Full length article

Effects of short-term fasting on the resistance of Nile tilapia (*Oreochromis niloticus*) to *Streptococcus agalactiae* infection

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ABSTRACT

Short-term feed deprivation or fasting is commonly experienced by aquaculture fish species and may be caused by seasonal variations, production strategies, or diseases. To assess the effects of fasting on the resistance of Nile tilapia to *Streptococcus agalactiae* infection, vaccinated and unvaccinated fish were fasted for zero, one, three, and seven days prior to infection. The cortisol levels of both vaccinated and unvaccinated fish first decreased and then increased significantly as fasting time increased. Liver glycogen, triglycerides, and total cholesterol decreased significantly after seven days of fasting, but glucose content did not vary significantly between fish fasted for three and seven days. Hexokinase (HK) and pyruvate kinase (PK) activity levels were lowest after seven days of fasting, while phosphoenolpyruvate carboxykinase (PEPCK) activity levels varied in opposition to those of HK and PK. Serum superoxide dismutase (SOD) and catalase (CAT) activity levels first increased and then decreased as fasting time increased; SOD activity was highest after three days of fasting. Interleukin-1beta (*IL-1β*) and *IL-6* mRNA expression levels first increased and then decreased significantly, peaking after three days of fasting. However, suppressor of cytokine signaling-1 (*SOCS-1*) mRNA expression levels were in opposition to those of *IL-1β* and *IL-6*. Specific antibody levels did not vary significantly among unvaccinated fish fasted for different periods. Although specific antibody level first increased and then decreased in the vaccinated fish as fasting duration increased, there were no significant differences in the survival rates of fasted vaccinated fish after challenge with *S. agalactiae*. The final survival rates of vaccinated fish fasted for zero, one, three, and seven days were 86.67 ± 5.44%, 80.00 ± 3.14%, 88.89 ± 6.28%, and 84.44 ± 8.32%, respectively. Among the unvaccinated fish, the survival rate was highest (35.56 ± 3.14%) in the fish fasted for three days and lowest (6.67 ± 3.14%) in the fish fasted for seven days. Therefore, our results indicated that short-term fasting (three days) prior to an infection might increase the resistance of unvaccinated Nile tilapia to *S. agalactiae*.

1. Introduction

Starvation, which is a major threat to fish in natural environments, may result from fluctuations in food availability, water temperature, salinity, and oxygen concentration; fish may starve during spawning preparation or migration [1,2]. Farmed fish may also experience starvation due to insufficient feeding, uneven feeding, or high breeding densities. In general, fish tolerate starvation better than mammals. Some fish tolerant feed deprivation, fasting, and other adverse conditions using adaptive biochemical and physiological mechanisms [3–5]. Such strategies include the activation of the neuroendocrine system

through the release of stress-related hormones (catecholamines and cortisol) in the blood, as well as secondary responses, including hematological and biochemical changes [6,7]. The intricate balance among the nutritional conditions, antioxidant capacity, and immunity determines the physiological disease resistance of a given fish [8].

Interestingly, although starvation may reduce fish growth and survival, appropriate dietary modulations may prolong lifespan and enhance the disease resistance [9–11]. Experimental studies in fish and rodents have demonstrated that fasting influences the immune system. For example, intermittently-fasted mice had a significantly lower mortality rate after injection with *Salmonella typhimurium*, as compared

to mice fed regularly [12]. In addition, Mohapatra et al. (2017) showed that the short-term starvation of red sea bream prior to *Edwardsiella tarde* exposure effectively increased the resistance of the fish to bacterial infection [13].

Tilapia is an economically-important fish worldwide, due to its adaptability and high protein content. However, *Streptococcus agalactiae* infections lead to high mortality rates in this fish, causing significant economic losses for the tilapia aquaculture industry [14,15]. Decreasing food intake during the infectious phase has long been used as a remedial measure in both vertebrates and invertebrates [16–18]. During field investigations, we found that tilapia farmers typically stop or reduce feeding after *S. agalactiae* outbreaks, expecting this remedial measure to reduce streptococcosis-associated economic losses. However, it is unclear whether short-term fasting improves the resistance of tilapia to *S. agalactiae*. Therefore, in this study, we investigated whether fasting vaccinated and unvaccinated tilapia helped to stimulate physiological changes necessary to effectively resist *S. agalactiae* infection.

