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Ozone nanobubble modulates the innate defense system of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae*

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ABSTRACT

Ozone nanobubble ($NB–O_3$) is a promising technology for improving dissolved oxygen and reducing bacterial concentration in aquaculture systems. Here, we investigated the effects of $NB-O_3$ on the innate immunity of fish by monitoring the expression levels of nonspecific immune-related genes (*IL-1β, IL-2β*, *TNF-α*), heat-shock protein genes (*HSP70, HSP90-α*), and a bacteriolytic enzyme, C-type lysozyme, gene (*LYZ*) post-treatment with this technology. Following exposure to $NB-O_3$, the different tissues of Nile tilapia (*Oreochromis niloticus*) were collected over time for quantitative real-time PCR (qPCR) analysis. The expression of all the genes evaluated in the gills, the head kidney, and the spleen of the $NB-O_3$ treated group was significantly up-regulated compared to that in the untreated control group. The expression levels were the highest (approx. 2 to 4-fold) at 15 min and 3 h post-exposure and then decreased from 6 to 24 h. These findings suggested that $NB-O_3$ could switch on the innate immunity genes of Nile tilapia. Thus, we hypothesized that the $NB-O_3$-immune-activated fish would respond more effectively to subsequent bacterial infections, thereby improving survivability compared to that of untreated fish. To test this hypothesis, 3 h post $NB-O_3$ exposed fish and unexposed fish were challenged with a lethal dose of *Streptococcus agalactiae*. Interestingly, the survival rate of the $NB-O_3$ group was significantly higher than that of the non-treated controls, with a relative percent survival (RPS) of 60–70%. Together, these findings indicate, for the first time, that $NB-O_3$ may trigger the nonspecific defense system of the fish, thereby improving fish survivability during subsequent bacterial infections. This research identified another potential benefit of $NB-O_3$ in aquaculture for preventing infectious bacterial diseases.

1. Introduction

Water bubbles can be classified into three groups based on their sizes and characteristics. Macrobubbles have diameter varies from 100 μm to 2 mm, which rapidly reach the water surface and burst. Microbubbles (MBs) are smaller than macrobubbles, with a diameter size between 1 μm and 100 μm, which shrink in the water and dissolve afterward. By contrast, nanobubbles (NBs) or ultrafine bubbles are incredibly tiny gas bubbles with diameter < 1 μm, high internal pressure, negatively charged, and low buoyancy [1]. These distinctive physical properties allow NBs to be suspended for long periods in liquids. They exhibit high specific surface areas and high stagnant periods, which enhance the efficacy of mass transportation, physical uptake processes, and chemical reactions at the gas-liquid interface [1,2]. Therefore, NBs technology has

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been extensively explored in numerous fields, including engineering, medical, agriculture, food, and aquaculture [3–7].

Nanobubble technology is relatively new to the aquaculture industry. There are only a few studies describing the manner in which the unique properties and stability of NBs may be utilized to enhance growth performance of cultivated animals and reduce pathogen load in water [8]. Kurita et al. [9], showed that the survival rate of planktonic crustacean parasites in aquaculture tanks was decreased by approximately 63% following exposure to NB-O_3 for 25 min compared to that of the control groups. In addition, 4 d after exposure to NB-O_3, no negative impact on the survival rate of juvenile sea cucumbers (*Apostichopus japonicus*) and sea urchins (*Strongylocentrotus intermedius*) was observed, suggesting that NB-O_3 treatment is relatively safe for these marine aquaculture species. NB-O_3 is also effective at disinfecting *Vibrio parahaemolyticus* in brackish water, and preventing mortality caused by this pathogen in whiteleg shrimp (*Penaeus vannamei*) [10]. Our recent investigation on the acute impact of NB-O_3 on Nile tilapia and its pathogens demonstrated that a 10-min NB-O_3 treatment (834 ± 22 mV ORP) was effective at reducing the concentration of *Streptococcus agalactiae* and *Aeromonas veronii* in freshwater, and caused no acute mortality to Nile tilapia [21]. The use of NB-O_3 in tilapia aquaculture is promising, however, effects of NB-O_3 on the fish immune system remain uninvestigated.

Innate immunity, which constitutes front-line defense against infections, refers to a critical systemic response that inhibits pathogens and sustains homeostatic interactions [11–13]. Significant differences in the expression transcripts of nonspecific immune-related genes following a pathogenic infection are important indicators of the immune response in fish. Thus, studies of the nonspecific immune-related genes may lead to a better understanding of the immune mechanisms in finfish. In a continuous effort to understand how nanobubble technology affects fish health, we investigated the effects of NB-O_3 on the innate immunity of fish by monitoring the expression of nonspecific immune-related genes: interleukin 1 beta (*IL-1β)*; interleukin 2 beta (*IL-2β*); tumour necrosis factor alpha (*TNF-α*), which regulate pro-inflammatory cytokines [14–18], heat-shock protein genes (*HSP70, HSP90-α*), which are now supposed to play a larger-term function by modulating the immune system [19], and a bacteriolytic enzyme, C-type lysozyme gene (*LYZ*), which destroys the bacterial cell wall [20]. Subsequently, we determined whether NB-O_3 treatment improves survival rate of Nile tilapia following infection with *S. agalactiae*.

2. Materials and methods

2.1. Experimental fish

Apparently healthy juvenile Nile tilapia (*O. niloticus*) were obtained from a commercial tilapia hatchery in Thailand. Prior to the experiment, fish were acclimatized in 100 L fiberglass tanks, 68 cm (L) × 49 cm (W) × 30 cm (D) containing chlorine-free water at a temperature of 30 ± 1 °C for 14 d. They were fed on a 30% crude protein commercial tilapia pellet feed (5% biomass) twice a day. Air stones and cotton filters were placed in each tank. Water and filters were replaced twice a week during the experimental period, and water parameters (temperature, pH, nitrite, and ammonia) were checked daily. Ten fish were picked randomly for bacterial and parasitic testing to verify whether they were sufficiently healthy for further experimentation. All animal procedures were approved by the Thai Institutional Animal Care and Use Committee (Approval no. MUSC62-039-503).

