hemorrhage in the skin, eyes, base of the fins and intestine, and fluid accumulation in the abdominal cavity (Eldar et al., 1995; Romalde et al., 2008).

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This disease was reported in Iran for the first time in rainbow trout farms, and so far, only two species, including *Streptococcus iniae* and *Lactococcus garviae*, have been isolated as the causative agents of the disease, which cause great losses to these farms (Soltani et al., 2008). In many cases, fish tend to stop eating following an outbreak of disease, so drug therapy in food does not have much effect. Similarly, drug therapy through bath immersion becomes expensive for larger farms, resulting in significant losses for fish farms. Preventing diseases is the best way to manage farm health. In this regard, various methods have been proposed, such as using immune stimulants and vaccination (Van Doan et al., 2022). Vaccination is one of the most economical ways to control fish disease. Fish vaccines typically contain inactivated or killed pathogens or parts of pathogens that stimulate the fish's immune system.

Vaccines can be administered through injection, oral, or bathing methods. Although injection is the most effective method, farmers often use vaccines through bathing or oral methods due to some problems associated with the injection method (Bowald and Dalmo, 2019). It will be crucial to achieve the highest protection and vaccine effectiveness. Bathing time, vaccine dose, and fish size are important in the bath vaccination. Failure to consider any mentioned items may disrupt the vaccine's effectiveness (Du et al., 2017). Some methods have been proposed to increase antigen uptake for the immersion method, including using a hyperosmotic environment (Gao et al., 2016) or various adjuvants to increase vaccine absorption, etc. (Ji et al., 2019). In the present study, the performance and efficacy of the Double Streptococcus vaccine (*Streptococcus iniae* / *Lactococcus garviae*) have been investigated on great sturgeon in both immersion and injection methods, as well as the effects of using a hyperosmotic environment.

## Materials and Methods

**Fish preparation and rearing procedure:** A total of 120 great sturgeons, *Huso huso*, were obtained from a local farm with an average weight of  $27.4 \pm 1.7$  and divided into twelve 200 L fiberglass tanks, each

containing 140 L of dechlorinated tap water and equipped with central aeration. The fish were acclimatized to the experimental conditions for two weeks, after which they were reared for 8 weeks post-treatment. Throughout this period, the fish were fed twice a day with a commercial diet (Faradaneh Co, Iran) at a rate of 3% of the fish biomass. Additionally, 80% of the water in each tank was replaced daily. The water temperature during this period was  $21.4 \pm 3.5^\circ\text{C}$ , water hardness was  $186.2 \pm 0.33$  mg/l, dissolved oxygen was  $6.4 \pm 0.3$  mg/l, and the pH was measured as  $7.1 \pm 0.1$ .

**Grouping and vaccination:** The study was conducted with six experimental groups, each with two repetitions, including the control group, treatment 1: one-stage vaccination by bath method, treatment 2: hyperosmotic environment + one-step vaccination by bath method, treatment 3: two-step vaccination by bath method, treatment 4: hyperosmotic environment + two-step vaccination by bath method and treatment 5: vaccination by one-step injection method. The vaccine used in this study was the double Streptococcus vaccine (*Streptococcus iniae* / *Lactococcus garviae*), which was manufactured by Bujan Tech Pharmed Co., Iran, and is known as the GaroVac vaccine. For the hyperosmotic + vaccination groups, the fish of each treatment were placed in a 15 g/liter saltwater environment for 8 minutes. Immediately after that, vaccination was done using the bath method. For the vaccination by injection method, the fish were anesthetized with 100 mg/l eugenol (Sigma-Aldrich, St. Louis, MO, USA). They received 0.1 CC of the vaccine via intraperitoneal injection using an insulin syringe.

**Serological and hematological tests:** Blood sampling was done on the fish 50 days after the first stage of vaccination. The fish were first anesthetized with 100 ppm eugenol, and blood was collected using the caudal vein puncture with a 23-gauge needle and 2 cc syringes. The blood sample was then divided into two parts for further analysis. To perform serology tests, the samples were separated using a centrifuge at 8000 rpm for 15 min. Serum biochemical parameters such as glucose, total protein, ALP, AST, and ALT

were measured using medical laboratory diagnostic test kits from Pars Azmoun Co in Iran.