2. Materials and methods

2.1. Bacterial strain and vaccine preparation

In 2009, the virulent *S. agalactiae* strain THN0901 (serotype Ia) was isolated from infected tilapia during a typical streptococcosis outbreak at a fish farm in Hainan province, China. This *S. agalactiae* strain is fatal to tilapia [19]. *S. agalactiae* strain THN0901was cultured, harvested, and quantified, and an inactivated vaccine was prepared, as previously described [20]. The final concentration of inactivated bacteria in the vaccine was 8.0×10^9 CFU/ml.

2.2. Fish, fasting experiment and sampling

Prior to experimentation, all procedures involving animals were reviewed and approved by the Animal Ethics Committee of the Life Science Institute of Sun Yat-sen University (Guangdong, China). We obtained 720 healthy, male, juvenile Nile tilapia (mean weight: 12.0 ± 2.0 g) from the Yueguang Tilapia Breeding Farm, Guangdong Province, China. The fish were divided evenly among 24 glass tanks (30 fish per tank). Each tank contained about 110 L of water at about 30 °C. Fish were allowed to acclimatize for two weeks and fed twice daily with 3% of their body weight in commercial feed pellets (Guangdong Evergreen Feed Industry Co., Ltd, China; crude protein ≥35.0%, crude fibre ≤ 7.0%, and crude ash ≤ 16.0%). After the acclimatization period, the 24 glass tanks were randomly divided into a vaccinated set and an unvaccinated set (12 tanks per set). All fish in each tank were injected intraperitoneally with either 100 μl of prepared inactivated vaccine or 100 μl of PBS. At 21 days after injection, each tank was subjected to fasting for 0, 1, 3, or 7 days. Thus, the eight treatment groups, each comprising three replicate tanks, were as follows: PBS + Fas. 0 d; PBS + Fas. 1 d; PBS + Fas. 3 d; PBS + Fas. 7 d; Vac. + Fas. 0 d; Vac. + Fas. 1 d; Vac. + Fas. 3 d; and Vac. + Fas. 7 d.

At the end of the fasting period, three fish were randomly selected from each group (one fish per tank). Peripheral blood samples were taken, and serum from each sample was isolated by centrifugation (4000 rpm, 10 min). Biochemical parameters and specific antibody levels in the serum samples were measured. The livers and spleens of the sampled fish were collected under sterile conditions and immediately stored in liquid nitrogen. We measure the activity levels of enzymes important for glucose metabolism in each liver, and extracted RNA from each spleen. After sampling, 15 fish per tank were challenged with live *S. agalactiae*. All of the tanks were kept at 30 ± 0.5 °C, with pH 6.8 ± 0.3, dissolved oxygen at ≥ 7.0 mg/L, and nitrite at ≤ 0.5 mg/L. The water in each tanks was self-circulating.

2.3. Biochemical parameters

Serum cortisol concentrations were measured using a commercial enzyme linked immunoassay (ELISA) kit (Nanjing Jin Yi Bai Co., Ltd., China), following Wang et al. (2018) [20]. Serum glucose, triglyceride, and total cholesterol levels were measured with an automated hematology analyzer (Mindray BC-2900, China). We used assay kits (Nanjing Jiancheng Ins., China) to measure serum glycogen, as well as serum superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and lysozyme (LSZ) activity levels, following the manufacturer's instructions. We also used assay kits (Nanjing Jiancheng Ins., China) to measure levels of hexokinase (HK), pyruvate kinase (PK), and phosphoenolpyruvate carboxykinase (PEPCK) activity in the liver, following the manufacturer's instructions.

2.4. Analysis of immune-related gene expression

We extracted total RNA from the spleens, determined nucleic acid quality and concentration, synthesized cDNA, and performed real-time quantitative PCR (RT-qPCR) to quantify gene expression as described previously [20]. The relative expression levels of four immune-related genes, including suppressor of cytokine signaling-1 (*SOCS-1*), *SOCS-2*, interleukin-1beta (*IL-1β*), and interleukin-6 (*IL-6*), in the spleen were determined using RT-qPCR. *β-actin* was chosen as the internal standard [21,22]. All RT-qPCR primers were designed using Beacon Designer 17.0 software based on the gene sequences available in GenBank (Table 1). Each sample was run in triplicate. Dissociation-curve analyses were performed and showed a single peak in all cases. The relative expression of each gene was determined using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen [23].

2.5. ELISAs

To determine IgM antibody levels after exposure to *S. agalactiae*, ELISAs were performed using the mouse anti-tilapia (*O. niloticus*) IgM monoclonal antibody (Aquatic Diagnostics, UK), following the manufacturer's instructions. Details of these procedures have been previously described [20].

