Pre-treatment of Nile tilapia (*Oreochromis niloticus*) with ozone nanobubbles improve efficacy of heat-killed *Streptococcus agalactiae* immersion vaccine

Nguyen Vu Linh[1], Le Thanh Dien[2], Pattiya Sangpo[3], Saengchan Senapin[3,4], Anat Thapinta[5], Wattana Panphut[5], Sophie St-Hilaire[6], Channarong Rodkhum[1*], Ha Thanh Dong[5,7*]

[1] Center of Excellence in Fish Infectious Diseases (CE FID), Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

[2] Faculty of Technology, Van Lang University, Ho Chi Minh City, Vietnam

[3] Fish Health Platform, Centex of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, 10400, Thailand

[4] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani, 12120, Thailand

[5] Faculty of Science and Technology, Suan Sunandha Rajabhat University, Bangkok, 10300, Thailand

[6] Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon Tong, Hong Kong

[7] Department of Food, Agriculture and Bioresources, School of Environment, Resources and Development, Asian Institute of Technology, Pathum Thani, 12120, Thailand

[*] **Corresponding authors**:

C.Rodkhum (channarong.r@chula.ac.th)

H.T.Dong (htdong@ait.ac.th)

Highlights

- Immune response and efficacy of a heat-killed *Streptococcus agalactiae* immersion vaccine for Nile tilapia with and without pre-treatment with NB-O_3 were accessed.
- Bacterial antigen uptake in the NB-O_3-VAC compared to the AT-VAC groups was increased 1.32 and 1.80-fold at 3 and 6 h post-vaccination, respectively.
- Vaccinated group that received pre-treatment with NB-O_3 had slightly to significantly higher levels of *IgM*, *IgD*, and *IgT* mRNA expression; IgM levels; and survival rate.
- Pre-treatment with NB-O_3 may be a novel strategy for improving efficacy of immersion vaccine in aquaculture

Abstract

Nanobubble technology has shown appealing technical benefits and potential applications in aquaculture. We recently found that treatment with ozone nanobubbles (NB-O_3) activated expression of several immune-related genes leading to effective response to subsequent exposure to fish pathogens. In this study, we investigated whether pre-treatment of Nile tilapia (*Oreochromis niloticus*) with NB-O_3 can enhance specific immune responses and improve efficacy of immersion vaccination against *Streptococcus agalactiae*. Spleen and head kidney of fish in the vaccinated groups showed a substantial upregulation in expression levels of three immunoglobulin classes (*IgM, IgD,* and *IgT*) compared with the unvaccinated control groups. At day 21 post-immunization, the relative expression was greatest (approx. 3.2 to 4.1 folds). Both systemic and mucosal IgM antibodies were elicited in vaccinated groups. As the result, the cumulative survival rate of the vaccinated groups was found to be higher than that of the unvaccinated groups, with a relative percent survival (RPS) ranging from 52.9-70.5%. However, fish in the vaccinated groups that received pre-treatment with NB-O_3, bacterial antigen uptakes, expression levels of *IgM*, *IgD*, and *IgT*, as well as the specific-IgM antibody levels and percent survival, were all slightly or significantly higher than that of the vaccinated group without pre-treatment with NB-O_3. Taken together, our findings suggest that utilizing pre-treatment with NB-O_3 may improve the immune response and efficacy of immersion vaccination in Nile tilapia.

Keywords: Ozone nanobubble; Immersion vaccination; Immunoglobulin genes; Nile tilapia; Heat-killed vaccine

1. Introduction

Aquaculture has grown at an unprecedented rate worldwide over the past two decades [1]. This expansion has resulted in larger and more numerous farms within watersheds, which enhances the risk of host-dependent pathogen transmission and makes disease management more challenging [2-4]. Disease outbreaks are a major cause of economic losses in aquaculture, including Nile tilapia (*Oreochromis niloticus*) [5-8]. As a result of these developments, the use of chemotherapy to control diseases has increased. Recently, numerous antibiotics have been used in aquaculture to minimize losses due to bacterial diseases. When an antibiotic treatment is unsuccessful, the farmer typically switches to another antibiotic or increases the dose of the medication, both of which result in greater antibiotic usage [9], which is problematic for the development of antimicrobial resistance (AMR).

Disease prevention is the most rational approach to resolving the problems associated with antibiotic treatments, as it reduces the need for these products. Reduced antibiotic usage in aquatic systems will eventually decrease the risk of AMR in these sectors, which may directly affect AMR risk in human populations. Prevention of disease can be accomplished through a number of ways, which include reducing exposure to pathogens and/or improving host resistance to disease [10,11]. The latter can be achieved through vaccination [12]. To date, vaccination has been shown to be the most efficient method for combating pathogenic infections or conferring struggle to target pathogens. Numerous successful vaccines have been produced that provide effective protection in fish, including subunit vaccines, inactivated vaccines, DNA vaccines, and vaccines with live attenuation [13-16]. However, issues of vaccination also limit their use. Historically, immunizations have been administered through injection, which is time consuming and difficult to deliver to young fish [17]. While oral and immersion vaccinations are simple to administer with

74 minimum stress to the fish, they often generate limited immune responses [12,18-20]. Improving
75 immersion vaccines would go a long way to preventing infectious diseases on fish farms [12].
76 A new technology that injects nanobubbles into liquids and helps reduce bacterial counts in water
77 may reduce pathogen burdens on fish farms [21-24]. In our previous investigations, ozone
78 nanobubbles were found to be efficient in reducing bacterial concentrations in water and
79 upregulating the innate immune system of fish, resulting in increased fish survival during
80 pathogenic infections. When Nile tilapia were infected with a pathogenic multidrug-
81 resistant *Aeromonas hydrophila*, they displayed a higher survival rate when exposed to treatments
82 with ozone nanobubbles (NB-O_3) [25-27]. Given the stimulation of the innate immune system in
83 the treated groups, we hypothesized that this technology might improve the efficacy of immersion
84 vaccines. This study aimed to investigate: 1) whether treatment of Nile tilapia with NB-O_3 can
85 enhance specific immune responses to vaccine, and 2) whether simultaneous treatment with NB-
86 O_3 can improve the efficacy of immersion vaccination against *S. agalactiae*.