For hematological tests, the red blood cell (RBC) counts and white blood cell (WBC) counts were measured using a Neubauer hemocytometer after diluting the samples 100 times with a Dice solution. The hematocrit of the blood was measured using the standard microhematocrit method, and hemoglobin values were determined using the cyanomethemoglobin method with medical laboratory diagnostic test kits (Pars Azmoun, Iran). The hematological indices, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were calculated based on the routine formula (Dacie and Lewis, 2001).

**Bacterial challenge:** Ten fish from each group were used for bacterial exposure. *Streptococcus iniae* isolate PTCC 1887 (IROST, Iran) and *L. garvieae* isolate PTCC 1884 (IROST, Iran) were obtained from the Iranian Research Organization for Science and Technology. The bacteria were enriched separately in Nutrient broth medium (Merck, Germany) for 48 hours and then routinely cultured on Nutrient agar (Merck, Germany). 48 h after incubation, bacteria were suspended in sterile PBS (OD = 1 at 620 nm) and determined by viable counting on Nutrient agar as  $6.3 \times 10^8$  cfu ml<sup>-1</sup> for *S. iniae* and  $4.8 \times 10^8$  cfu ml<sup>-1</sup> for *L. garvieae*. The suspensions were diluted 10-fold in PBS, and 0.1 ml of each was injected (via intraperitoneal injection) into the fish. Mortality was recorded daily during 14 days post-challenge.

**Antibody titer:** Specific antibody levels against *S. iniae* and *L. garvieae* were separately tested using agglutination tests in a 96-well microtitre plate. At first, the serum samples were heat-inactivated according to Barnes et al. (2002) by placing them in a water bath at 44°C for 20 min, then serial dilutions were made (two-fold) in PBS, and a suspension of formalin-killed bacteria was added to each well. The plates were incubated for 1 hour at room temperature and left overnight at 4°C. The antibody titers were measured in the highest serum dilution that produced a positive agglutination reaction (Swain et al., 2007;

Barnes et al., 2002).

**Statistical analysis:** SPSS 18 and Excel 2010 software were used for statistical analysis. A one-way analysis of variance (ANOVA) was used to determine significant differences, followed by Duncan's post hoc test, with a 95% confidence interval. All data were expressed as mean  $\pm$  standard deviation.

## Results

Figure 1 shows the results of bacterial exposure in different groups of vaccinated fish. Based on the results, 80% of the fish in the control group died within 12 days after exposure. In addition, the lowest death rate was observed in the group vaccinated by injection, which was 20%. The mortality was recorded at the same rate in vaccinated fish by the two-step immersion in treatments 2 and 3. No significant difference in hematological parameters was recorded between the vaccinated fish and the control group ( $P \leq 0.05$ ) (Table 1).

Figure 2 shows some serology indices in different treatments. Based on the results, the highest level of total protein was measured in the fish that received the vaccine via the injection method and then in the two-step immersion vaccinated fish ( $P \geq 0.05$ ). No significant difference in total protein levels was found in fish vaccinated via one-step immersion (e.g., treatments 4 and 5) compared to the control group (Fig. 2a). The lowest albumin levels were measured in the fish that received a one-step immersion vaccine (e.g., treatments 4 and 5). The highest level of plasma immunoglobulin was recorded in fish vaccinated by injection. In addition, the value of this parameter in unvaccinated fish was significantly lower than that of all vaccinated fish (Fig. 2b). No significant difference ( $P \geq 0.05$ ) in plasma glucose levels was recorded in different groups of fish in this study (Fig. 2c).

The results of ALP, ALT, and AST enzymes are demonstrated in Figures 2d-f. Based on the results, the levels of alkaline phosphatase (ALP) and alanine aminotransferase (ALT) in different treatments had no significant differences ( $P \geq 0.05$ ) (Figs. 2d, e). In the aspartate aminotransferase (AST), the level in the fish of the vaccinated group by injection method was

Table 1. Haematological parameters in different treatments.

