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Immune Responses and Protective Efficacy Against Streptococcosis Following Polyvalent Inactivated Vaccine Injection in the Nile Tilapia, *Oreochromis niloticus*

Amira El-daim*, Eman A. Abdel Gawad, Amel M. El Asely,
Amany A. Abbass, Adel A. Shaheen
Department of Aquatic Animals medicine, Faculty of Veterinary Medicine, Benha University, Egypt

***Corresponding author: amiramohamed2577@yahoo.com**

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ABSTRACT

One of the most significant bacterial diseases affecting the Nile tilapia is streptococcus, which has a significant negative impact on the global economy. As a result, vaccination is seen to be the best strategy for controlling bacterial disease in farmed fish. The objective of the current study was to assess the effects of formalin and autoclaved polyvalent vaccinations on immunological responses, oxidative stress biomarkers, antioxidant defense, and streptococcal protection in the Nile tilapia. Healthy fish (30±5g) were intraperitoneally vaccinated with 0.1ml of polyvalent vaccine, containing either formalin or autoclaved inactivated *streptococcus agalactiae*, *streptococcus iniae* and *lactococcus garvieae*, while the control group was injected with sterile saline. Serum samples were withdrawn 14 days post-immunization to assess immunoglobulin M (IgM), lysozyme activity (LYZO), alkaline phosphatase (AKP) and acid phosphatase (ACP). In addition, oxidative stress biomarkers [malondialdehyde (MDA), nitric oxide (NO)], hepatic antioxidant activities [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx)] were recorded. All groups were separately challenged by virulent strains of *S. agalactiae*, *S. iniae* and *L. garvieae* at the end of the immunization period (14 days). The results showed that IgM level and LYZO, AKP and SOD activities were significantly increased in both vaccinated groups, compared to the control group. The relative percent survival of the immunized Nile tilapia varied from 88.8- 94.0%. These results confirmed that both polyvalent formalin and autoclaved inactivated vaccine boost immune response, antioxidant activity and confer an excellent protective effect against streptococcal infection in the Nile tilapia.

INTRODUCTION

Oreochromis niloticus is a globally important freshwater farmed species **(FAO, 2020)**. Customers in Egypt prefer this fish because of its superior meat quality, reasonable cost and lack of intramuscular spines. The Nile tilapia aquaculture production reached 86% in 2020. However, since tilapia business has quickly grown, bacterial disease outbreaks have recently been on the rise, making disease control one of our fish farms' biggest concerns **(GAFRD, 2020)**.

According to **Zhang *et al.* (2018)**, streptococcal infection was identified as a significant obstacle to the sustainable expansion of tilapia aquaculture; it is a disease of public health significance **(Baiano & Barnes 2009)**. The bacterial species *S. agalactiae, S. dysgalactiae, S. iniae, L. garvieae, S. parauberis,* and *S. faecalis* cause streptococcosis in aquatic animals **(Abdel-Gawad *et al.,* 2007; Osman *et al.,* 2017; Mishra *et al.,* 2018; Pierezan *et al.,* 2020)**. Streptococcus is an opportunistic pathogen that is frequently present in freshwater and saltwater. It is adaptable to a wide variety of hosts and can cause abnormal behavior, bone abnormalities, body ulcers and a high mortality rate (**Wang *et al.,* 2018; Younes *et al*., 2019)**. In order to protect both animals and humans against infections, it is crucial to create techniques for reducing the prevalence and occurrence of this disease. Regrettably, the prolonged use of various chemicals and antibiotics in aquaculture to treat bacterial diseases has led to the buildup of drug residues, the emergence of antibacterial resistance, environmental contamination and negative effects on the health of both fish and people **(Watts *et al.,* 2017; Heydari *et al.,* 2020)**. Thus, in order to combat infectious diseases, vaccines must be used instead of antibiotic therapy.

Vaccination has emerged as a crucial method of protection against a variety of viral pathogens infecting aquatic species. Effective vaccinations against infectious diseases have been developed as a result of numerous studies, including live-attenuated, inactivated and genetically modified vaccines **(Li *et al*., 2015; Sukenda *et al*., 2018; Youssef *et al*., 2022)** To produce higher and longer protection, several vaccinations contained non-oil adjuvants **(Kole *et al*., 2019; Zhang *et al*., 2020a)** or mineral oil adjuvants **(Abu-Elala *et al*., 2019)**. In the meanwhile, the vaccination employed may be monovalent, bivalent or polyvalent **(Hayat *et al*., 2020; Mohammadi *et al*., 2021)**. Most inactivated vaccinations currently available are created by physically or chemically inactivating virulent wild strains **(Tkachenko *et al*., 2014; Bactol *et al*., 2018)**. Nevertheless, inactivation, particularly when done with chemicals, may lower the immunogenicity and effectiveness of the vaccine **(Grabowski *et al*., 2004).** Moreover, oral, intraperitoneal, and subcutaneous delivery methods have an impact on vaccine efficiency **(Dong *et al*., 2020)**. Notably, the most popular technique for generating efficient systemic adaptive immunity is injection **(Plant & Lapatra, 2011)**.