2.2. Experimental design for NB-O_3 treated Nile tilapia and sample collection

In the first trial, a total of 60 fish (5.34 ± 0.32 g) were divided into two groups: NB-O_3 treatment (30 fish) and control groups (30 fish), respectively. Each tank consisted of 50 L of freshwater. The temperatures were maintained at 30 ± 1 °C using a heater. The treatment tank was treated with NB-O_3 for 10 min using a nanobubble generator (model: aQua+075MO; maker: AquaPro Solutions Pte Ltd, Singapore) as described previously [21]. The temperature (T^o), dissolved oxygen (DO), pH, and oxidation-reduction potential (ORP) were measured using a multi-parameter meter (YSI Professional Plus, YSI Incorporated, Ohio, USA) during the running process (Table S1).

In order to examine the expressions of immune genes in different tissues of Nile tilapia, five fish (five biological replications) were randomly sampled from each tank at different time-point, including before treatment (0 min) and post-treatment (15 min, 3, 6, 12, and 24 h). The fish were euthanized by an overdose of clove oil (250 ppm) prior to sample collection. The gills, head kidney, and spleen from each fish were collected and separately placed into 1.5 mL Eppendorf tubes containing 200 μL Trizol (Invitrogen, USA) and then stored at −20 °C until needed for analysis (Fig. S1a). In this study, apart from the gills that exposed directly to NB-O_3 during the treatment, we also investigated systemic immune responses in the two other important lymphoid organs (head kidney and spleen) of Nile tilapia.

2.3. Experimental design for NB-O_3 treated fish following bacterial infections

The bacterial isolate *S. agalactiae* 2803 used in this study was collected from a natural disease outbreak in Nile tilapia in 2018 [21]. The isolated bacterium was propagated in Tryptic Soy Broth; TSB (Becton Dickerson, USA) for 18 h at 28 °C with constant shaking at 150 rpm. The culture was adjusted to $OD_{600nm} = 1.0$ (~10^9 CFU/mL), and enumeration was done using the conventional plate count method on Tryptic Soy Agar; TSA (Becton Dickerson, USA).

In trial 1, there were three treatment groups including NB-O_3 treatment (as described above), positive control, and negative control. The latter two groups were treated with normal aeration using an air-stone. Each tank consisted of 35 fish in a volume of 50 L of freshwater. The water temperature in each tank was maintained at 30 ± 1 °C using a heater. Water parameters were recorded before and after the 10 min NB-O_3 treatment (Table S2). Three hours post NB-O_3 treatment, the fish were transferred into new 50 L tanks equipped with aeration and 500 mL *S. agalactiae* (~10^9 CFU/mL) was added into each tank to attain a final concentration of 1.47×10^7 CFU/mL that was retrospectively verified by plate count method on *Streptococcus agalactiae* Selective Agar; SSA (HiMedia, India). Positive control group was performed in the same manner while the negative control group received 500 mL of sterilized TSB media without bacteria. At time points of 3 and 6-h post-challenge, tissue samples including the gills, head kidneys, and spleens were collected from five random biological replicates from each group. All samples were immediately preserved in 200 μL Trizol and then stored at −20 °C until analysis was conducted. Remaining 20 fish from each tank were monitored daily for up to 14 days, mortality rates and water parameters were recorded daily (Table S3). Re-isolation of bacteria from the challenged fish tissues (head kidney and spleen) was performed using SSA. The protective efficacy was determined by comparing the cumulative mortalities of the challenged and control groups. The relative percent survival (RPS) was calculated according to the following equation: RPS = [1 – (% mortality in challenge/% mortality in control)] × 100. To confirm the result of survival in challenge test was repeatable, the second trial (trial 2) was performed in the same manner, using 20 fish per tank and focused on monitoring only survival rate.

To investigate whether fish that survived the challenge experiment developed specific antibody IgM (a humoral component of adaptive immune system), fish serum was obtained from five fish per each group at day 14 post-challenge for enzyme-linked immunosorbent assay (ELISA). Approximately 0.8 mL of blood was withdrawn from the fish caudal vein using a syringe with a 23-G needle and kept at room temperature for 2 h. The samples were centrifuged at 5.000 rpm for 15 min.

The sera were collected and stored at −20 °C until used. ELISA test was performed as described below.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from 40 to 50 mg of samples using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA quantity (OD = 260 nm) and purity (OD_{260nm}/OD_{280} nm ratio, range 1.90–2.14) were determined using a spectrophotometer NanoDrop One (Thermo Fisher Scientific, UK). The first complementary DNA (cDNA) strand was produced from 2 μg of the isolated total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad, USA) according to the procedure described in the product manual. Afterward, the reaction was stored at −20 °C until further use.

2.5. Gene expression study by quantitative real-time PCR

Expression levels associated with different immune genes were assessed via quantitative real-time PCR (qPCR). The primers used are listed (Table 1). SYBR green reagent (iTaq™ Universal SYBR™ green Supermix; Bio-Rad, Hercules, CA, USA) was used in the qPCR reaction. First, the qPCR was performed in a 20 μL reaction mixture containing 2 × SYBR Green, 10 mM of each primer, and 1 μL of the above cDNA template. The qPCR amplification cycles were performed using a CFX Connect™ Real-time System (Bio-Rad). Cycling conditions were 94 °C for 15 s, 40 cycles of denaturation at 95 °C for 30 s, annealing at the optimal temperature of each primer as indicated (Table 1) for 30 s, and a final extension at 72 °C for 30 s. Melting curves were obtained in the 55–85 °C range with 0.1 °C increments per second to evaluate for the specificity of all qPCR products. Results were analyzed using the CFX Manager™ Software (Bio-Rad) for the quantification cycle (C_q) determination. Representative melting curves analyses were shown in Fig. S2. The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method [22]. The transcript levels of each target gene were obtained as C_q values and normalized to that of the *EF-1α* as an internal reference.