2.6. S. agalactiae challenge

After fasting, 15 fish per tank were intraperitoneally injected with the median lethal dose (LD_{50}: 0.85×10^6 CFU/fish) of live *S. agalactiae* at 30 °C. Mortality was recorded for 14 days after the challenge. We re-isolated *S. agalactiae* strain THN0901 from all dead fish to confirm bacterial identity.

2.7. Statistical analysis

All treatment groups were represented in triplicate. Data were expressed as means ± standard deviation (SD). Differences between

Table 1
Primers used for RT-PCR analysis.

Gene	Sequence (5′ to 3′)	Accession No.
SOCS-1 F	TTCTTCACGCTGTCCTACCACG	KR149237.1
SOCS-1 R	GCAAAGAGTGTTTGGAAAGACCG	
SOCS-2 F	AACAACACCGGAGCTGTGGAA	KR149238.1
SOCS-2 R	TGCAGGATCTCTTTGGCTTCA	
IL-1β F	ATTGTCGTCCTGTCTATC	GBAY01004231.1
IL-1β R	AATGTCATCATGGTATTGC	
IL-6 F	ACAGAGGAGGCGGAGATG	XM_019350387
IL-6 R	GCAGTGCTTCGGGATAGAG	

Abbreviations: SOCS-1: suppressor of cytokine signaling-1; SOCS-2: suppressor of cytokine signaling-2; IL-1β: interleukin-1beta; IL-6: interleukin-6.
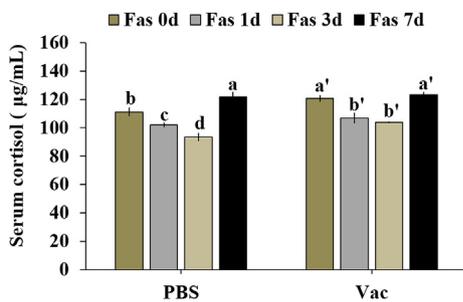
Fig. 1. Serum cortisol levels in Nile tilapia post-fasting. Different lowercase letters (a–d or a'–b') indicate significant differences in serum cortisol levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).

groups were analyzed using one-way analyses of variance (ANOVAs) and Duncan's tests in SPSS 19.0 (IBM, Armonk, NY, USA). The results were considered as significant at $p < 0.05$.

3. Results

3.1. Serum cortisol levels

To evaluate the post-fasting stress response of Nile tilapia, serum cortisol levels were measured after different fasting durations (Fig. 1). In the unvaccinated fish, serum cortisol levels first decreased and then increased as fasting duration increased; serum cortisol levels were lowest after three days of fasting ($p < 0.05$). Similar trends were observed in the vaccinated fish, but there were no significant differences in serum cortisol levels between one and three days of fasting ($p > 0.05$).

3.2. Biochemical parameters

After fasting, glucose, liver glycogen, triglyceride, and serum total cholesterol levels were measured (Fig. 2). In both vaccinated and

unvaccinated fish, glucose levels decreased significantly after three and seven days of fasting ($p < 0.05$). Liver glycogen and triglyceride levels decreased significantly as fasting duration increased, with the lowest levels observed after seven days of fasting ($p < 0.05$). Serum total cholesterol levels fluctuated similarly to glucose levels, decreasing significantly after three and seven days of fasting ($p < 0.05$).

3.3. Activity levels of key glucose metabolism enzymes

To evaluate the activity levels of key glucose metabolism enzymes in Nile tilapia after fasting, we measured the activity levels of enzymes associated with glycolysis (HK and PK) and gluconeogenesis (PEPCK) after different fasting durations (Fig. 3). In both vaccinated and unvaccinated fish, HK activity levels decreased significantly as fasting duration increased ($p < 0.05$). HK and PK activity levels were lowest after seven days of fasting ($p < 0.05$). However, PEPCK activity levels fluctuated in opposition to those of HK and PK and increased significantly after seven days of fasting ($p < 0.05$).

3.4. Activity levels of non-specific immune enzymes

Activity levels of non-specific immune enzymes were showed in Fig. 4. Serum SOD and CAT activity levels first increased and then decreased as fasting was prolonged. SOD activity was highest after three days of fasting ($p < 0.05$). GSH-Px activity decreased significantly after seven days of fasting ($p < 0.05$). Similarly, LSZ activity was lowest after seven days of fasting ($p < 0.05$), but there were no significant differences in LSZ activity levels over the first three days of fasting ($p > 0.05$).