The use of vaccines in fish farms faces many difficulties, including the lack of vaccination programs, high import vaccine costs, the limited range of cross protection, high mortality rates associated with stress during fish handling, high anesthetic costs and the requirement for trained personnel and equipment **(Zhang *et al*., 2020a)**. Yet, Egypt's existing plan favors the development of efficient vaccines to combat bacterial diseases threatening large-scale tilapia farming in multiple farms **(Abu-Elala *et al*., 2019)**. In order to provide the Nile tilapia with the best protection, vaccination should be delivered when the fish are 21 days of old with weights between 5 and 30g, when enough immunity has time to develop **(Evans *et al*., 2004 b)**. In the current study, we evaluated

the formalin and autoclaved inactivated polyvalent streptococcal vaccine's impact on the Nile tilapia's immune response, oxidative stress, antioxidant defense and disease resistance. To our knowledge, this is the first study to examine the effectiveness of the polyvalent autoclaved inactivated vaccine against streptococcosis in the Nile tilapia.

MATERIALS AND METHODS

1. Fish rearing management

Healthy *O.niloticus* of average body weight 30±5g was obtained from private farm, Sharqia governorate, Egypt. Fish were transferred, maintained in glass aquarium (90×30×40 cm) and acclimated for 14 days at wet Laboratory of Aquatic Animal medicine, Faculty of Veterinary Medicine, Benha University, Egypt. Throughout the experimental period, fish specimens were kept at a water temperature of 26 ± 2°C, pH (7.2 ± 0.24) and dissolved oxygen (5.12 ± 0.2mg/L). Twice daily, a commercial feed containing 30% protein was supplied to all fish until it appeared satiated (Aler-Aqua, Egypt). After fish arrival, random fish samples were screened for parasitic and bacterial infection to confirm their health statue and ensure pathogenic microorganism free **(Austin & Austin, 2017)**. This research was conducted according to the guidelines of the Committee of Animals Welfare and Research Ethics of Benha University, Faculty of Veterinary Medicine, Egypt (BUFVTM: 19-10-22).

2. Bacterial strains and adjuvant

Virulent strains of *S. agalactiae*, *L. garvieae* and *S. iniae* were used in this study. *S. agalactiae* and *L. garvieae* were previously isolated from disease outbreaks in the Egyptian *O.niloticus* farms (unpublished data); meanwhile, *S. iniae* strain was gifted from Fish Diseases Department, Faculty of Veterinary Medicine, Beni-suef University, Egypt. Montanide™ ISA 71 VG (Seppic, Paris, France) was used in this study as mineral oil-based adjuvant for vaccine preparation.

3. Polyvalent inactivated vaccine preparation

S. agalactiae, *S. iniae*, and *L. garvieae* bacterial strains were subcultured in brain heart infusion (BHI) broth (Himedia, India) and incubated at 30 °C for 24-48 hours. Then, to determine the equivalent number of colony forming units (CFU)/ml, the culture broth of each bacterial strain was serially ten-fold diluted in sterile physiological saline. 0.1ml of each culture was then plated on tryptic soya (TSA) agar (Oxoid, UK). The final concentration of produced bacterial strains was adjusted to OD600nm =1.3 (equivalent to10^8 CFU mL−1) for the vaccine formulation.

Bacterial inactivation was physically perfomed by autoclaving and chemically by formalin according to the methods of **Bactol *et al* (2018)** and **Youssef *et al* (2022)**, respectively. Briefly, the cultures were incubated at 25°C for 24h while continuously stirring in a 0.5% formalin (Himedia, India) (37% formaldehyde). Using sterile phosphate buffer (PBS) saline, the cell pellets from the inactivated bacterial culture were washed twice after being centrifuged at 1800g for 30min. (pH 7.4). The cell pellets had a final

concentration of 1×10^8 CFU $ml^{-1}$when they were re-suspended in sterile PBS and kept at 4°C. Bacterial culture was incubated at 121°C for 15min to create autoclaved inactivated bacterial strains, which were then stored at 4°C until needed. Placing an amount of 100µl of formalin-inactivated and autoclaved bacterial suspensions on TSA, followed by incubating them for 72h at 30°C served as the sterility test.