2.6. Determination of serum antibody by enzyme-linked immunosorbent assay (ELISA)

The 96 well EIA/RIA Plates (Costar®, Corning Inc., USA) were coated with 100 μL/well of formalin-killed *S. agalactiae* whole-cell antigen (OD_{600nm} = 1.0, equivalent to ~10^8 CFU/well) in carbonate coating buffer (pH 9.6) and incubated at 4 °C overnight. The wells were washed three times with 1 × PBS, containing 0.05% Tween-20 (PBST) (Amresco, USA). To identify working dilution for ELISA test, 2-fold serial dilutions of pooled serum samples from five fish collected at day 14 post challenge were used for preliminary test. Subsequently, the appropriate dilution for ELISA analysis was found to be 1:512. The tested serum samples were then individually diluted in PBST containing 5% skim milk (PBSTM) and incubated with the antigen coated plates for 1 h at room temperature. After washing five times with PBST, anti-Tilapia IgM secondary antibody [23] diluted with PBSTM (1:200) was added and incubated for 1 h, followed by washing with PBST and commercial goat anti-mouse antibody horseradish peroxidase (HRP) conjugate (Thermo Fisher Scientific, USA) diluted in PBSTM (1:3000) was added into each well for 1 h. After washing, 3, 3′, 5, 5′- tetramethylbenzidine (TMB) substrate (EMD Millipore Corp, USA) was added, and the wells were incubated for 10 min with gentle shaking. Finally, 100 μL of 2 M H_2SO_4 was added into each well to stop the reaction prior to the absorbance measurement at 450 nm using the SpectraMax iD3 microplate reader (Molecular Devices, LLC, USA).

2.7. Statistical analysis

All statistical analyses in this study were performed using the software SPSS ver20.0 (IBM Corp., Armonk, NY, USA). Innate immune-related gene expression and OD_{450nm} value from ELISA assay were subjected to a homogeneity of variance test, followed by one-way analysis of variance (ANOVA). The Duncan (variance homogeneity with $p < 0.05$) or Dunnett's T3 (variance homogeneity with $p > 0.05$) multiple comparison tests were used to compare arithmetic means. In all experiments, p was set at < 0.05.

3. Results

3.1. Innate immune response of Nile tilapia upon exposure to NB-O_3

3.1.1. Pro-inflammatory cytokines and lysozyme expression levels

The qPCR was performed to investigate the effects of NB-O_3 on the expression of pro-inflammatory cytokines and lysozyme genes encoding *IL-1β*, *IL-2β*, *TNF-α*, and *LYZ* in the gills, head kidneys, and spleens at 15 min, 3 h, 6 h, 12 h, and 24 h following treatment, with 0 min (before treatment) as the control. The different expression patterns of the examined genes on gills, head kidney, and spleen tissues were observed (Fig. 1). *IL-1β* expression was slightly up-regulated in the gills and head kidney from 15 min to 6 h post-treatment (Fig. 1a and b). In the spleen, the expression of *IL-1β* in the NB-O_3 groups was markedly up-regulated to 1.98-fold at 15 min post-treatment, which slightly decreased thereafter (Fig. 1c). *IL-2β* expression in the gill was increased from 15 min to 3 h post-treatment in the NB-O_3 compared to that in the control groups (Fig. 1d). *IL-2β* expression in the head kidney and spleen of the NB-O_3 group was up-regulated to the highest level (1.92- and 2.01-fold) at 15 min post-treatment and then slightly decreased at 3 h (Fig. 1e and f). *TNF-α* expression in gills and head kidney was significantly up-regulated to 1.75- and 2.94-fold at 3 h post-treatment of NB-O_3 groups, down-regulation was then observed thereafter. There was an increase of

Table 1
Primers used to quantify relative gene expression in this study.

Target gene	Oligo sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
IL-1β	F: AAGATGAATTGTGGAGCTGTGTT R: AAAAGCATCGACAGTATGTGAAAT	175	55	[53]
IL-2β	F: GCAGTACCGAACCCTTCAAA R: GAGATTTGGTCGATGGGCTG	128	55	this study
TNF-α	F: CTTCCCATAGACTCTGAGTAGCG R: GAGGCCAACAAAATCATCATCCC	161	60	[54]
LYZ	F: AAGGGAAGCAGCAGCAGTTGTG R: CGTCCATGCCGTTAGCCTTGAG	151	60	[55]
HSP70	F: CATCGCCTACGGTCTGGACAA R: TGCCGTCTTCAATGGTCAGGAT	107	59	[55]
HSP90-α	F: ATTGCTCAGCTGATGTCCCT R: GTGGGATCCGTCAAGCTTTC	128	56	this study
EF-1α	F: CTACAGCCAGGCTCGTTTCG R: CTTGTCACTGGTCTCCAGCA	139	60	[56]

*F: forward primer, R: reverse primer, bp: base pair.