3.5. Analysis of immune-related gene expression

To compare immune-related gene expression profiles between vaccinated and unvaccinated fish after different fasting durations, the relative gene expression levels of *SOCS-1*, *SOCS-2*, *IL-1β*, and *IL-6* in the spleen were measured with RT-qPCR (Fig. 5). In both vaccinated and unvaccinated fish, *SOCS*-1 mRNA levels first decreased and then
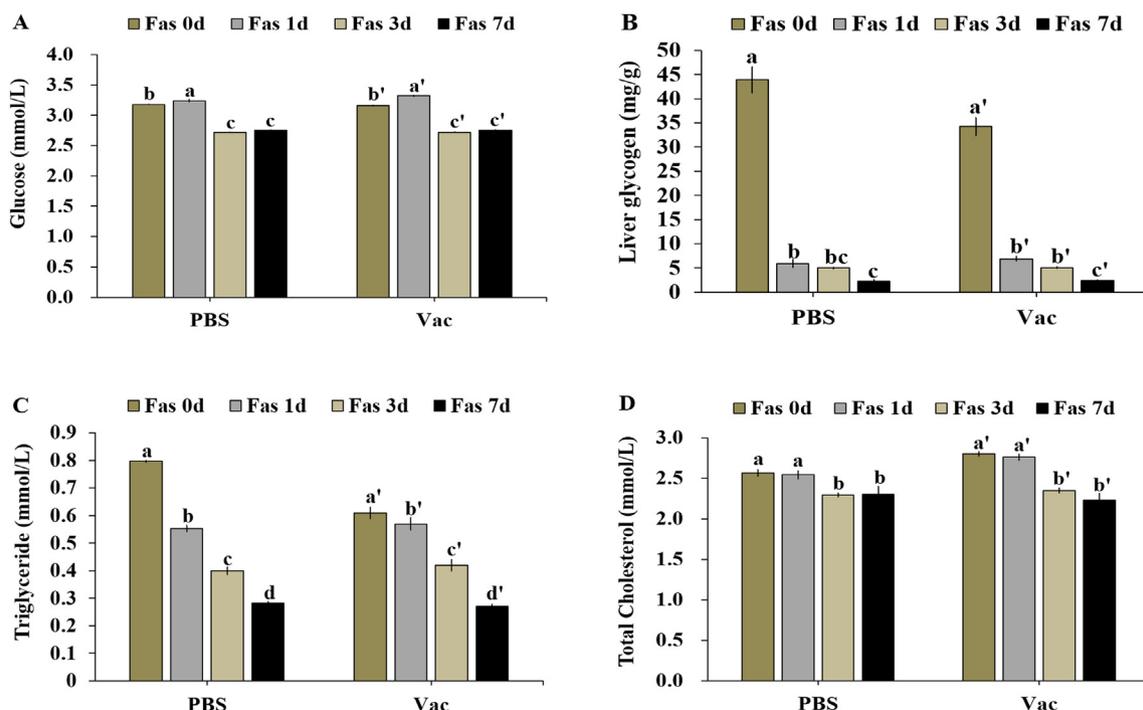