	Control	Treatment1	Treatment2	Treatment3	Treatment4	Treatment5
<b>RBC (<math>\times 10^6</math> cell/mm<sup>3</sup>)</b>	0.749 $\pm$ 0.012	0.726 $\pm$ 0.094	0.801 $\pm$ 0.054	0.724 $\pm$ 0.012	0.714 $\pm$ 0.016	0.781 $\pm$ 0.085
<b>Hemoglobin (mg/dl)</b>	7.9 $\pm$ 0.4	7.7 $\pm$ 0.7	8.1 $\pm$ 0.2	8.8 $\pm$ 0.6	7.9 $\pm$ 0.5	8.4 $\pm$ 0.6
<b>Hematocrit (%)</b>	24.3 $\pm$ 3.9	26.5 $\pm$ 2.4	24.3 $\pm$ 5.2	23.3 $\pm$ 4.5	25.8 $\pm$ 1.8	26.9 $\pm$ 4.2
<b>MCV (fl)</b>	324.7 $\pm$ 24.5	333.9 $\pm$ 11.3	307.6 $\pm$ 41.1	334.1 $\pm$ 19.6	340.5 $\pm$ 39.7	324.2 $\pm$ 43.7
<b>MCH (pg)</b>	110.4 $\pm$ 11.2	113.2 $\pm$ 6.1	99.8 $\pm$ 15.2	117.7 $\pm$ 7.9	113.2 $\pm$ 12.2	98.4 $\pm$ 13.2
<b>MCHC (g/dl)</b>	33.8 $\pm$ 1.2	34.9 $\pm$ 1.9	32.4 $\pm$ 2.0	33.5 $\pm$ 1.4	33.3 $\pm$ 1.9	33.5 $\pm$ 1.1

Control: without vaccination, Treatment 1: vaccinated fish by injection method, Treatment 2: vaccinated fish by hyperosmotic solution + two-step immersion, Treatment 3: vaccinated fish by two-step immersion, Treatment 4: vaccinated fish by hyperosmotic solution + one-step immersion, and Treatment 5: vaccinated fish by two-step immersion.

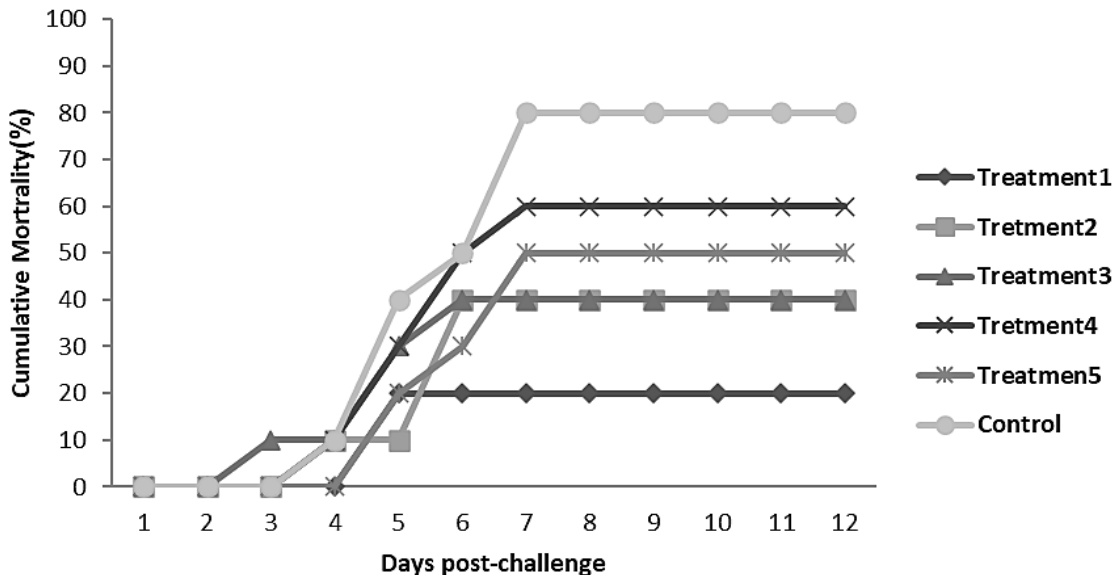


Figure 1. Cumulative mortality of *Huso huso* exposed to bacteria via interaperitoneal injection (Control: without vaccination, Treatment1: vaccinated fish by injection method, Treatment2: vaccinated fish by hyperosmotic solution + two-step immersion, Treatment3: vaccinated fish by two-step immersion, Treatment4: vaccinated fish by hyperosmotic solution + one-step immersion, and Treatment5: vaccinated fish by two-step immersion).

significantly higher than the other groups ( $P \geq 0.05$ ); the enzyme levels in other vaccinated groups and the control group had no significant difference (Fig. 2f).