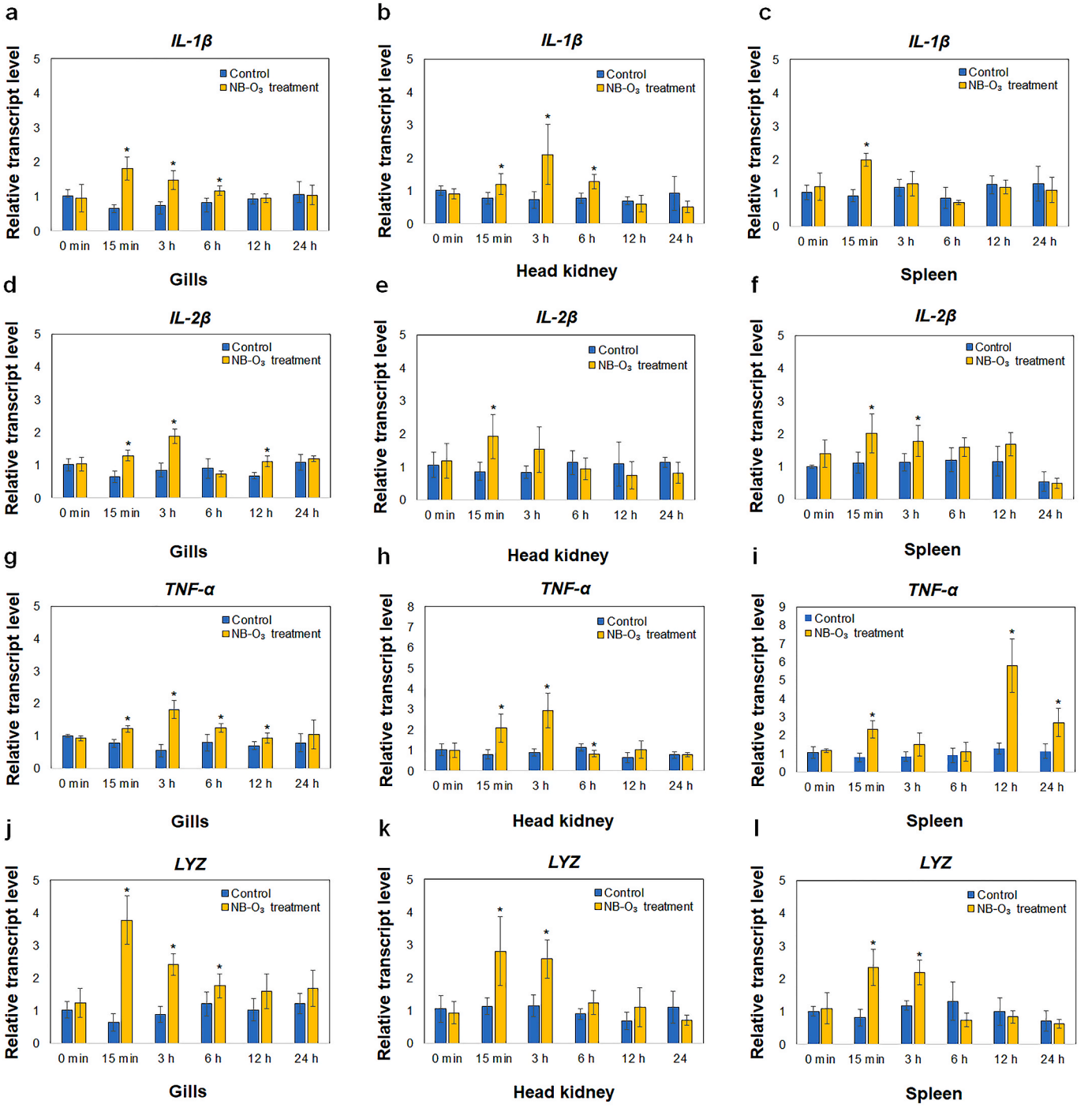

Fig. 1. Comparative expression of *IL-1β*, *IL-2β*, *TNF-α*, and *LYZ* in the gills, head kidney, and spleen of control and NB-O_3 treated fish (n = 5) at 0 min, 15 min, 3 h, 6 h, 12 h and 24 h after treatment. The expression of target genes was normalized using *EF-1α*. Transcript levels of control at 0 min were set as 1. Error bars represent standard deviation and * indicate *t*-test $p < 0.05$.

TNF-α in the spleen at 15 min and it peaked at 12 h post-treatment (6.31-fold) (Fig. 1i). C-type lysozyme (*LYZ)* expression significantly increased and showed the highest level (3.77-fold) in the gills, followed by 2.80-fold increase in the head kidney and 2.35-fold in the spleen at 15 min post-treatment, and then decreased at 3 h post-treatment in all the investigated tissues (Fig. 1j, k, l).

3.1.2. *Expressions of antioxidant and stress-related genes*

HSP70 expression in the gills, head kidney, and spleen was up-regulated from 15 min to 3 h post-treatment and later down-regulated at 6 h post-treatment (Fig. 2a–c). The expression patterns of *HSP90-α* were similar to those of *HSP70*, with a stable decrease at 6 h in the gills, head kidney, and spleen tissues post-treatment (Fig. 2d–f).

3.2. *Innate immune response of NB-O_3 treated fish to subsequent infection with S. agalactiae*

3.2.1. *Pro-inflammatory cytokines and lysozyme expression levels*

The results of qPCR showed that the expressions of target genes were significantly up-regulated in the NB-O_3 treatment groups compared to those in the control groups (Fig. 3). *IL-1β* expression in the gills and head kidney of the NB-O_3 groups was up-regulated at both time points (3- and

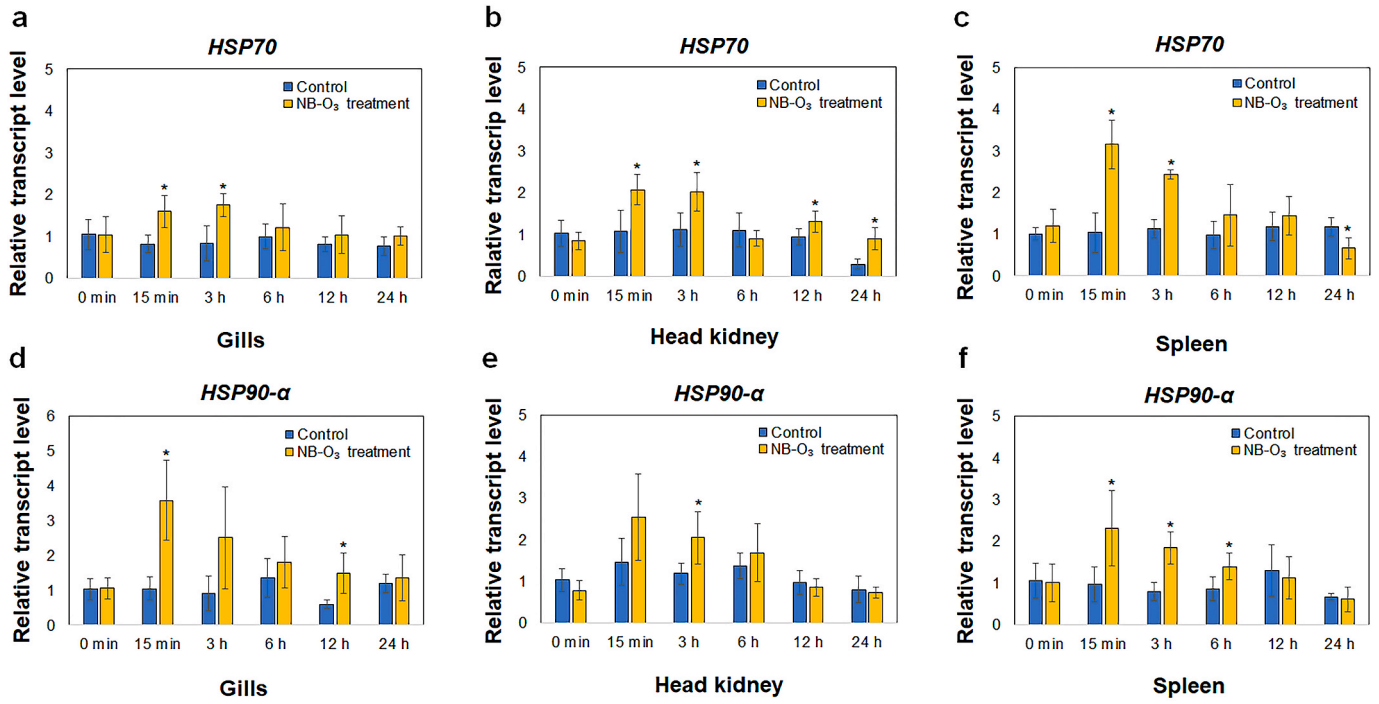

Fig. 2. Comparative expression profiles of *HSP70* and *HSP90-α* in the gills, head kidney, and spleen of control and treated fish (n = 5) at 0 min, 15 min, 3 h, 6 h, 12 h and 24 h after treatment. The expression of target genes was normalized using *EF-1α*. Transcript levels of control at 0 min were set as 1. Error bars represent standard deviation and * indicate *t*-test $p < 0.05$.