Fig. 2. Levels of glucose (A), liver glycogen (B), triglyceride (C), and total cholesterol (D) levels in Nile tilapia post-fasting. Different lowercase letters (a–d or a'–d') indicate significant differences in biochemical parameter levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).
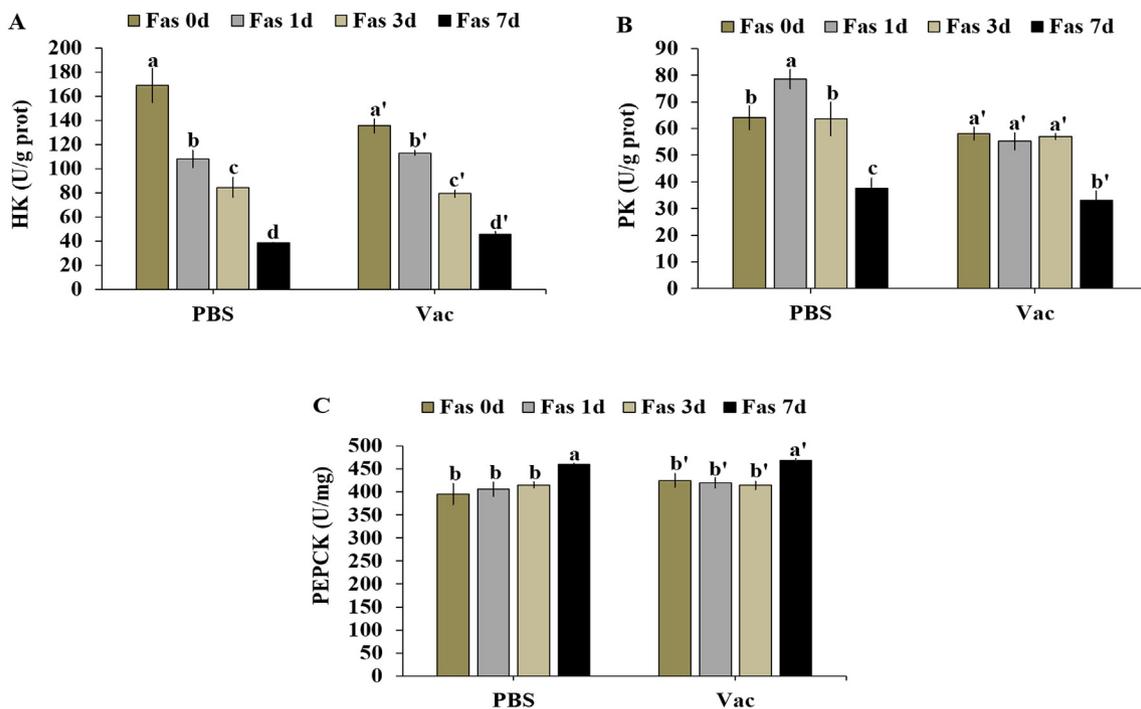
Fig. 3. Activity levels of the liver (A) HK, (B) PK, and (C) PEPCK activities in Nile tilapia post-fasting. Different lowercase letters (a–d or a'–b') indicate significant differences in liver enzyme activity levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).

increased as fasting duration increased; *SOCS*-1 mRNA expression was lowest after three days of fasting and was highest after seven days of fasting ($p < 0.05$). *SOCS*-2 mRNA levels did not differ significantly over the first three days of fasting ($p > 0.05$), but decreased significantly after seven days of fasting ($p < 0.05$). *IL-1β* and *IL*-6 mRNA expression levels fluctuated in opposition to those of *SOCS-1*, which were first increased and then decreased as fasting duration increased. *IL-1β* and *IL*-6 mRNA expression levels were highest after three days of

fasting ($p < 0.05$).

3.6. Serum antibody levels

Serum specific antibody levels were measured in the vaccinated and unvaccinated fish after different durations of fasting (Fig. 6). In the unvaccinated fish, there were no significant differences in specific antibody levels among the different fasting durations ($p > 0.05$). In the
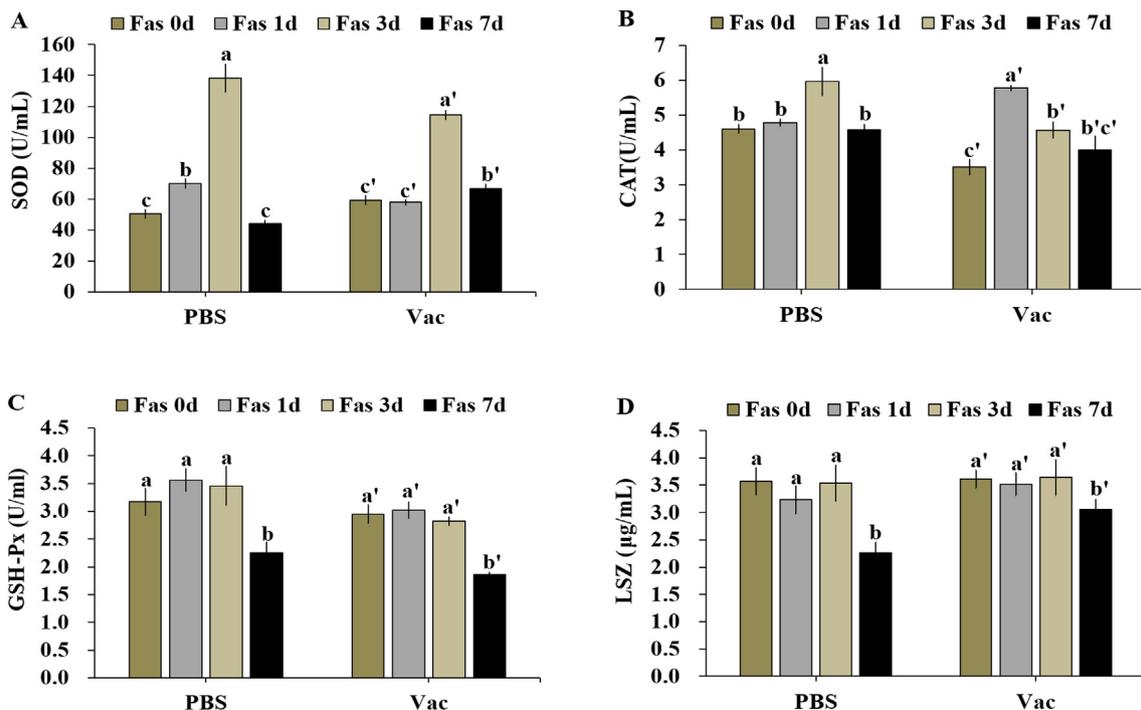