The counts of WBC in vaccinated fish by the injection method were higher than those of others ( $P \geq 0.05$ ). These values were not significantly different in one-step immersion vaccinated and non-vaccinated fish (Fig. 2g).

As shown in Figures 2h and 2j, the bacterial agglutination titre for *S. iniae* and *L. garvieae* bacteria was observed in all the vaccinated fish. In addition, no agglutination titres were recorded in the control group for the two bacteria. Based on these results, the highest titres belonged to the groups of fish vaccinated by the injection method for both bacteria ( $P \geq 0.05$ ). The fish vaccinated by the two-step immersion method (Treatments 2 and 3) had higher titres than those

vaccinated by the one-step immersion method (Fig. 2h, 2j). The antibody titers for each bacterium were low for the fish of the one-step vaccinated groups (Treatments 4 and 5, and there was no significant difference with the control group ( $P > 0.05$ )).

**Discussions**

In the present study, the double-streptococcus (*S. iniae* / *L. garvieae*) vaccine was effective for controlling streptococcus infection in the great sturgeon. In fish farms, vaccination using the injection method is not very popular; instead, farmers prefer using the bath or oral method for vaccination. Unfortunately, these methods are less effective than the injection method (Bogwald and Dalmo, 2019). The effectiveness of vaccination can be influenced by various factors, such as the time of vaccination (in the immersion method),