6 h post-challenge) but only significantly different from the control group at time 3 h (Fig. 3a and b). The expression in the spleen of NB-O_3 was significantly increased at 3 h post-challenge, whereas a significant change was observed in the air treatment at 6 h time point compared to that in the NB-O_3 and control groups (Fig. 3c). The highest level of the *IL-2β* transcript (4.04 folds) was measured in the gills at 3 h post-infection in the NB-O_3 treatment group (Fig. 3d). It was significantly different in the head kidney and spleen of the NB-O_3 group at 3 h post-challenge compared to that in the air treatment and control groups (Fig. 3e and f). *TNF-α* expression of the NB-O_3 group was significantly up-regulated in all investigated tissues at 3 h post-challenge compared with that in the air treatment and control groups (Fig. 3h and i). Up-regulation of *TNF-α* at 6 h was the only observed effect in head kidney of the NB-O_3 treatment (Fig. 3h). Similar to that of *TNF-α*, the expression of the *LYZ* in different tissues was distinctive. There was a significant difference between the NB-O_3 treatment and the other groups in gills, head kidney and spleen (Fig. 3j, k, i). The highest level of *LYZ* transcripts was observed in the spleen (Fig. 3i). *LYZ* expression was lowest in the gills at 6 h post-challenge (Fig. 3j).

3.2.2. *Expressions of antioxidant and stress-related genes*

The expression patterns of *HSP70* and *HSP90-α* in the gills, head kidney, and spleen of Nile tilapia were determined at 3 and 6 h post-challenge with *S. agalactiae*. At 3 h post-challenge, the expression levels in the NB-O_3 treatment group were the highest level compared to that of the other groups (Fig. 4). Notably, up-regulated transcripts were found in the gill tissue of NB-O_3 treatment group at 3 h post-challenge, whereas the expression transcripts in all treatments remained largely unchanged at 6 h post-challenge (Fig. 4a). *HSP70* expression levels in the head kidney and spleen of the NB-O_3 treatment group were significantly upregulated 3 h after being challenged, while gene expression was nearly steady and no alteration was observed in all bacterial challenged groups after 6 h (Fig. 4b and c). *HSP90-α* expression in the gills, head kidneys, and spleens of the NB-O_3 group was significantly upregulated at 3 h post-challenge. By contrast, *HSP90-α* expression remained roughly unchanged in all tissues of all bacterial challenged groups at 6 h post-challenge (Fig. 4d–f).

3.3. *NB-O_3 treatment before S. agalactiae infection improve survivability of Nile tilapia*

Fish in the NB-O_3 treatment groups demonstrated lower mortalities (15–20%) than those in the positive control air treatment groups (50%) post-challenge with *S. agalactiae*. No mortality was observed in the negative controls following 14 d of experiment. *Streptococcus agalactiae* was re-isolated from representative moribund and dead fish in both positive control and NB-O_3 treatment groups by inoculating specimens of the spleen and head kidney on SSA. The mortality patterns based on the two independent challenged experiments were consistent (Fig. 5a and b). In the positive control group, mortalities were observed from day 2 (Fig. 5b) or day 3 (Fig. 5a) to day 8 post-infection, with mortality rates reaching 50% and no mortality was observed thereafter. In one of the NB-O_3 treatment groups, mortality was first observed from day 4 post-infection (Fig. 5a), with the mortality rates reaching 15% at day 7 post-infection, following which no additional mortality was recorded until day 14. In the second NB-O_3 treatment group mortality was observed at day 3 (Fig. 5b), with mortality rates reaching 20% at day 10 post-infection, and no mortality was recorded until day 14. Relative percentage survival (RPS) of the NB-O_3 treatment groups was 60–70%.

ELISA assay revealed that surviving fish from both NB-O_3 treatment and positive control (Air treatment) groups developed significantly higher levels of specific IgM response at 14 d post-challenge compared to that in negative control group ($p < 0.05$) (Fig. 6). The serum from fish in the NB-O_3 treatment group had the highest ELISA optical density (OD) readings (0.14 ± 0.03), followed by OD readings of serum from fish in the air treatment group (0.12 ± 0.03). In contrast, the lowest level (0.06 ± 0.01) was recorded in the negative control (Fig. 6).

4. Discussion

Current application of NB-O_3 in aquaculture is still limited due to lack of knowledge of the advantages of this technology. Previous studies