87 **2. Materials and Methods**

88 **2.1 Animals and ethical issues**

89 A total of 360 apparently healthy Nile tilapia fish were provided by a tilapia hatchery (Department
90 of Fisheries, Thailand). Experimental fish were maintained in fiberglass tanks (100 L), which were
91 continuously aerated for 2 weeks and equipped with a cotton filter before the vaccination trials.
92 Prior to conducting additional experiments, 10 randomly selected fish were subjected to bacterial
93 and parasite examinations to guarantee their health. The Thai Institutional Animal Care and Use
94 Committee authorized all animal operations (approval no. MUSC64-024-573).

95 **2.2 Bacterial culture and heat-killed vaccine preparation**

96 *Streptococcus agalactiae* strain 2809, identified from a tilapia field outbreak, was used for this

research (Centex Shrimp, Mahidol University, Thailand). It was retrieved from frozen glycerol stocks and cultivated for 24 h at 28 °C on tryptic soy agar (TSA, Becton, Dickinson and Company, USA), followed by culturing in 100 mL of tryptic soy broth (TSB, Becton, Dickinson and Company, USA) for 18 h. The bacterial cells were inactivated using the heat-killed method at 56 °C for 30 min in a water bath [28]. To confirm bacterial inactivation, an aliquot of 0.1 mL of killed bacterial suspension was plated onto TSA and *S. agalactiae* selective agar bases (SSA, HiMedia, India) and incubated for 48 h at 28 °C. The absence of bacterial growth indicated successful inactivation. The heat inactivated *S. agalactiae* without adjuvant was used as immersion vaccine in this study.

2.3 Fish vaccination and challenge

Two weeks after acclimation, 360 fish (15.62 ± 0.45 g) were randomly allocated into 4 experimental groups: ozone nanobubbles without vaccine (NB-O_3-noVAC) group (G1), ozone nanobubbles with vaccine (NB-O_3-VAC) group (G2), an air-stone with vaccine (AT-VAC) group (G3), and an air-stone without vaccine (AT-noVAC) group (G4). Each treatment was performed in duplicates with 45 fish per tank. Firstly, the nanobubble tanks (G1, G2) were subjected to NB-O_3 for 10 min according to a previously reported protocol [27]. After 3 h, the vaccinated groups (G2, G3) were immunized with heat-killed *S. agalactiae* vaccine (1.56×10^9 CFU/mL) by adding 1 L of inactivated vaccine to each tank containing 50 L of water and 45 fish to reach a final concentration of 1.67×10^7 CFU/mL. The other non-vaccinated groups (G1, G4) were carried out in the same manner using TSB without inactivated bacteria as the control group. Following a 12-hour immersion vaccination, fish were transferred to new aeration tanks and maintained at 30 ± 1 °C for 21 d.

The efficiency of vaccines against *S. agalactiae* was assessed using an experimental challenge

with *S. agalactiae* on day 21 after vaccination. The experimental trials were conducted in 100 L dechlorinated tap water tanks, including 20 fish per tank. The fish immunized with heat-killed vaccines (n = 20) were injected intraperitoneally with *S. agalactiae* at a dose of 10^7 CFU/fish. By contrast, the NB-O_3-noVAC and AT-noVAC groups received injections of 0.1 mL of 1× PBS. Fish mortality was monitored for 14 d (Fig. 1).

2.4 Sample collection

To investigate the uptake of vaccine into fish bodies of Nile tilapia through gills, six representative fish (six biological replicates/group) were selected at different periods (3 and 6 h) post-immersion vaccination. Prior to sample collection, the fish were euthanized with a lethal dose of clove oil (250 ppm). The gill tissues from each fish were collected and stored in 95% ethanol at a ratio of 1:10 (v/v) tissue:ethanol until further analysis.

To collect the mucus and serum samples for enzyme-linked immunosorbent assay (ELISA), six random fish were selected at different intervals: days 0 (baseline), 7, 14, and 21. Mucus samples were collected by gently rubbing the fish in a plastic bag that contained 1 mL of 1× PBS and 0.02% sodium azide [29]. Blood samples were obtained from the tail vein of fish using a syringe fitted with a 23-G needle. The serum and mucus were then collected and kept at –20 ºC for further analysis.

To analyze mRNA expression of three immunoglobulin genes encoding *IgM, IgD*, and *IgT*, the fish tissues (spleen and head kidney) were collected at different periods (days 7, 14, and 21) after immunization. Investigated tissues (40–50 mg) from six randomly chosen fish (mentioned earlier) were collected and stored in sterile tubes supplemented with 200 µL Trizol (Invitrogen, USA) at –20 ºC until examinations.