Fig. 4. Serum (A) SOD, (B) CAT, (C) GSH-Px, and (D) LSZ activity levels in Nile tilapia post-fasting. Different lowercase letters (a–c or a'–c') indicate significant differences in serum enzyme activity levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).
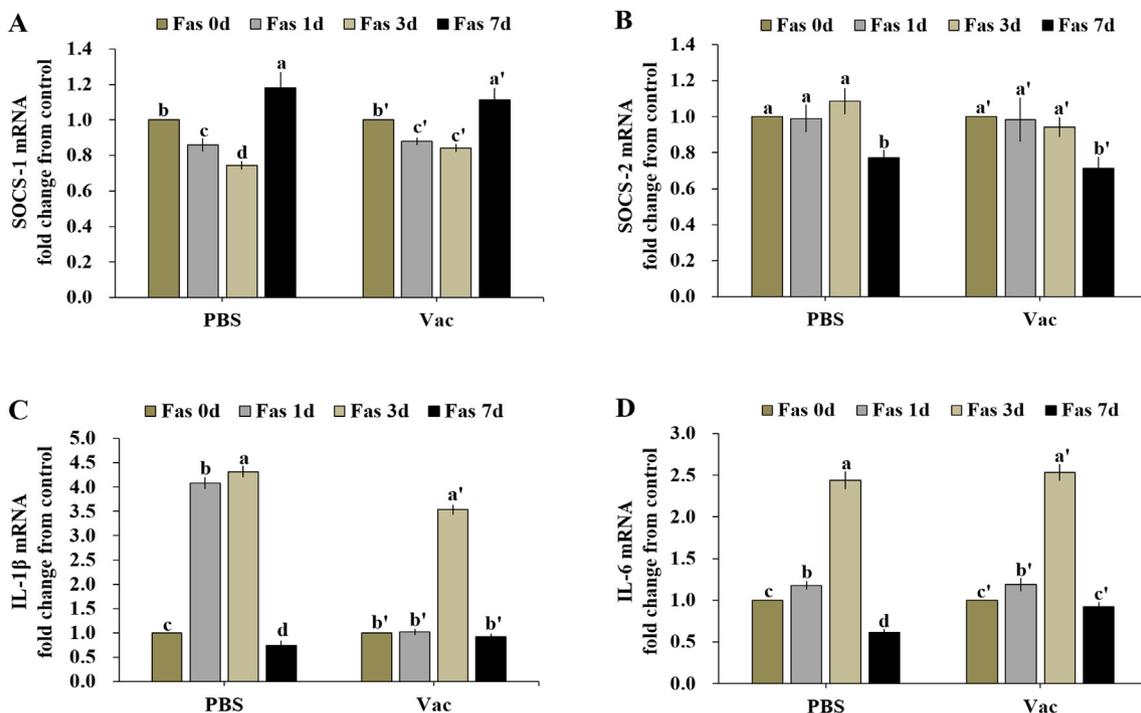
Fig. 5. The relative expression levels of *SOCS-1* (A), *SOCS-2* (B), *IL-1β* (C), and *IL-6* (D) in the Nile tilapia spleen post-fasting. Different lowercase letters (a–d or a'–c') indicate significant differences in relative gene expression levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).
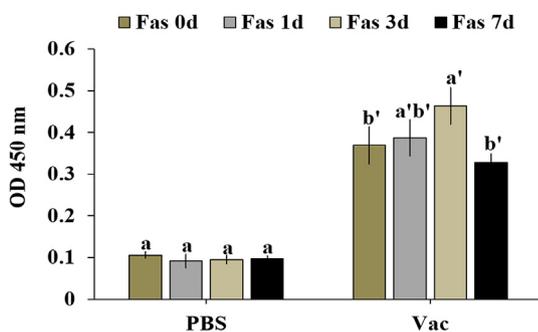

Fig. 6. Serum antibody levels in Nile tilapia post-fasting. Different lowercase letters (a or a'–b') indicate significant differences in serum antibody levels among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).