The completely inactivated bacterial cells [*S. agalactiae, S. iniae, L. garvieae* (10^8 CFU/ml final concentrations)] were mixed at a ratio of 1:1:1 in volume as polyvalent inactivated culture. Finally, the polyvalent vaccine was prepared by mixing an equal volume (50/50) of polyvalent inactivated culture and Montanide adjuvant at room temperature under moderate agitation until completely mixed.

4. Experimental set up

Two hundred and seventy *O. niloticus* individuals were divided randomly into three groups, with 90 fish individual for each. Each group had three replicates / 30 each. The first group 1; G1 (control) was IP injected with 0.1ml sterile normal saline; the second group 2; G2 was IP injected with 0.1ml formalin inactivated polyvalent (FIP) vaccine and the third group 3; G3 was IP injected with 0.1ml autoclaved inactivated polyvalent (AIP) vaccine. After ten days of first immunization, all vaccinated groups were given a booster dose. Fish samples were fed a basic commercial food (Aler-Aqua, Egypt) at a rate of 3% of body weight twice daily during the immunization study (9:00 a. m. and 16:00 p.m.). About 50% of the water was replaced three times per week with well-aerated, dechlorinated water.

5. Samples collection

Blood samples were collected 14 days post vaccination (n=9 fish per group). Fish were anesthetized using MSS222 at a dose of 30mg/l water, and blood samples were obtained from caudal blood vessels using a 1-mL syringe with a 25-G needle. After that, samples were kept overnight to coagulate at a refrigerator temperature of 4°C. Centrifugation was used to separate the serum for 10 minutes at 3000 rpm. The obtained serum was combined into three samples, each of which was kept at -20°C until the immunological parameters were assessed. Liver specimens from vaccinated and control groups were also taken 14 days after vaccination and homogenized in phosphate buffer saline (PBS) (pH 7.4) at a ratio of 1:10 (w/v), using electrical homogenizer (Heidolph, Germany). The homogenates were centrifuged at 4°C at 4000 ×g for 15min, and the supernatants were stored at –20°C until analysis of oxidative stress biomarker, antioxidant enzymes activities and nitric oxide level.

6. Immunological parameters assay

The serum bactericidal activity was measured using a modified version of **Kajita *et al* (1990)** by **El-Asely *et al.*, (2014)**. Briefly, *A. veronii* were suspended in PBS and diluted to a concentration of 1×10^7/ml. A Bürker-Türk hemocytometer cell counting chamber (Hirschmann, Eberstadt, Germany) was used to adjust the necessary bacterial

cell concentration. Afterwards, a bacterial suspension in an identical amount was combined with 500µl of serum, and the mixture was incubated for 60min at 25°C. A control group was likewise incubated for 60 minutes at 25°C with a bacterial solution in the same buffer. Both the sera-bacterial suspensions and the control were diluted at a ratio of 1:10 in PBS following incubation. The incubated mixture (100 µl) was cultivated on TSA plates in triplicate, and the numbers of colonies were counted after incubation for 24h at 25°C by using colony counter.

The serum IgM level and lysozyme activity were measured following the manufacturer's instructions of commercial ELISA kit (Sunlong Biotech co. China).

Acid phosphatase (ACP) and alkaline phosphatase (AKP) were colorimetrically determined at a wavelength of 510nm according to **Kind and King (1954)** and **Belfield and Goldberg (1971)**, respectively.

7. Antioxidant activities, oxidative stress biomarker and nitric oxide analysis

Superoxide dismutase (SOD) and catalase (CAT) activities were measured according to **Fossati *et al.* (1980)**. Glutathione reductase (GR) activity was analyzed according to the methodology of **Satoh (1978)**, and glutathione peroxidase (GPx) activity was calculated according to the method of **Moin (1986)**. Lipid peroxidation was determined by estimating malondialdehyde (MDA) content as oxidative stress indicator according to the method of **Kamyshnikov (2004)**. Nitric oxide (NO) level was estimated according to **Rajaraman *et al* (1998)**.