2.5 qPCR assay for quantifying *S. agalactiae*

Quantitative PCR was performed according to the protocol reported by Leigh et al. [30]. Primers *SagroEL-F/R*, which targets the *groEL* gene of *S. agalactiae* (accession number EU003621). A 142-bp product amplified from *S. agalactiae* 2809 was cloned into pGEM and the recombinant plasmid, namely pSNB1, was used as a positive control and for standard curve construction. Serial dilutions of the pSNB1 plasmid spiked with 200 ng of tilapia DNA were used to construct a standard curve for quantifying *S. agalactiae.* Fish gill DNA was isolated using the conventional phenol-chloroform method [31,32] and 200 ng of each DNA sample was subjected to qPCR assays using the CFX Connect™ Real-time System (Bio-Rad, USA). The resulting C_q value was used to compute bacterial DNA in the fish gills using the equation: copy number = $10^{(Ct - Intercept)/Slope}$. qPCR for each template was performed in triplicate and calculated as bacterial load per 1 µg DNA template.

2.6 qPCR assay for immune gene expression study

Total RNA was isolated from tissue samples (spleen and head kidney) using the Trizol method according to the manufacturer's procedures. First-strand complementary DNA synthesis and qPCR were performed following the procedures reported by Linh et al. [27]. The primers specific for tilapia *IgM, IgD*, *IgT,* and *β-actin* used for qPCR are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative gene expression data [33]. Transcript levels of AT-noVAC groups on day 7 were set at 1.

2.7 Serum and mucus antibody assays

The mucosal and systemic IgM antibody levels of Nile tilapia were detected using ELISA. Mucus and serum ELISA assays were performed in the same manner as previously reported [27], with minor modifications. Briefly, mucus or serum samples were collected from six representative fish

of each time point (days 0, 7, 14, and 21). Two-fold serial dilutions were performed to determine the optimal dilution. The ELISA dilutions for mucus and serum were 1:16 and 1:512, respectively.

2.8 Statistical analysis

SPSS program (ver. 22.0) was used to conduct all statistical analyses. The Kaplan–Meier method was used to evaluate the survival rates in challenge trials, and a log-rank test was used to compare the treatment groups. One-way analysis of variance (ANOVA) was used to evaluate expression of immunoglobulin genes. Duncan's post hoc tests were used to compare mean values. The Kruskal–Wallis test was used to examine the ELISA data. Bonferroni test was used to compare various groups. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 Quantification of bacterial uptake into the fish gills after immunization

The qPCR for *S. agalactiae* performed in the current research showed a detection limit of 100 copies/μL of target template with an amplification efficiency of 90.2% and an $R^2 = 0.991$. The mean $C_q \pm$ standard deviation (SD) for the detection limit was 36.64 ± 0.54 (Fig. 2). That is to say, samples with $C_q \leq 36.14$ were considered *S. agalactiae* positive. The mean bacterial uptake ± SD in the NB-O_3-VAC groups was 2032.40 ± 2053.45 and 5669.50 ± 2763.31 per 1 μg DNA template, whereas a lower value (1539.61 ± 585.91 and 3223.92 ± 970.96) was observed in the AT-VAC groups at 3 and 6 h post-immunization. The bacterial DNA present in the gills in the NB-O_3-VAC was 1.32 and 1.80-fold higher compared to that in the AT-VAC groups after 3 and 6 h of vaccination, respectively (Table 2).

3.2 Expression of specific immune-related genes

Different expression levels of three immunoglobulin genes encoding *IgM, IgD*, and *IgT* in the spleen and the head kidney were observed for the different treatment groups (Fig. 3). On day 14, a significant increase in *IgM* expression in the spleen (approximately 2.5 folds) was observed in the vaccinated groups that received pre-treatment with NB-O_3 (NB-O_3-VAC) compared with the unvaccinated groups (NB-O_3-noVAC and AT-noVAC), while no significant difference was observed between any of the treatment groups on day 7 or day 21 post-vaccination. A significant upregulation in the spleen of *IgD* (approximately 2-fold) was found in the vaccinated groups that received pre-treatment with NB-O_3 (NB-O_3-VAC) at day 21 compared with the unvaccinated groups (NB-O_3-noVAC and AT-noVAC), whereas no significant differences were observed in the spleen among all treatment groups at day 7 or 14 post-vaccination. On day 7 post-vaccination, neither the vaccinated nor the unvaccinated groups demonstrated substantial changes in *IgT*

197 expression in the spleen of fish. *IgT* expression showed a higher relative change in both vaccinated
198 groups compared to the unvaccinated groups at all time points. However, these differences were
199 not statistically significant. At days 14 and 21 post-vaccination, *IgT* expression was substantially
200 increased (approx. 2.8–4.1 folds) in the spleen of the NB-O_3-VAC and AT-VAC groups compared
201 to the fish in the NB-O_3-noVAC and AT-noVAC groups. The highest expression was obtained in
202 the NB-O_3-VAC group (approximately 4.1-fold) at day 21 post-vaccination (Fig. 3A).
203 In the head kidney tissues, the relative expression of *IgM* was significantly increased in both the
204 AT-VAC and NB-O_3-VAC groups at day 14 (approx. 1.8–2.2 folds) and 21 (approx. 2.2–3.6 folds)
205 post-vaccination, with significantly higher levels in the NB-O_3-VAC fish. *IgM* expression in the
206 NB-O_3-VAC group was considerably upregulated compared with *IgM* expression in the AT-VAC
207 group on day 21 post-vaccination. On day 7 post-vaccination, no significant variations in *IgM*
208 expression were observed in any groups.
209 On days 7, 14, and 21 after immersion vaccination, *IgD* expression was essentially constant, and
210 no alteration was detected in any of the treatment groups. There was a considerable increase in *IgT*
211 of the AT-VAC and NB-O_3-VAC groups (approx. 1.9–3.6 folds) compared with the NB-O_3-
212 noVAC and AT-noVAC groups at day 21 post-vaccination. Notably, expression levels of fish in
213 the NB-O_3-VAC groups were much higher than those of fish in the AT-VAC groups (Fig. 3B).
214 **3.3 Analysis of specific-IgM antibody response**
215 The systemic antibody response of the vaccinated fish showed substantially greater levels ($P <$
216 0.05) of specific IgM antibodies in their serum by indirect ELISA methods on days 7, 14, and 21
217 compared to fish that were not vaccinated. No significant specific antibody titer was detected in
218 fish pre-vaccination (at day 0); however, the production of IgM serum in the vaccinated fish that
219 received pre-treatment with NB-O_3 was, on average slightly higher than that of vaccinated fish