Fig. 7. Survival rates (%) of Nile tilapia over 14 days post-intraperitoneal challenge with *S. agalactiae*. Different lowercase letters (a–c or a') indicate significant differences in survival rate among fasting durations within the unvaccinated (PBS) and the vaccinated (Vac) groups ($p < 0.05$).

vaccinated fish, specific antibody levels increased significantly after three days of fasting ($p < 0.05$) and then returned to initial values ($p > 0.05$).

3.7. Survival rates of Nile tilapia after S. agalactiae infection

To evaluate the effects of fasting on the resistance of Nile tilapia to *S. agalactiae* infection, vaccinated and unvaccinated fish were fasted and then challenged with *S. agalactiae*. Post-infection survival rates were calculated (Fig. 7). Nile tilapia survival was recorded daily for 14 days after the intraperitoneal injection. Massive tilapia mortalities occurred over the first three days post-challenge. The survival rates of unvaccinated fish fasting for zero, one, three, and seven days were 13.33 ± 5.44%, 20.00 ± 5.45%, 35.56 ± 3.14%, and 6.67 ± 3.14%, respectively. And the survival rates of vaccinated fish fasting for zero, one, three, and seven days were 86.67 ± 5.44%, 80.00 ± 3.14%, 88.89 ± 6.28%, and 84.44 ± 8.32%, respectively. No significant differences were identified among the fish fasted fore different durations in the vaccinated group ($p > 0.05$). However, significantly more unvaccinated fish in the group fasted for three days
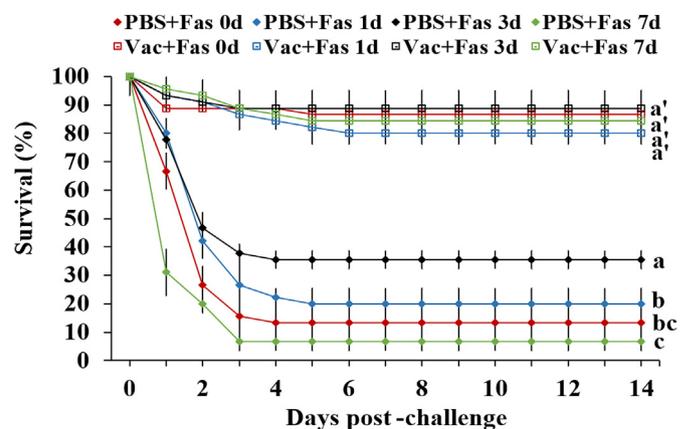
survived, as compared to other groups ($p < 0.05$). The survival rate was lowest in the unvaccinated fish fasted for seven days, and there were no significant differences in the survival rates of unvaccinated fish fasted one and seven days ($p > 0.05$).

4. Discussion

Feeding regimen alterations are common practice in the modern aquaculture industry, predicated in part on studies indicating that fasting is well tolerated by most fish species [10,18,24]. Indeed, some studies have shown that food deprivation strategies can be employed in response to seasonal change, overproduction, or disease outbreaks [16,25,26]. For example, food deprivation reduced the mortality rates of channel catfish (*Ictalurus punctatus*) exposed to the bacterial pathogens *Edwardsiella ictaluri* and *Flavobacterium columnnare* [26,27]. In the vaccinated and unvaccinated Nile tilapia studied here, it was clear that

both biochemical parameters and the mRNA expression levels of immune-related genes were affected by fasts of varying durations, as was the resistance of this fish to *S. agalactiae* infection.