8. Challenge test

Two weeks post-vaccination, 120 fish from the vaccinated groups were randomly divided into six groups. Prior to the challenge, all fish were starved for 24 hours then, 20 fish in each group were separately IP challenged with virulent strains of *S. agalactiae*, *S. iniae* and *L. garvieae* ($1x10^8$ CFU/ml). Meanwhile, 80 fish of control non- vaccinated group were divided into two sub-groups. The first subgroup (control positive) (n= 60 fish) was IP injected with 0.1ml of virulent bacterial strains ($1x10^8$ CFU/ml). The second subgroup (control negative) (n=20 fish) was IP administrated with 0.1ml of PBS. For ten days after the challenge, necropsy findings, cumulative mortality, and relative percentage survival (RPS) were recorded. RPS was determined using the formula mentioned by **Amend (1981):** RPS = [1− (mortality in vaccinated group/mortality in control group)] × 100%. Bacterial re-isolation from kidneys of the moribund- challenged fish was carried out to achieve Koch postulate.

9. Statistical analysis

Using the SPSS 26.0 statistical analysis program, one-way analysis of variance (ANOVA) and Duncan post-hoc testing were used to examine the statistical significance between the control and vaccine groups. Data were presented as the mean ± SEM. A *P*-value < 0.05 was considered significant.

RESULTS

1. Innate immunity components

Data in Table (1) display the serum innate immune parameters in vaccinated and control groups. IgM level and LYZO activity were significantly improved ($P< 0.05$) in all vaccinated groups, compared to control groups. Interestingly, serum LYZO and IgM in the autoclaved vaccinated group were higher than formalin vaccinated and control groups.

Serum bactericidal activity and AKP recorded a significant increase ($P< 0.05$) in all vaccinated groups, compared to control. Although, serum ACP showed improvement in all vaccinated fish, no statistical difference was detected, compared to the control fish.

Table 1. Immunological parameters of the Nile tilapia vaccinated with formalin inactivated polyvalent vaccine (FIPV) and autoclaved inactivated polyvalent vaccine (AIPV) for 14 days

Group	IgM(µg/ml)	Lyzo(U/ml)	BA (µg/l)	AKP (U/l)	ACP (U/l)
Control	$1.13\pm.09^{b}$	$1.59\pm.03^{a}$	$17.48\pm.024^{b}$	$6.53\pm.06^{b}$	$4.12\pm.01^{b}$
G 2 (FIPV)	$1.58\pm.02^{ab}$	$2.08\pm.02^{b}$	$20.67\pm.06^{a}$	$8.46\pm.04^{a}$	$4.43\pm.02^{b}$
G 3 (AIPV)	$1.93\pm.01^{a}$	$2.61\pm.02^{ab}$	$21.6\pm.09^{a}$	$9.17\pm.04^{a}$	$4.89\pm.01^{ab}$

Different superscripts in the same columns show significant differences among groups ($P < 0.05$). All data appear as mean and SE (n = 3/replicate).

2. Antioxidant activities, oxidative biomarker and nitric oxide

Liver SOD activity was significantly increased ($P< 0.05$) in all vaccinated groups, compared to the control activity level. In contrast, CAT activity was significantly decreased in autoclaved vaccinated groups, compared to the control group. GR and GP_X activities did not significantly differ ($P> 0.05$) with the control fish. The level of lipid peroxidation in the liver specimens of vaccinated fish did not significantly differ ($P> 0.05$) from that recorded in the control. The activities of hepatic antioxidant enzymes in response to vaccine treatment are presented in Table (2). Moreover, all vaccinated samples exhibited no significant differences in NO level, compared with the control level.

3. Challenge trial

Clinical signs of streptococcal infection were exhibited in all challenged groups, with less pronounced in the vaccinated groups. These include corneal opacity, darkness of skin, lethargy in swimming, exophthalmia & loss of appetite. Moreover, re-isolation of bacteria from kidneys of moribund fish revealed Gram- positive streptococci. Necropsy

finding of the challenged Nile tilapia revealed congested spleen, liver and kidney, with the presence of abdominal fluids in some fish. Fish vaccinated with AIPV and challenged with *S. agalactiae*, *S. iniae* and *L. garvieae* recorded the highest RPS (94.0%), compared to the group vaccinated with FIPV, which had RSP 94.0%, 88.8%, 93.3%, respectively (Fig 1, 2, 3).