that did not receive pre-treatment with NB-O_3 on day 7 (0.138 ± 0.005 vs. 0.116 ± 0.006), day 14 (0.144 ± 0.02 vs. 0.138 ± 0.015), and day 21 (0.146 ± 0.06 vs. 0.09 ± 0.005) post-vaccination, respectively. Similar to pre-vaccination fish, no significant systemic antibody response was detected in any of the unvaccinated fish on any of the sample days (Fig. 4A).

Analysis of IgM levels in the mucous membranes of tilapia after vaccination displayed a similar pattern to the systemic antibody response; however, lower $OD_{450\ nm}$ reading values of mucosal antibody titers were observed on days 7, 14, and 21 post-vaccination compared to the systemic antibody response measurements. Specific-IgM antibody responses in the vaccinated groups that received pre-treatment with NB-O_3 were all slightly higher than those of the vaccinated group without pre-treatment with NB-O_3 on days 7, 14, and 21. On day 14 post-vaccination, the mucosal IgM of fish in the vaccinated groups (NB-O_3-VAC) had significantly higher antibody levels than those in the unvaccinated groups (NB-O_3-noVAC and AT-noVAC). On the other days, all differences were not statistically significant (Fig. 4B).

3.4 Cumulative survival of vaccinated fish after challenging with *S. agalactiae*

Mortality was noted on day 3 post-challenge and persisted until day 6 and day 9 post-challenge for fish in the NB-O_3-VAC and AT-VAC groups, respectively. However, fish mortality occurred earlier in the unvaccinated groups than in the vaccinated groups (on day 2) and persisted until day 8 post-challenge. The NB-O_3-VAC group had the highest RPS of 70.5 ± 8.31%, followed by the RPS in the AT-VAC group (52.9 ± 16.63%) (Fig. 5A). This study demonstrated a significant difference ($P = 0.003$) between the NB-O_3-VAC and the AT-noVAC groups. The AT-VAC groups also displayed a substantial difference compared to the AT-noVAC groups ($P = 0.02$) (Fig. 5B). Fish that died during the challenge experiment exhibited typical clinical signs of *S. agalactiae* infection. *Streptococcus agalactiae* was isolated from a representative group of moribund and dead

243 fish on SSA.

244 **4. Discussion**

245 Based on the results of antigen uptake, antibody assay, expression levels of three immunoglobulin
246 genes, and RPS, this study indicated that pre-treatment with NB-O_3 improved immune responses
247 in Nile tilapia to heat-killed *S. agalactiae* immersion vaccine. This approach represents a
248 promising strategy for the prevention of streptococcus infections in Nile tilapia. Recently, several
249 advantages of NB-O_3 have been reported in terms of reducing the concentration of certain
250 pathogenic bacteria and increasing dissolved oxygen (DO) in water. It has been suggested that NB-
251 O_3 could act as an "immunostimulant" to activate the fish innate immune system against bacterial
252 infections [27] and improve the survivability of Nile tilapia challenged with *Aeromonas*
253 *hydrophila* [25]. Notably, the findings of our investigation revealed that better bacterial antigen
254 uptake into the gill tissues after 3 and 6 h immunization is achieved with fish in the vaccinated
255 group that received a pre-treatment with NB-O_3 (NB-O_3-VAC) compared with fish in the AT-
256 VAC group. It is possible that ozone nanobubbles in the NB-O_3-VAC groups activated innate
257 immune system, which modulated immune cells in response to infections [25-27]. When these
258 innate immune cells are stimulated, they perform several response mechanisms to antigens,
259 including phagocytosis, degranulation, and production of cytokines, which may trigger and/or
260 recruit other leukocytes (e.g., neutrophils, macrophages, and dendritic cells) to the fish gills, and
261 it is highly tempting to speculate that pre-treatment with NB-O_3 might cause a temporary
262 disruption of epithelial cells, leading to reducing barrier, increasing antigen uptake, and producing
263 a greater immune response. Another possible explanation for the improved antigen absorption may
264 be the physical and biological properties of ozone nanobubbles that require further investigation.
265 Antigens may have been taken up by the gills or skins during immersion vaccination and processed

by the innate immune system (e.g., phagocytes), where subsequent responses resulted in adaptive immune system and protected the immunized fish [34]. Indeed, pre-treatment with NB-O_3 appears to have aided in the uptake of bacterial antigens during immersion vaccination. Several studies have previously demonstrated that enhanced antigen uptake results in increased protection [35,36]. These findings indicate that NB-O_3 may be useful for improving the efficiency of immersion immunization in Nile tilapia.