During the stress response, the hypothalamic-pituitary-interstitial axis is activated, resulting in a transient or persistent increase in the levels of some neuroendocrine hormones, including cortisol. Cortisol is a reliable indicator of stress, which plays an important role in the regulation of various physiological and biochemical responses, including growth, metabolism, and immunity [28,29]. Here, serum cortisol levels first decreased and then increased as fasting duration increased, suggesting that fasting for a short while reduced cortisol production [6], but that fasting for more than seven days might cause starvation stress in tilapia [30,31]. The stress responses are energy-dependent processes. In response to environmental changes, cortisol increases glucose levels by activating liver gluconeogenesis; this adaptive mechanism increases host energy demand [32]. Here, PEPCK activity increased significantly after seven days of fasting, while and HK and PK activity levels decreased significantly. These results suggested that starving Nile tilapia maintain energy by increasing the activity levels of enzymes important for gluconeogenesis and reducing the activity levels of key enzymes associated with glycolysis. The significant decrease in liver glycogen observed here after one day of fasting might be due to the breakdown of glycogen by the body, releasing glucose and thus maintaining blood glucose levels in the early stages of starvation [33,34]. Here, triglyceride and total cholesterol levels decreased as fasting duration increased, further indicating that lipids may act as energy supplements for Nile tilapia during starvation [35].

SOD, CAT, and GSH-Px are important antioxidants in the non-specific immune system, and these molecules play vital roles in ROS transfer for protecting both membranes and DNA from damage [36,37]. Previous study has shown that excessive ROS are produced during early starvation due to the high levels of lipid and fatty acid oxidation [38]. Therefore, during the early stages of starvation (here, the first three days of fasting), Nile tilapia may protect cells from oxidative damage by activating antioxidant enzymes. However, Nile tilapia might also reduce energy expenditure by reducing enzyme activity, as reflected by the increase and then decrease of antioxidant enzyme activity as fasting duration increased. LSZ activity is closely related to the bodily immunity [39]. In both vaccinated and unvaccinated Nile tilapia, LSZ activity levels decreased significantly after seven days of fasting, suggesting that fasting for longer than seven days may have inhibitory effects on the immune response.

To further determine the possible effects of fasting on Nile tilapia immune capacity, we measured the mRNA expression levels of immune-related genes in the spleen as well as serum specific antibody levels. We found that, similar to cortisol levels, *SOCS*-1 mRNA expression levels first decreased and then increased as fasting duration increased. Philip et al. (2012) demonstrated that cortisol increased the abundance of *SOCS* mRNA in rainbow trout hepatocytes, mediated by glucocorticoid receptor activation [40]. The *SOCS* genes are negative regulators of the Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway [41]. Cortisol-dependent stimulation of these genes creates resistance to immunomodulatory cytokines, including *IL-1β*, *IL-6*, and *IL-8* [39,42]. Therefore, the significant increase in *SOCS*-1 mRNA expression observed in Nile tilapia after seven days of fasting might be closely related to cortisol concentration. The *SOCS-1* gene might downregulate *IL-1β* and *IL-6* expression via the JAK/STAT signaling pathway. Thus, our results indicated that cytokine expression levels were negatively correlated with cortisol levels. Moreover, *SOCS-2* expression levels were opposite to those of *SOCS-1*: a significant decrease *SOCS-2* expression was observed in both vaccinated and unvaccinated fish after seven days of fasting. These expression profiles might indicate that, in Nile tilapia, *SOCS-1* is more likely to play roles in innate immune regulation, while *SOCS-2* might be more involved in metabolic regulation [43,44].

A previous study showed that the Nile tilapia *IL-6* gene increased

antibody production and was involved in host defense against *S. agalactiae* infection [45]. However, we found no positive correlation between specific antibody levels and *IL-6* mRNA expression levels in unvaccinated Nile tilapia, possibly because tilapia B cells have different stages of differentiation (plasma-like cells, plasmablasts and activated B cells, and resting and partially activated B cells), and secretory antibodies are primarily produced in the plasma-like B cells [45]. Although there were no significant differences in specific antibody levels among unvaccinated fish fasted for different periods, the highest survival rate among the unvaccinated tilapia challenged with *S. agalactiae* was observed in the fish fasted for three days, while the lowest survival rate was observed in the fish fasted for seven days. The results of survival rate might reflect the comprehensive antibacterial defense produced by the non-specific immune system of unvaccinated Nile tilapia after fasted.

In conclusion, our results indicated that short-term fasting (three days) prior to infection might increase the resistance of unvaccinated Nile tilapia to *S. agalactiae* infection. However, fasting for up to seven days had no significant effect on the *S. agalactiae* resistance of vaccinated Nile tilapia.

Acknowledgements

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