Table 2. Antioxidant activity in liver tissue of the Nile tilapia vaccinated with formalin inactivated polyvalent vaccine (FIPV) and autoclaved inactivated polyvalent vaccine (AIPV) for 14 days

Group	SOD (U/g)	CAT (U/g)	GSH (µmol/g)	GPX (ng/g)	MDA (nmol/g)	NO (µmol/g)
Control	33.47±.09^{b}	15.50±.06^{ab}	3.20±.06^{a}	1.76±.06^{a}	40.2±.10^{a}	3.20±.03^{a}
G1 (FIPV)	43.76±.08^{a}	13.22±.07^{ab}	2.74±.03^{bc}	1.59±.01^{a}	40.51±.02^{a}	3.59±.02^{a}
G 2 (AIPV)	45.00±.08^{a}	12.27±.03^{b}	2.74±.07^{ab}	1.79±.08^{a}	41.68±.03^{a}	3.29±.02^{a}

Different superscripts in the same columns show significant differences among groups ($P< 0.05$). All data appear as mean and SE (n = 3).

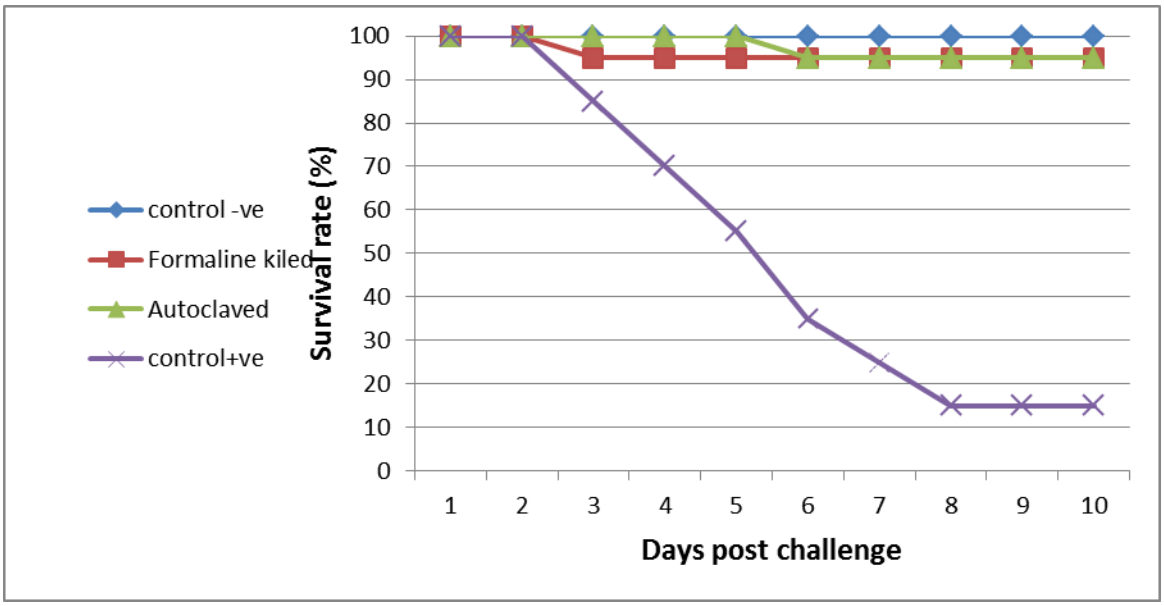

Fig. 1. Survival rate of formalin inactivated and autoclaved *S. agalactiae* vaccine ten days post challenge