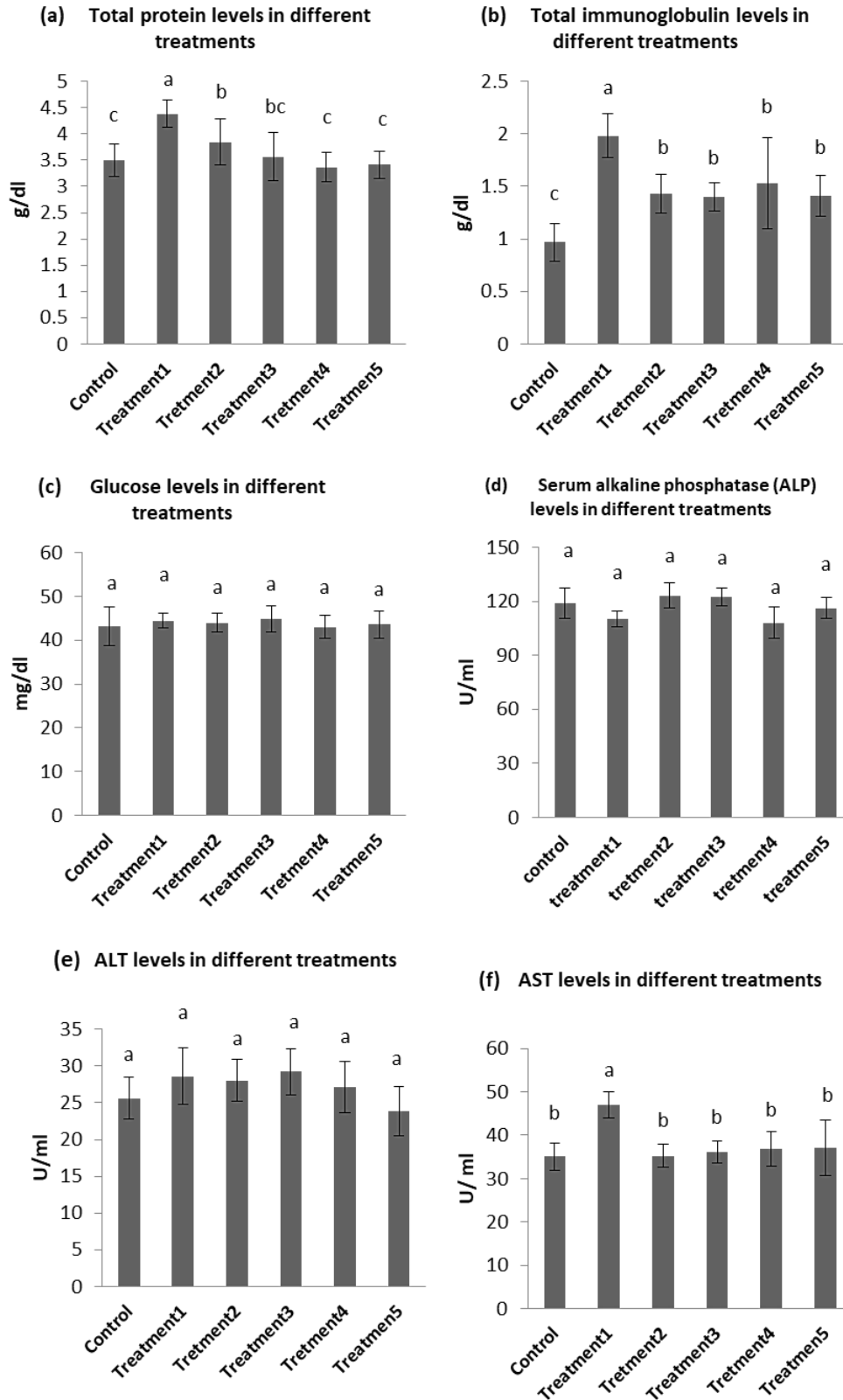


Figure 2. The values of serum biochemical parameters, including (a) total protein, (b) total immunoglobulin, (c) glucose, (d) ALP, (e) ALT, (f) AST, (g), (h), and (J) values in different treatments (Control: without vaccination, Treatment 1: vaccinated fish by injection method, Treatment 2: vaccinated fish by hyperosmotic solution + two-step immersion, Treatment 3: vaccinated fish by two-step immersion, Treatment 4: vaccinated fish by hyperosmotic solution + one-step immersion, and Treatment 5: vaccinated fish by two-step immersion).