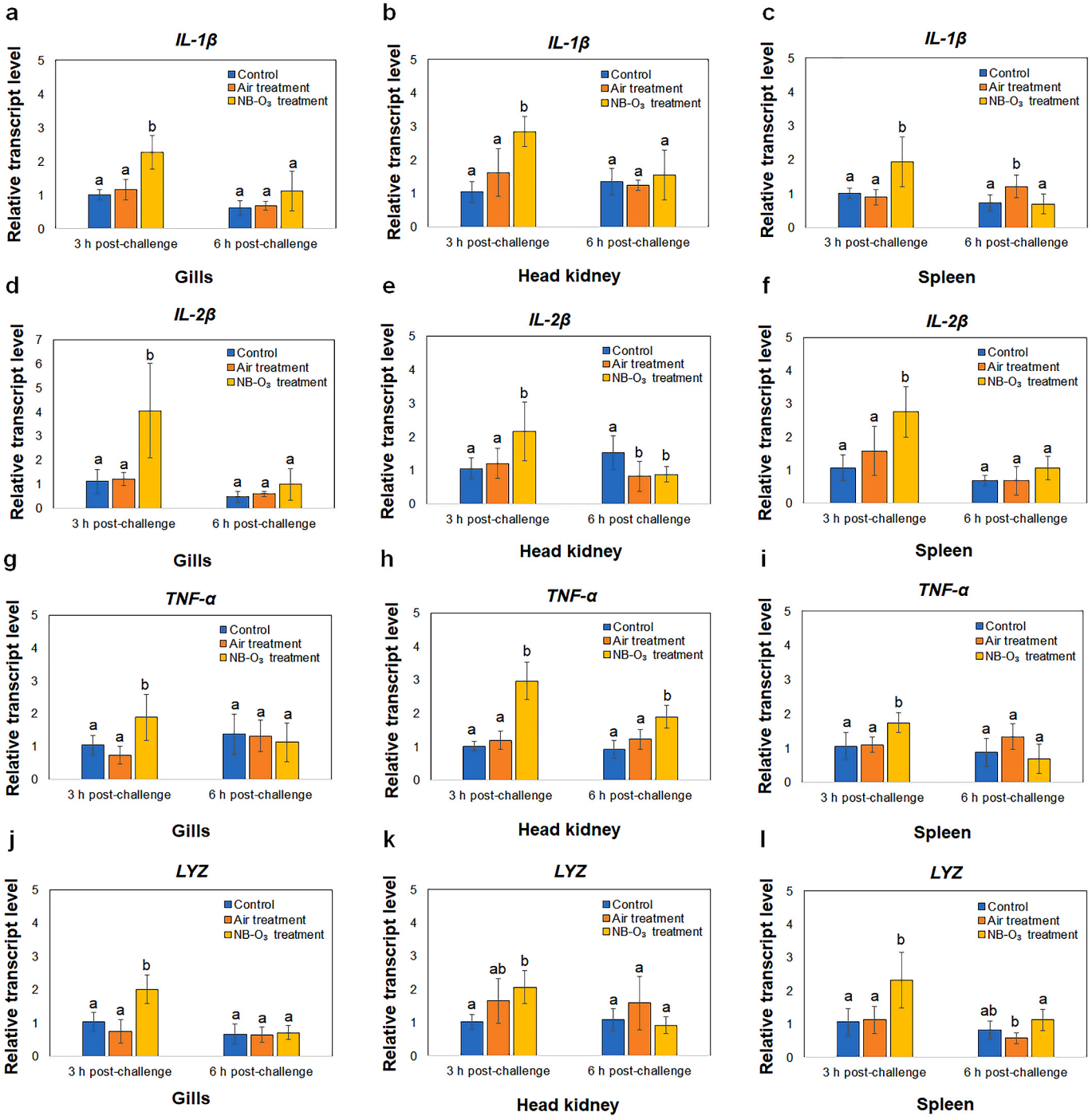

Fig. 3. Comparative expression profiles of *IL-1β*, *IL-2β*, *TNF-α*, and *LYZ* in the gills, head kidney, and spleen of control and treated fish (n = 5) at 3 h and 6 h following *S. agalactiae* challenge. The expression of target genes was normalized using *EF-1α*. Transcript levels of control at 0 min were set as 1. Error bars represent standard deviation and values with different superscript letters showed significant differences ($p < 0.05$) at different samples of each sampling point in post-hoc multiple range tests.

reported several benefits of NB-O_3 in respect of reducing concentration of certain pathogenic bacteria and increasing dissolved oxygen (DO) in water while not negatively impacting the health of aquaculture species [21,24,25]. In a continuous effort to explore further benefits of this technology in aquaculture, this study discovered that a short exposure of 10-min NB-O_3 treatment, which produced 2–3 × 10^7 NBs with majorities of bubble size less than 130 nm [21], triggered significant up-regulation (roughly 2 to 6-fold) of six immune-related genes of the innate defense system of Nile tilapia, including pro-inflammatory cytokines genes (*IL-1β, IL-2β, TNF-α*), heat-shock protein genes (*HSP70, HSP90-α*), and a bacteriolytic enzyme, C-type lysozyme gene (*LYZ*). These genes play important roles in activation of the immune system and facilitate early responses to infections [26]. They induce the immune response by stimulating lymphocytes or enhancing the release of other cytokines that can activate natural cells and macrophages, resulting in their clearance, as well as response against pathogens [26,27]. Interestingly, upregulations of these genes in all examined organs occurred relatively fast, as early as 15 min-post NB-O_3 treatment and lasted for 12–24 h in the gills, the head kidney, and the spleen, suggesting that a dose of NB-O_3 effectively stimulated the expression of multiple genes involved in the