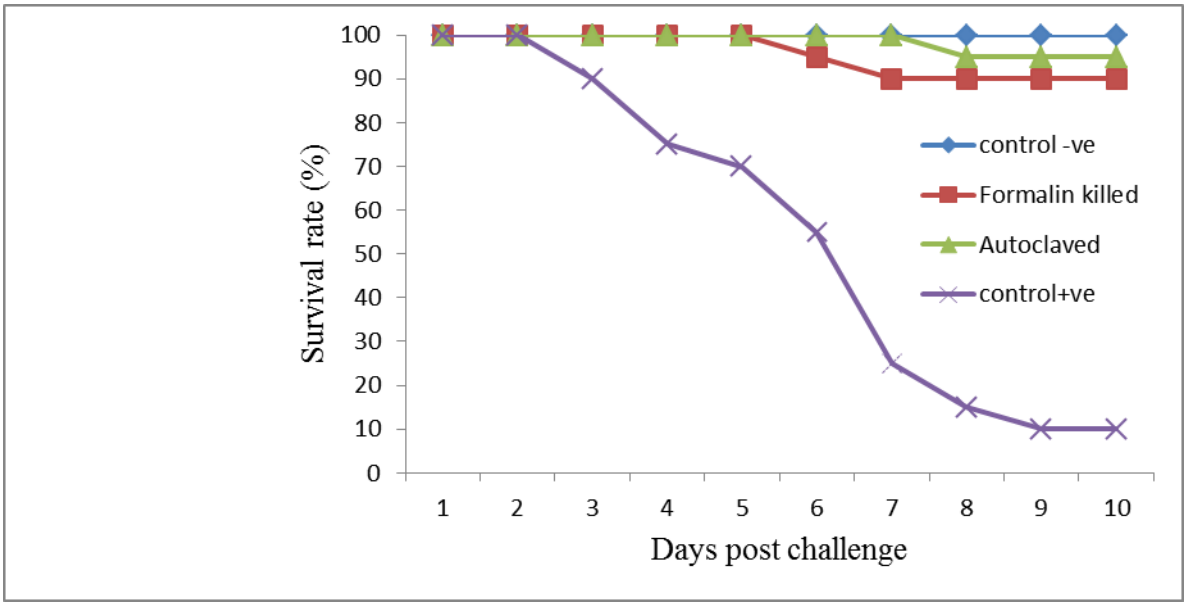

Fig. 2. Survival rate of formalin inactivated and autoclaved *S. inaie* vaccine ten days post challenge

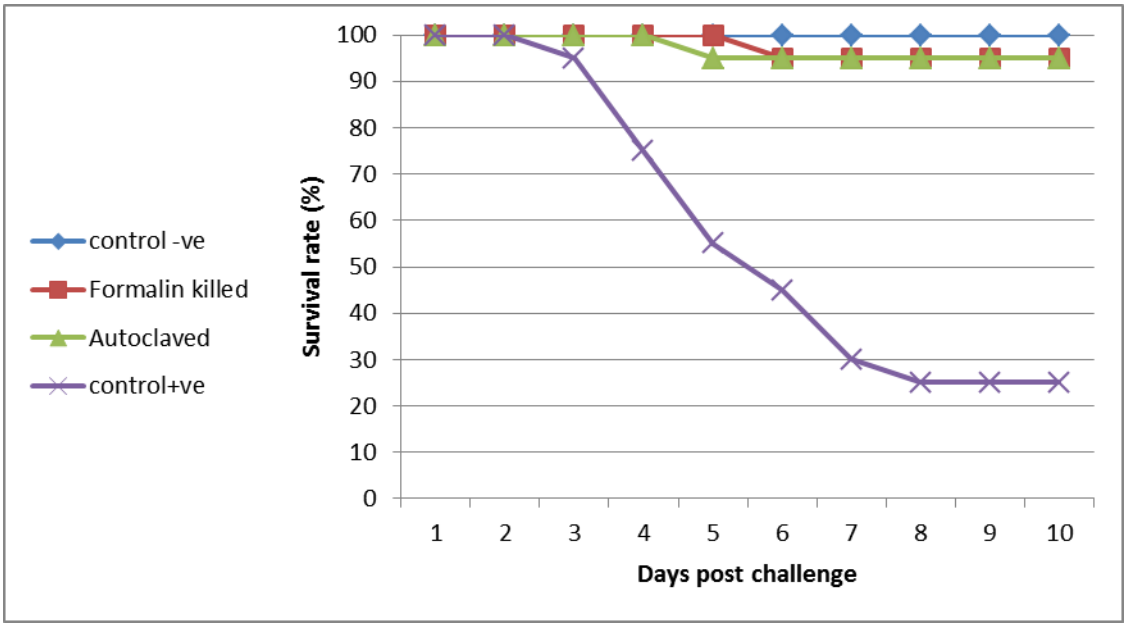

Fig. 3. Survival rate of formalin inactivated and autoclaved *L. gravaie* vaccine ten days post challenge

DISCUSSION

S. agalactiae, S. iniae, and *L. gravaie,* were believed to be the main causes of streptococcal septicemia, which can result in high mortality rates and significant financial losses in aquaculture **(Wang *et al.*, 2020)**. The most effective method for preventing and controlling streptococcus infection is still vaccination **(Evans *et al.*, 2004a)**. Fish farms frequently use inactivated vaccines because of their quick development times, security and cheaper labor expenses **(Sobh & Bonilla, 2016)**. Earlier research has shown that fish can produce antibodies, protecting them from a variety of bacterial infections when given injectable inactivated formalin-killed vaccinations **(Liu *et al.*, 2016**; **Hayat *et al.*, 2020**; **Kong *et al.*, 2022)**.