In the current study, our data showed that fish in the vaccinated groups that received pre-treatment with NB-O_3 had an increase in mRNA expression of three immunoglobulin classes (*IgM*, *IgD*, and *IgT*) compared to other treatment groups, including vaccinated fish that were not treated with NB-O_3. Immunoglobulins (Ig) are important players in adaptive immune responses because they recognize and eliminate infections through a variety of mechanisms [37]. The immunoglobulin M (IgM) is the most abundant Ig in the plasma and the primary participant in systemic immunity, whereas the immunoglobulin T (IgT) is found in mucosal secretions and represented the primary Ig in mucosal immunity. The immunoglobulin D (IgD) is presumably involved in vertebrate immune responses, its relevance in Nile tilapia, however, remains unclear [38-40]. Specific immune gene expressions (*IgM*, *IgD*, and *IgT*) of fish in the vaccinated groups that received pre-treatment with NB-O_3 were all slightly or significantly higher compared to fish in the other groups, reflecting the important role of NB-O_3 in transporting vaccine antigens to the lymphoid organs and subsequent induction of mucosal and/or systemic immunity. Interestingly, our works demonstrate for the first time that immersion immunization induces a mucosal IgD response in Nile tilapia. The considerable elevations of *IgM*, *IgD*, and *IgT* in the spleens or head kidneys suggest that these immunoglobulins may play an essential role in defending fish against *S. agalactiae* infection.

The interaction between the cumulative survival of fish-immunized and antibody titers in the

mucus and serum of Nile tilapia [41], Asian seabass [42], and Atlantic cod [43] highlights the critical role of antibody-mediated immunity in protecting fish against streptococcal infections. This concept was consistent with our observation; there were higher survival in the groups of fish with the higher antibody titers and these fish were more common in the NB-O_3 treatment group [27]. The results obtained in this study are in agreement with a previous study, which induced significant protection in Asian seabass (*Lates calcarifer*), demonstrated by an RPS value ranging from 75–85% in both monovalent and bivalent vaccine groups after challenge with the inactivated *S. agalactiae* and *S. iniae* [42] or better RPS value (59.3 59.3% and 77.8%) was achieved with fish in the vaccinated group supplemented with adjuvants (aluminum hydroxide and FIA) compared with fish in the other groups without adjuvants [44]. Our previous study reported that pre-treatment with NB-O_3 stimulated expression of immune-related genes of the fish innate immune system [27]. This may explain better responses of adaptive immune system after vaccination. Furthermore, the high RPS obtained in the current study in the NB-O_3 group may be partially due to the contribution of specific immune mechanisms and the induction of mucosal and systemic IgM antibodies, as evidenced by upregulation of *IgM* in the spleens or head kidneys and an increase of total IgM in the mucus and serum of vaccinated fish.

There are still many questions to be addressed regarding the mechanisms of gene regulation as well as the mechanism of immunological responses in Nile tilapia. Further studies are needed to corroborate these findings and develop a deeper understanding of the mechanism of the immune stimulation observed in this study.

In conclusion, this study reported that pre-treatment with NB-O_3 is a promising strategy for enhancing the efficacy of immersion vaccines against bacterial infections in tilapia, which has potential to be applied in aquaculture on a large scale.

Data availability

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

Author contributions

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

Disclaimers

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

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research project received financial support from the UK government - Department of Health and Social Care (DHSC), Global AMR Innovation Fund (GAMRIF), and the International Development Research Center (IDRC), Ottawa, Canada. Nguyen Vu Linh has been supported by

334 the Chulalongkorn University, Ratchadapisek Somphot Fund for Postdoctoral Fellowship. The
335 authors like to express their gratitude to Mr. Nguyen Dinh-Hung for his technical support.

References

[1] FAO, The State of World Fisheries and Aquaculture, Sustainability in action, Rome, (2020), https://doi.org/10.4060/ca9229en.

[2] R. Miller, H. Harbottle, Antimicrobial Drug Resistance in Fish Pathogens, Microbiol. Spectrum 6 (1) (2018) 6–1, https://doi.org/10.1128/microbiolspec.ARBA-0017-2017.

[3] T. Xie, X. Xu, Q. Wu, J. Zhang, J. Cheng, Prevalence, Molecular Characterization, and Antibiotic Susceptibility of *Vibrio parahaemolyticus* from Ready-to-Eat Foods in China, Front. Microbiol. 7 (2016) 1–10, https://doi.org/10.3389/fmicb.2016.00549.

[4] O.E. Heuer, H. Kruse, K. Grave, P. Collignon, I. Karunasagar, F.J. Angulo, Human health consequences of use of antimicrobial agents in aquaculture, Clin. Infect. Dis. 49 (8) (2009) 1248–1253, https://doi.org/10.1086/605667.

[5] P.T. Woo, Protective immunity in fish against protozoan diseases, Parassitologia 49 (3) (2007) 85–191, https://pubmed.ncbi.nlm.nih.gov/18410078/.

[6] Y. Muktar, S. Tesfaye, B. Tesfaye, Present status and future prospects of fish vaccination: a review, J. Veterinar. Sci. Technol. 7 (2) (2016) 299, http://doi.org/10.4172/2157-7579.1000299.

[7] S.E. Ali, M.D. Jansen, C.V. Mohan, J. Delamare-Deboutteville, H. Charo-Karisa, Key risk factors, farming practices and economic losses associated with tilapia mortality in Egypt, Aquaculture 527 (2020) 735438, https://doi.org/10.1016/j.aquaculture.2020.735438.