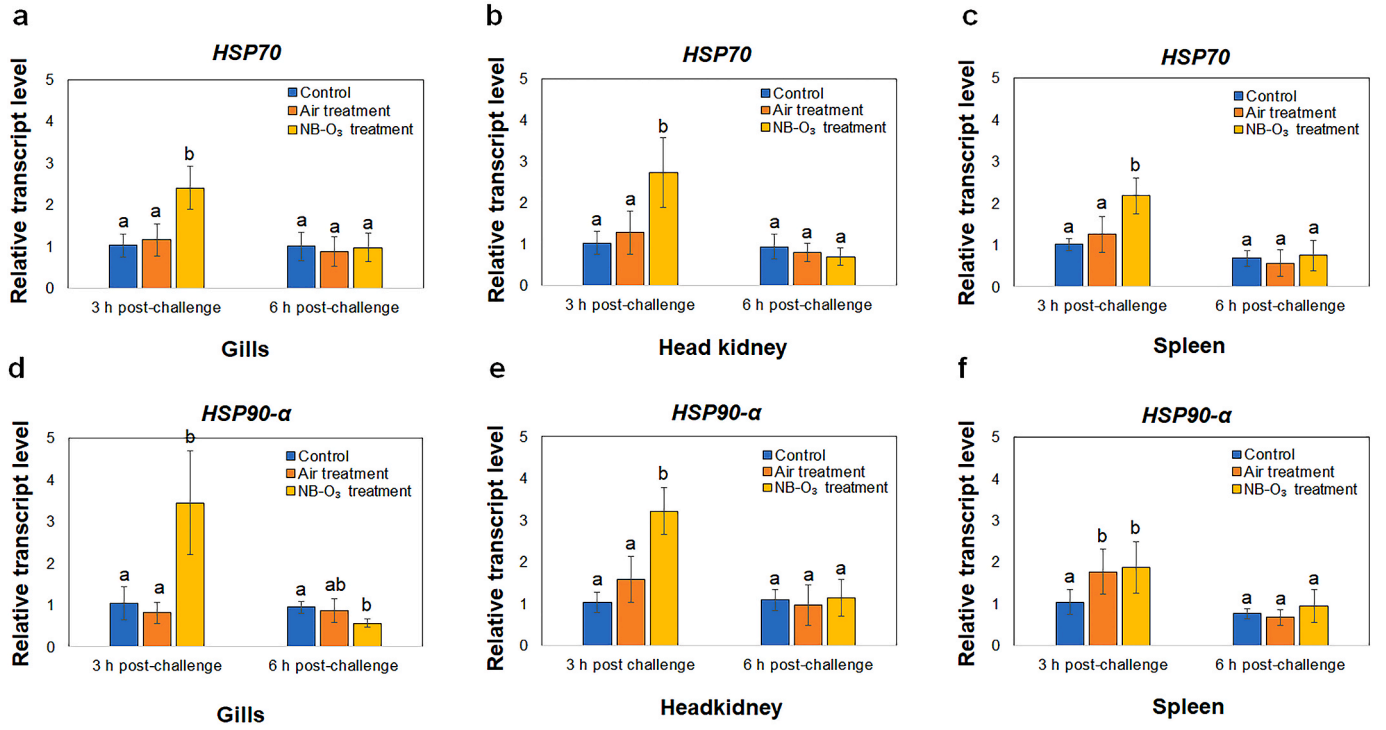

Fig. 4. Comparative expression profiles of *HSP70* and *HSP90-α* in the gills, head kidney, and spleen of control and treated fish (n = 5) at 3 h and 6 h following *S. agalactiae* challenge. The expression of target genes was normalized using *EF-1α*. Transcript levels of control at 0 min were set as 1. Error bars represent standard deviation and values with different superscript letters showed significant differences ($p < 0.05$) at different samples of each sampling point in post-hoc multiple range tests.

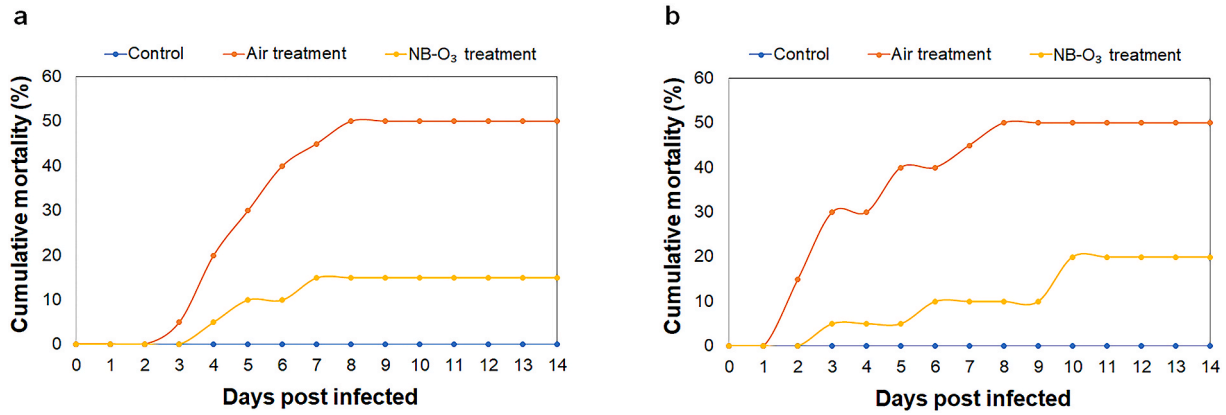

Fig. 5. Cumulative mortality observed in the different groups following challenge by immersion in *S. agalactiae*. Negative control, no challenge with bacteria; Air treatment (positive control), challenge with *S. agalactiae*; $NB\text{-}O_3$ treatment, upon exposure to $NB\text{-}O_3$ for 10 min followed by challenge with *S. agalactiae*. The experiments were performed in two independent trials (n = 20), a, trial 1; b, trial 2.

systemic frontline defense system of Nile tilapia.

Similar upregulation of nonspecific immune-related genes was reported in Nile tilapia after intraperitoneal injection of Cr-glucan and Yb-glucan immunostimulants. The expression levels of pro-inflammatory cytokine genes, *IL-1β* and *TNF-α*, were upregulated approximately 3-10-fold in both glucan treatments. An elevated level of these 2 genes was detected at 6 h post-challenge, then declined thereafter [28]. Two different doses of β-1, 3-glucan orally administered to Nile tilapia for 5 d also stimulated the production of cytokines, including TNF-α and IL-1β antibody reactive proteins in fish plasma [29]. The 45-min-long β-glucan treatment, which was administered through a bath to fry of rainbow trout (*Oncorhynchus mykiss*) enhanced pro-inflammatory cytokine transcript levels. However, repeated baths did not induce any change [30]. Ji et al. [31], reported that β-glucan could play an important role in regulating stress- and immune-related factors in rainbow trout following *Aeromonas salmonicida* challenge. Lysozyme and *HSP70* expressions in the head kidney of the β-glucan treated groups were higher and occurred earlier compared to that in other groups. Compared with those of previous studies, our findings have demonstrated that the $NB\text{-}O_3$ treatment could modulate expression of a vast array of the innate immune genes of Nile tilapia, similar to that of immunostimulants. These findings open up a potential application of $NB\text{-}O_3$ as a novel, inexpensive treatment for fish.