In fish's defense against infection, the non-specific immune system is more important than the specific immune system **(Behera & Swain, 2013)**. Specific antibody production is one of the significant parameters of the immune response of fish **(Abdelhamid *et al.*, 2021; Akter *et al.*, 2022)**

The finding of the present study revealed significant increase in serum IgM level in the vaccinated groups, compared to the unvaccinated group. This result is in accordance with several previous studies showing that vaccination can efficiently improve the immune response of the Nile tilapia against streptococcosis **(Ismail *et al.*, 2016; Abu-Elala *et al.*, 2019; Hayat *et al.*, 2020; Monir *et al.*, 2020)**.

The innate immune system's key enzyme and fish lysozyme can split the cell walls of Gram-positive bacteria (peptidoglycan) **(Saurabh & Sahoo, 2008)**. Moreover, it functions as an opsonin and stimulates phagocytes and the complement system. In our investigation, 14 days after immunization, serum lysozyme activity significantly increased in all vaccinated groups. This suggests a strengthening of the fish immune system and increased resistance to infections, which can raise metabolic activity, lower mortality and boost fish survival rates **(Mohammadi *et al.*, 2021)**. Similarly, **Wang *et al.* (2020)** recorded higher increase in LYZO activity of the vaccinated Nile tilapia using *S. iniae* and *S. agalactiae* formalin killed bacteria. Moreover, **Huang *et al* (2014)** observed that the formalin inactivated *S. iniae* vaccine enhances innate defense mechanisms in farmed grouper, *Epinephelus coioides*.

An essential measure to consider when assessing a non-specific response to suppress bacterial growth is serum bactericidal activity **(Biller-Takahashi *et al.*, 2013)**. It is understood that the rise in protective proteins in the serum, such as immunoglobulins, complement system proteins, acute phase proteins, cytokines, lysozyme, transferrin and lectins, which are typically elevated after infection or vaccination, reflects the increased serum bactericidal activity. In this study, all vaccine groups considerably outperformed the unvaccinated control group in terms of serum bactericidal activity. The increased lysozyme activity and IgM level may be responsible for this improvement. Previous investigations recorded a considerable improvement in serum bactericidal activity after vaccination in many fish species **(Silva *et al.*, 2009; Kitiyodom *et al.*, 2021)**. In contrast,

Halimi *et al*. (2020) observed no significant difference in serum bactericidal activities in rainbow trout, *Oncorhynchus mykiss*, orally vaccinated with chitosan-alginate coated vaccines against *L. garvieae* and *S. iniae.* The serum complement activity of Asian seabass *(Lates calcarifer)* fingerlings immunized against *S. iniae* and *Vibrio harveyi* was not significantly altered according to **Mohammadi *et al.* (2021)**. These variations may result from the type of vaccine, the length of time and method of immunization, as well as the fish species.

Two important enzymes, ACP and AKP, are known to play a crucial part in the immunological response of fish by hydrolyzing and digesting foreign invaders **(He *et al*., 2017)**. These actions serve as potent indicators of macrophage activation and improve pathogen detection and phagocytosis **(Zhang *et al.*, 2020b)**. As presented in this study, the autoclaved inactivated polyvalent vaccine could significantly enhance AKP activity, whereas ACP showed no significant difference compared to control activity. Vaccinated Nile tilapia against *S. iniae* **(Wang *et al*., 2020)** and yellow catfish *(Pelteobagrus fulvidraco)* with bivalent inactivated *Aeromonas veronii* and *Edwardsiella ictaluri* vaccine **(Kong *et al*., 2022)** both showed a considerable increase in ACP nd AKP activates.

As far as we are aware, there has never been a study done on the use of an autoclaved inactivated vaccination against streptococcosis. Intriguingly, as compared to the FIPV and control groups, the AIPV showed higher increases in innate immune markers. As a result, it may be concluded that AIPV generate greater antigenicity than FIPV whole cell vaccines. This might be as a result of formalin's cross-linking characteristics, which might have decreased antigenicity **(Grabowski *et al*., 2004)**. Similarly, **Bactol *et al* (2018)** showed that autoclave-killed whole cell *Aeromonas hydrophila* vaccine produces significant response in the Nile tilapia, compared to the formalin killed vaccine.

Reactive oxygen species (ROS) are quickly removed by fish body antioxidants under normal physiological conditions **(Halliwell, 1994)**. The body's antioxidant system is unable to remove ROS after the overproduction of these molecules and oxidative stress results **(Oruc, 2010; Engwa, 2018)**. Oxidative stress may be induced under the influence of vaccination, leading to membrane and DNA damage **(Ural, 2013; Tkachenko *et al*., 2014)**.