[8] M.D. Jansen, H.T. Dong, C.V. Mohan, Tilapia lake virus: a threat to the global tilapia industry?, Reviews in Aquaculture 11 (3) (2019) 725–39, https://doi.org/10.1111/raq.12254.

[9] Y. Shen, H. Zhou, J. Jiao Xu, Y. Wang, Q. Zhang, R. Timothy, T.R. Walsh, B. Shao, C. Wu, Y. Hu, L. Yang, Z. Shen, Z. Wu, Q. Sun, Y. Ou, Y. Wang, S. Wang, Y. Wu, C. Cai, J. Li, J. Shen, R. Zhang, Y. Wang, Anthropogenic and environmental factors associated with high incidence of

359 *mcr-1* carriage in humans across Chin, Nature Microbiology 3 (9) (2018) 1054–1062,
360 https://doi.org/10.1038/s41564-018-0205-8.
361 [10] D. Price, J. Sanchez, J. McClure, S. McConkey, R. Ibarra, S. St-Hilaire, Assessing
362 concentration of antibiotics in tissue during oral treatments against piscirickettsiosis, Prev. Vet.
363 Med. 156 (2018) 16–21, https://doi.org/10.1016/j.prevetmed.2018.04.014.
364 [11] A.E. Toranzo, J.L. Romalde, B. Magariños, J.L. Barja, Present and future of aquaculture
365 vaccines against fish bacterial diseases, Options Mediterr. 86 (2009) 155–176,
366 http://om.ciheam.org/article.php?IDPDF=801069.
367 [12] S. Yun, S.S. Giri, H.J. Kim, S.G. Kim, S.W. Kim, J.W. Kang, S.J. Han, J. Kwon, W.T. Oh,
368 C. Chi, J.W. Jun, Enhanced bath immersion vaccination through microbubble treatment in the
369 cyprinid loach, Fish & Shellfish Mmmunology 91 (2019) 12–28,
370 https://doi.org/10.1016/j.fsi.2019.05.021.
371 [13] T. Langemann, V.J. Koller, A. Muhammad, P. Kudela, U.B. Mayr, W. Lubitz, The Bacterial
372 ghost platform system: production and applications, Bioeng Bugs 1 (2010) 326–336,
373 https://doi.org/10.4161/bbug.1.5.12540.
374 [14] J. Ma, T.J. Bruce, E.M. Jones, K.D. Cain, A review of fish vaccine development strategies:
375 Conventional methods and modern biotechnological approaches, Microorganisms 7 (11) (2019)
376 569, https://doi.org/10.3390/microorganisms7110569.
377 [15] S. Jorge, O.A. Dellagostin, The development of veterinary vaccines: a review of traditional
378 methods and modern biotechnology approaches, Biotechnology Research and Innovation 1 (1)
379 (2017) 6–13, https://doi.org/10.1016/j.biori.2017.10.001.
380 [16] H. Su, J. Su, Cyprinid viral diseases and vaccine development, Fish & Shellfish Immunol. 83
381 (2018) 84–95, https://doi.org/10.1016/j.fsi.2018.09.003.

382 [17] C.M. Caipang, J.B. Lucanas, C.M. Lay-yag, Updates on the vaccination against bacterial
383 diseases in tilapia, *Oreochromis* spp. and Asian seabass, *Lates calcarifer*, AACL Bioflux 7 (3)
384 (2014) 184–93, http://bioflux.com.ro/docs/2014.184-193.pdf.
385 [18] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal immunity and B cells in teleosts: effect of
386 vaccination and stress, Front. Immunol. 6 (2015) 354, https://doi.org/10.3389/fimmu.2015.00354.
387 [19] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal immunity and B cells in teleosts: effect of
388 vaccination and stress, Frontiers in Immunology 6 (2015) 354,
389 https://doi.org/10.3389/fimmu.2015.00354.
390 [20] J. Bøgwald, R.A. Dalmo, Review on immersion vaccines for fish: An update 2019,
391 Microorganisms 7 (12) (2019) 627, https://doi.org/10.3390/microorganisms7120627.
392 [21] A. Agarwal, W.J. Ng, Y. Liu, Principle and applications of microbubble and nanobubble
393 technology for water treatment, Chemospher 84 (9) (2011) 1175–1180,
394 https://doi.org/10.1016/j.chemosphere.2011.05.054.
395 [22] G. Kaushik, A. Chel, Microbubble technology: emerging field for water treatment. Bubble
396 Science Engineering & Technology, 5 (1-2) (2014) 33–38,
397 https://doi.org/10.1179/1758897914Y.0000000010
398 [23] P. Khan, W. Zhu, F. Huang, W. Gao, N.A. Khan, Micro–nanobubble technology and water-
399 related application, Water Supply 20 (6) (2020) 2021-35, https://doi.org/10.2166/ws.2020.121.
400 [24] J. Zhu, H. An, M. Alheshibri, L. Liu, P.M.J. Terpstra, G. Liu, V.S.J. Craig, Cleaning with
401 Bulk Nanobubbles, Langmuir. 32 (43) (2016) 11203–11211,
402 https://doi.org/10.1021/acs.langmuir.6b01004.
403 [25] L.T. Dien, N.V. Linh, P. Sangpo, S. Senapin, S. St-Hilaire, C. Rodkhum, H.T. Dong, Ozone
404 nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged

405 with a pathogenic multi-drug-resistant *Aeromonas hydrophila*, Journal of Fish Diseases 44 (9)
406 (2021) 1435–47, https://doi.org/10.1111/jfd.13451
407 [26] C. Jhunkeaw, N. Khongcharoen, N. Rungrueng, P. Sangpo, W. Panphut, A. Thapinta, S.
408 Senapin, S. St-Hilaire, H.T. Dong, Ozone nanobubble treatment in freshwater effectively reduced
409 pathogenic fish bacteria and is safe for Nile tilapia (*Oreochromis niloticus*), Aquaculture 534
410 736286, https://doi.org/10.1016/j.aquaculture.2020.736286.
411 [27] N.V. Linh, W. Panphut, A. Thapinta, S. Senapin, S. St-Hilaire, C. Rodkhum, H.T. Dong,
412 Ozone nanobubble modulates the innate defense system of Nile tilapia (*Oreochromis niloticus*)
413 against *Streptococcus agalactiae*, Fish & Shellfish Immunology 112 (2021) 64–73,
414 https://doi.org/10.1016/j.fsi.2021.02.015.
415 [28] B.K. Hvalbye, I.S. Aaberge, M. Løvik, B. Haneberg, Intranasal immunization with heat-
416 inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection,
417 Infection & Immunity 67 (9) (1999) 4320-5, https://doi.org/10.1128/IAI.67.9.4320–4325.1999.
418 [29] N. Abu Nor, M. Zamri-Saad, I.S. Md Yasin, A. Salleh, F. Mustaffa-Kamal, M.F. Matori, M.N.
419 Azmai, Efficacy of whole cell inactivated *Vibrio harveyi* vaccine against Vibriosis in a marine red
420 hybrid Tilapia (*Oreochromis niloticus* x *O. mossambicus*) model, Vaccines 8 (4) (2020) 734,
421 https://doi.org/10.3390/vaccines8040734.
422 [30] W.J. Leigh, R.N. Zadoks, J.Z. Costa, A. Jaglarz, K.D. Thompson, Development and
423 evaluation of a quantitative polymerase chain reaction for aquatic *Streptococcus agalactiae* based
424 on the *groEL* gene, Journal of Applied Microbiology 129 (1) (2020) 63–74,
425 https://doi.org/10.1111/jam.14556.
426 [31] S. Senapin, H.T. Dong, W. Meemetta, A. Siriphongphaew, W. Charoensapsri, W.
427 Santimanawong, W.A. Turner, C. Rodkhum, B. Withyachumnarnkul, R. Vanichviriyakit, *Hahella*

428 *chejuensis* is the etiological agent of a novel red egg disease in tilapia (*Oreochromis* spp.)
429 hatcheries in Thailand, Aquaculture 454 (2016) 1–7,
430 https://doi.org/10.1016/j.aquaculture.2015.12.013
431 [32] W. Meemetta, J.A. Domingos, H.T. Dong, S. Senapin, Development of a SYBR Green
432 quantitative PCR assay for detection of Lates calcarifer herpesvirus (LCHV) in farmed
433 barramundi, Journal of Virological Methods 285 (2020) 113920,
434 https://doi.org/10.1016/j.jviromet.2020.113920
435 [33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using realtime
436 quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, Methods 25 (4) (2001) 402–408.
437 https://doi.org/10.1006/meth.2001.1262.
438 [34] J.A. Knight, Free radicals, antioxidants, and the immune system, Ann. Clin. Lab. Sci. 30 (2)
439 (2000) 145–158, https://pubmed.ncbi.nlm.nih.gov/10807157/.
440 [35] D.F. Amend, D.C. Fender, Uptake of bovine serum albumin by rainbow trout from
441 hypersmotic solutions: a model for vaccinating fish, Science 192 (4241) (1976) 793–4,
442 https://doi.org/10.1126/science.1265480.
443 [36] R.L. Thune, J.A. Plumb, Evaluation of hyperosmotic infiltration for the administration of
444 antigen to channel catfish (*Ictalurus punctatus*), Aquaculture 36 (1-2) (1984) 1–8,
445 https://doi.org/10.1016/0044-8486(84)90049-8.
446 [37] J.S. Marshall, R. Warrington, W. Watson, H.L. Kim, An introduction to immunology and
447 immunopathology, Allergy Asthma Clin. Immunol. 14 (2) (2018) 1–10,
448 https://doi.org/10.1186/s13223-018-0278-1.