Despite the fact that the mechanism of fish immune modulation of $NB\text{-}O_3$ remains unknown, it is possible that physical interaction between the oxidative $NB\text{-}O_3$ and fish tissue, especially the gills may activate the innate recognition components through various cell receptors, stimulate production of cytokines and chemokines as well as the complement

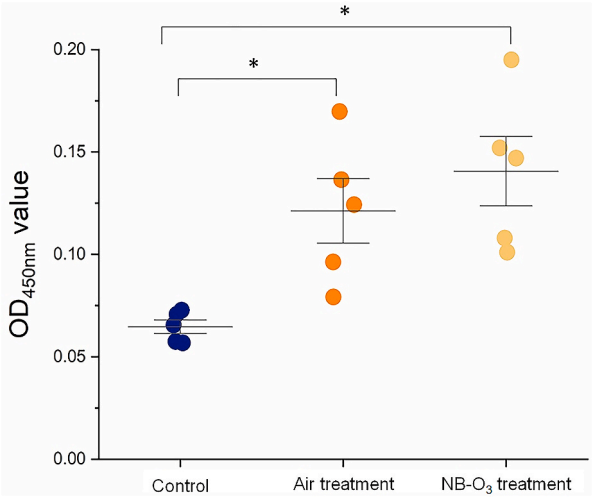

Fig. 6. Specific antibody (IgM) titer in serum of surviving fish at day 14 of the challenge experiment determined by indirect ELISA. Serum antibody titer was assayed with 1:512 dilutions, measured at OD 450 nm. Data represents the mean ± SD, one dot represents one biological replicate (n = 5). Significant differences are indicated by asterisk (*$p < 0.05$).

system. As hypothesized, the results in the challenge component of this study revealed that the NB-O_3 immune activated fish challenged with a lethal dose of *S. agalactiae*, responded better than the other groups, evidenced by significantly higher (approx. 2 to 4-fold) transcription levels of all six nonspecific immune genes. Overall, most of the genes upregulated as early as 3 h post-challenge in the gills, head kidney, and spleen and downregulated at 6 h, suggesting that early upregulation of these genes might facilitate effective response of the host defense system against a pathogen. Our immunological findings may explain the significantly higher survival rate (80–85%) in our challenge test compared to positive (untreated) control (50%) in this study. Particularly, in this study, the high expression level of *IL-2β* and *HSP90-α* in the gills, *TNF-α* and *IL-1β* in the head kidney were notably at 3 h post-challenge.

IL-1β is a cytokine involved in defense against foreign microbe invasion and tissue injury. *IL-1β* induces immune responses by stimulating lymphocytes releasing other cytokines [32]. *TNF-α* is one of the pro-inflammatory cytokines associated with detection of microorganisms and inflammatory reactions in fish [33–35]. *TNF-α,* which is usually the first cytokine to be secreted in pro-inflammatory cases, induces downstream *IL-1β* expression [30]. *IL-2β* is another crucial immunomodulatory cytokine that enhances the development, differentiation, and activation of T cells in particular [36].

Lysozymes are known for their lytic reaction to bacteria. C-type lysozymes are known to be active in the response against bacteria [37]. Transcriptional expression of C-type lysozyme is rapidly triggered in response to bacterial infections [38,39]. Previous studies showed that the C-type lysozymes of tilapia were effective in lysing both Gram-negative and Gram-positive strains. Bacterial challenge is interpreted as an acute stress stimulus by tilapia, which secretes cortisol to promote glucose synthesis and fat degradation, leading to the production of energy and specific proteins, such as lysozyme, which help enhance immunity level [20]. In the current analysis, C-type lysozyme expression was found to be upregulated in the gills, head kidney, and spleen of the NB-O_3 treated groups at 3 h post-challenge.

Heat shock protein genes are stress-associated genes that activate the host immune response. The current analysis indicated that the expression levels of *HSP70* and *HSP90-α* in each of the tissues of NB-O_3 treated groups were substantially increased at 3 h post-challenge (Fig. 4) relative to the control group not treated with NB-O_3; however, we also observed a slight increase in the air treated fish. *HSP70* counters bacterial infections by stabilizing the cells against damage due to pathogen proliferation, enhancing the proper folding of synthesizable cell proteins, and encouraging the retention and refolding of partially denatured proteins, which accounted for the post-challenge increase in *HSP70* expression observed in our study [40,41]. *HSP90-α* is involved in host immunity, and increased expression of this gene has been observed in fish during bacterial infections [42,43]. In the present study, *HSP90-α* expression in all tissues of the NB-O_3 treated groups was significantly increased at 3 h post-exposure compared to the non-treated group. However, this was followed by a decrease 6 h post NB-O_3 exposure. This type of fluctuation in expression levels has also been reported by previous studies (44–46). Up-regulations of expression levels were observed in the gills of shrimps challenged with *Vibrio harveyi* at 3, 12, and 24 h, whereas downregulation was observed at 48-h post-challenge [44]. Similar patterns were observed when *HSP90-α* expression was induced in the softshell clam hemocytes at 1-h following *V. splendidus* challenge and was subsequently down-regulated thereafter [45]. Initial upregulation may be due to bacterial multiplication and toxicity since *HSP90-α* is required to move bacterial toxins from the endosomal compartments into the cytosol [46]. However, the downregulation evident at later stages indicated a role for this gene during the initial periods of infection.

In accordance with previous studies that reported for the efficacy of immunostimulants in protecting the host in various fish pathogen models, our study suggests that the immune response to NB-O_3 exposure was similar with the response observed by other researchers when fish were given immunostimulants and challenged with bacteria [47–52].

Apart from effective responses of nonspecific immune-related genes, the present study also revealed that fish which survived exposure to *S. agalactiae* by immersion produced specific antibody IgM. The number of fish with OD readings above 0.14 on the ELISA test was higher in the NB-O_3 treatment group than in the untreated treatment group. These findings open up potential application of NB-O_3 as an immunomodulator in enhancing the fish immune system against bacterial infection.

Although this study reported an additional benefit of using NB-O_3 in aquaculture, several questions remain i.e. mechanisms and/or pathways that mediate the immune response in fish associated with NB-O_3 exposure, and the fish immune response kinetics to multiple NB-O_3 exposures. Further studies should explore these issues and field trials may be required to gain better understanding on feasibility of this technology in mitigating bacterial disease in aquaculture farms.

Data availability

The authors declare that they do not have any shared data available.

CRediT authorship contribution statement

Nguyen Vu Linh: Investigation, Methodology, Formal analysis, Writing - original draft, Software, Resources. **Le Thanh Dien:** Investigation, Methodology. **Wattana Panphut:** Supervision, Validation. **Anat Thapinta:** Supervision, Validation. **Saengchan Senapin:** Data curation, Writing - review & editing. **Sophie St-Hilaire:** Conceptualization, Writing - review & editing, Funding acquisition. **Channarong Rodkhum:** Supervision, Validation. **Ha Thanh Dong:** Conceptualization, Data curation, Writing – review & editing, Supervision, Validation, Funding acquisition, Project administration.

Disclaimers

The views expressed herein do not necessarily represent those of IDRC or its Board of Governors.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.02.015.

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