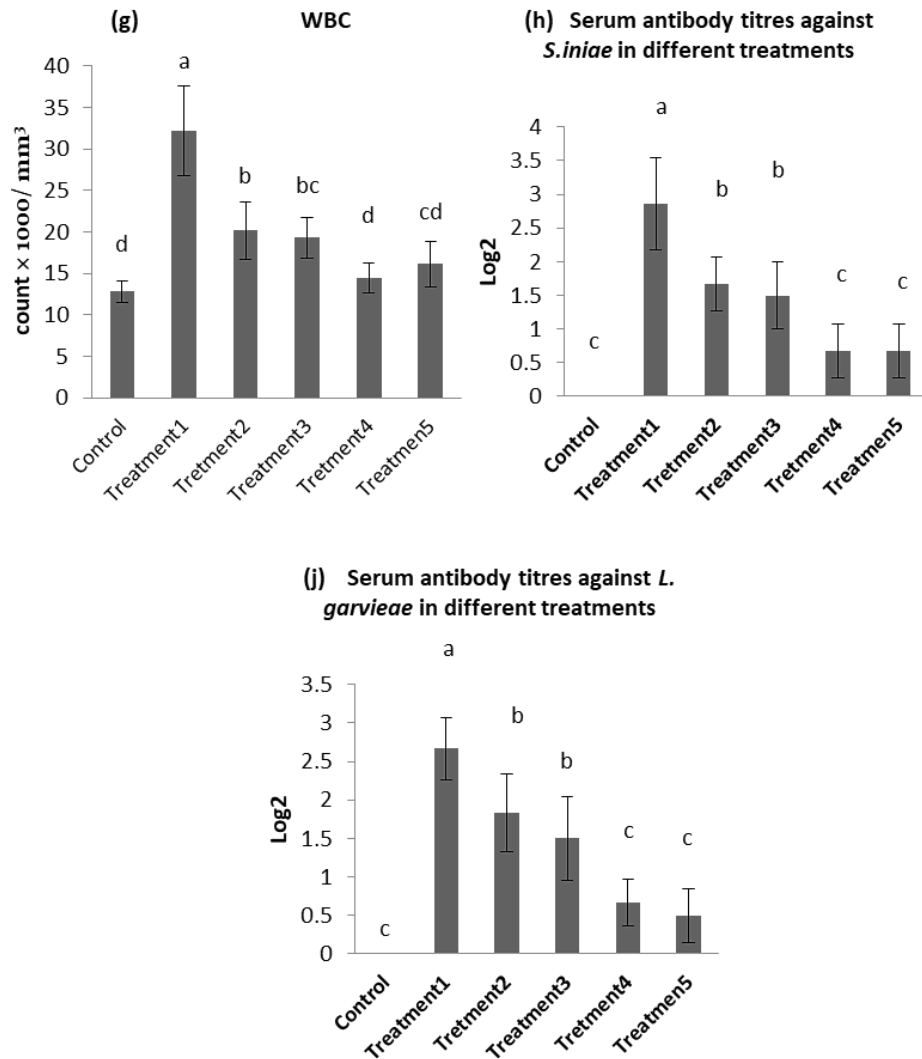


Figure 2. To be continued.

the vaccine dose, and the immune status of the fish (Du et al., 2017). To increase vaccination effectiveness, various methods such as using hyperosmotic environments before vaccination to increase the absorption of antigens have been suggested (Gao et al., 2016). In several studies, using hyperosmotic solutions before vaccination has shown a positive effect (Huising et al., 2003). In the case of olive flounder (*Paralichthys olivaceus*), using a hyperosmotic solution led to a significant increase in the efficacy of *Edwardseilla tarda* vaccine (Gao et al., 2016).

Based on the results, 80% of unvaccinated fish in the control group died after bacterial exposure. This mortality rate was 20% for vaccinated fish via the injection method, 40% for the vaccinated fish via the

two-step immersion method, and 50-60% for the fish vaccinated with the one-step immersion method. Therefore, based on the results, the two-step vaccination by the bath method had acceptable efficiency in great sturgeon, but the one-step bath vaccination was not very effective. This issue can also be traced in the bacterial antibody titer tests, i.e., in the fish that received a one-step vaccine by the bath method (in both the simple vaccination method and the hyperosmotic solution + vaccination method), despite observing the antibody titer, there was no significant difference compared to the control group. In the bacterial challenges, they did not have an acceptable efficiency in one-step bath vaccination, so one-step vaccination does not protect this fish against the mentioned bacteria; therefore, at least a two-step

vaccination in the bath method is suggested. In a previous study, it was found that using a hyperosmotic solution along with the vaccine by bath method significantly improved the efficacy of the vaccine in rainbow trout (Namrudi et al., 2023). However, in this study, using a hyperosmotic solution did not affect the increase in antibody titers and clinical disease control when directly challenged with the bacteria in question. Therefore, further research is required to determine whether NaCl can be used as a hyperosmotic solution in beluga sturgeon. This study had no significant effect on the efficiency of using 15 ppt NaCl as a hyperosmotic solution for bath vaccination methods.

ALP, AST, and ALT enzymes are found in liver hepatocyte cells and some kidney and intestinal cells. An increase in their level in the blood serum typically indicates damage to these tissues. These enzymes are usually measured to evaluate liver and kidney health (Giannini et al., 2005). In the current study, intraperitoneal injection of vaccine in great sturgeon did not increase or decrease the levels of ALP, AST, and ALT enzymes, indicating no side effects in these tissues post-injection. In a similar study, intraperitoneal injection of *Yersinia rockeri* vaccine in juvenile rainbow trout (5-10 g) led to a significant increase in the levels of these enzymes in the blood serum (Mazandarani et al., 2022). Additionally, intraperitoneal injection of double *streptococcus / lactococcus* vaccine (the same vaccine) significantly increased ALP, ALT, and AST enzyme levels in the blood serum of rainbow trout (Namroudi et al., 2023). Based on our findings, it can be concluded that vaccination via the injection method in great sturgeon weighing about 30 grams has fewer side effects compared to juvenile rainbow trout (5-10 g). Therefore, for this weight range of great sturgeon (about 30 g), the injection method of vaccine can be suggested.

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