The antioxidant enzymes SOD, CAT, and GPx serve as the first line of defense against oxidative stress by converting superoxide radicals into hydrogen peroxide, which is then converted into water and oxygen molecule **(Ural, 2013)**. In the present work, SOD was significantly increased in the vaccinated Nile tilapia; a finding which concurs with that of **Wang *et al.* (2020)** who recorded an elevation in SOD activity of the Nile tilapia vaccinated against *S. iniae* and *S. agalactiae*. However, CAT activity in the present study decreased in autoclaved vaccinated fish, compared to the formalin vaccinated and unvaccinated control groups. The decreased CAT activities may indicate the reduced

capacity to scavenge hydrogen peroxide free radicals produced in the liver tissue of vaccinated Nile tilapia. According to **Tkachenko *et al* (2014)**, rainbow trout immunized against furunculosis showed noticeably different responses from antioxidant enzymes, and the immunization may cause oxidative stress, mostly in the gill and liver tissues.

Glutathione reductase that is NADPH-dependent is required for GPx to have access to glutathione disulfide **(Sagara *et al.*, 1998**. According to our findings, there were no significant variations in liver tissue in the GR and GPx vaccinated groups, compared to the unvaccinated control group. In a similar manner, rainbow trout immunized against *Yersinia ruckeri* did not exhibit any significant changes in the liver antioxidant GPx during the first month following immunization **(Tkachenko *et al.*, 2017)**. On the other hand, in Asian seabass fingerlings immunized with monovalent and bivalent vaccinations against *S. iniae* and *Vibrio harveyi*, the level of GR was markedly boosted **(Mohammadi *et al.*, 2021)**. The type of vaccination, fish species, tissue response, length of the vaccine, and method of administration could all contribute to cause this variation.

MDA level is considered as lipid peroxidation biomarker, and its significant increase indicates oxidative damage due to excessive free radicals **(Tkachenko *et al.*, 2014)**. In the present study, hepatic MDA revealed no significant changes in vaccinated groups, indicating the safety of formalin and autoclaved inactivated polyvalent vaccine for the Nile tilapia, since it did not induce any stress. Similarly, Asian seabass showed non-significant increases in serum MDA after 30 days of receiving the *S. iniae* vaccine injection, but increased 60 days later according to **Mohammadi *et al.* (2021).**

NO is a signalling molecule that is crucial for many physiological processes, including growth and iron availability **(Ramirez *et al.*, 2011)**. Along with controlling fish immune responses, nitric oxide also has an antibacterial effect **(Villamil *et al*., 2002)**. NO production of vaccinated fish in the current study did not differ significantly from unvaccinated fish. Increased non-specific immunity and antioxidant enzyme systems, which stop the formation of any hazardous and unstable compounds may be responsible for this outcome **(Yeh & Klesius, 2013)**. The Nile tilapia was exposed to pathogenic strains of *S. agalactiae, S. iniae*, and *L. garvieae* to test the vaccinations' ability to protect against infection. In our study, the RPS of the vaccinated Nile tilapia with autoclaved inactivated polyvalent vaccine showed higher protection level (94.0%) against all bacterial strains. Meanwhile, the protection level was 88.8%, 93.3%, 94.0% in the formalin vaccinated group challenged with *S. iniae, L. garvieae*, and *S. agalactiae*, respectively. Similar to the finding of **Wang *et al* (2020)** and **Linh *et al.* (2022)** in Nile tilapia vaccinated with formalin-killed cells challenged with streptococcus species Therefore, with the obtained high protection level against various pathogens causing streptococcus infection, we considered that the formalin and autoclaved inactivated polyvalent vaccine performed excellent defense as reported by **Chettri *et al* (2015)** who suggested that vaccine protection levels should be greater than 80% to be considered excellent.

CONCLUSION

According to the results of this study, the Nile tilapia can increase their resistance to streptococcal infections by immunizing them with formalin and autoclaved inactivated polyvalent vaccinations. Thus, the vaccinated fish had lower mortality and greater RPS than the challenged vaccinated group. The polyvalent inactivated vaccines administered intraperitoneally are a good choice according to the results of this study for controlling of streptococcosis in the Nile tilapia aquaculture sector. In our opinion, autoclaved inactivated polyvalent vaccine is preferable to formalin polyvalent inactivated vaccine in terms of immune response and protective efficacy.

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