449 [38] G. Kato, T. Takano, T. Sakai, T. Matsuyama, C. Nakayasu, *Vibrio anguillarum* bacterin
450 uptake via the gills of Japanese flounder and subsequent immune responses, Fish Shellfish
451 Immunol. 35 (5) (2013) 1591–1597, https://doi.org/10.1016/j.fsi.2013.09.007.
452 [39] R. Korbut, F. Mehrdana, P.W. Kania, M.H. Larsen, D. Frees, I. Dalsgaard, LvG. Jørgensen,
453 Antigen uptake during different life stages of zebrafish (*Danio rerio*) using a GFP-tagged Yersinia
454 ruckeri, PloS One 11 (7) (2016) e0158968, https://doi.org/10.1371/journal.pone.0158968.
455 [40] B. Wang, P. Wang, Z.H. Wu, Y.S. Lu, Z.L. Wang, J.C. Jian, Molecular cloning and expression
456 analysis of IgD in Nile Tilapia (*Oreochromis niloticus*) in response to *Streptococcus agalactiae*
457 stimulus, Int. J. Mol. Sci. 17 (3) (2016) 348, https://doi.org/10.3390/ijms17030348.
458 [41] H.M. Munang'Andu, J. Paul, Ø. Evensen, An overview of vaccination strategies and antigen
459 delivery systems for *Streptococcus agalactiae* vaccines in Nile tilapia (*Oreochromis niloticus*),
460 Vaccines, 4 (4) (2016) 48, https://doi.org/10.1016/j.dci.2021.103993.
461 [42] N.G. Lan, K.R. Salin, S. Longyant, S. Senapin, H.T. Dong, Systemic and mucosal antibody
462 response of freshwater cultured Asian seabass (*Lates calcarifer*) to monovalent and bivalent
463 vaccines against *Streptococcus agalactiae* and *Streptococcus iniae*, Fish & Shellfish Immunology
464 108 (2021) 7–13, https://doi.org/10.1016/j.fsi.2020.11.014.
465 [43] J. Stenvik, M. Schrøder, K. Olsen, A. Zapata, T.Ø. Jørgensen, Expression of immunoglobulin
466 heavy chain transcripts (VH-families, IgM, and IgD) in head kidney and spleen of the Atlantic cod
467 (*Gadus morhua* L.), Developmental & Comparative Immunology 25 (4) (2001) 291–302,
468 https://doi.org/10.1016/s0145-305x(00)00056-2.
469 [44] M. Noia, F. Fontenla-Iglesias, A. Valle, V. Blanco-Abad, J.M. Leiro, J. Lamas,
470 Characterization of the turbot *Scophthalmus maximus* (L.) myeloperoxidase. An insight into the

471 evolution of vertebrate peroxidases, Dev. Comp. Immunol. 118 (2021) 103993,
472 https://doi.org/10.1016/j.dci.2021.103993.
473 [45] E. Wangkaghart, S. Deville, B. Wang, P. Srisapoome, T. Wang, C.J. Secombes, Immune
474 Response and Protective Efficacy of Two New Adjuvants, MontanideTM ISA 763B VG and
475 MontanideTM GEL02, Administered with a *Streptococcus agalactiae* Ghost Vaccine in Nile
476 tilapia (*Oreochromis niloticus*), Fish & Shellfish Immunology 116 (2021) 19–29,
477 https://doi.org/10.1016/j.fsi.2021.06.014.
478 [46] J. Velázquez, J. Acosta, J.M. Lugo, E. Reyes, F. Herrera, O. González, A. Morales, Y. Carpio,
479 M.P. Estrada, Discovery of immunoglobulin T in Nile tilapia (*Oreochromis niloticus*): A potential
480 molecular marker to understand mucosal immunity in this species, Developmental & Comparative
481 Immunology 88 (2018) 124–36, https://doi.org/10.1016/j.dci.2018.07.013.
482 [47] K. Shahin, A.P. Shinn, M. Metselaar, J.G. Ramirez-Paredes, S.J. Monaghan, K.D. Thompson,
483 R. Hoare, A. Adams, Efficacy of an inactivated whole-cell injection vaccine for Nile tilapia,
484 *Oreochromis niloticus* (L), against multiple isolates of *Francisella noatunensis* subsp. *orientalis*
485 from diverse geographical regions, Fish & Shellfish Immunology 89 (2019) 217–27,
486 https://doi.org/10.1016/j.fsi.2019.03.071.
487
488

Tables and Figures

Table 1: Primers used in this study

Primers	Oligo sequence (5′ - 3′)	Product size (bp)	Annealing temperature (°C)	Reference
IgM	F: GGATGACGAGGAAGCAGACT R: CATCATCCCTTTGCCACTGG	122	59	[45]
IgD	F: AACACCACCCTGTCCCTGAAT R: GGGTGAAAACCACATTCCAGC	127	61	[40]
IgT	F: TGACCAGAAATGGCGAAGTATG R: GTTACAGTCACATTCTCTGGAATTACC	128	56	[46]
β-actin	F: CCACACAGTGCCCATACTACGA R: CCACGCTCTGTCAGGATCTTCA	144	60	[47]

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

493 **Table 2**: Quantification of *Streptococcus agalactiae* DNA on gill tissues by quantitative
494 polymerase chain reaction (qPCR)

Samples	3 h post-vaccination		6 h post-vaccination	
	Mean C_q	Bacterial loads/1µg DNA template	Mean C_q	Bacterial loads/1µg DNA template
NB-O_3 and vaccine groups				
NB-O_3-VAC-1	32.55	5445.77	32.07	7419.79
NB-O_3-VAC-2	36.74	-	33.83	2393.43
NB-O_3-VAC-3	35.82	664.35	31.56	10270.22
NB-O_3-VAC-4	35.82	664.35	32.82	4550.15
NB-O_3-VAC-5	35.34	905.39	32.83	4531.08
NB-O_3-VAC-6	33.77	2482.13	32.73	4852.34
Mean		2032.40 ± 2053.45		5669.50 ± 2763.31
AT and vaccine groups				
AT-VAC-1	37.13	-	33.20	3570.26
AT-VAC-2	36.26	-	33.21	3559.03
AT-VAC-3	34.30	1765.36	33.33	3303.40
AT-VAC-4	35.54	793.42	34.77	1304.34
AT-VAC-5	33.97	2178.44	33.21	3559.03
AT-VAC-6	34.64	1421.23	33.01	4047.45
Mean		1539.61 ± 585.9		3223.92 ± 970.96

495 *"-", under the detection limit*

496

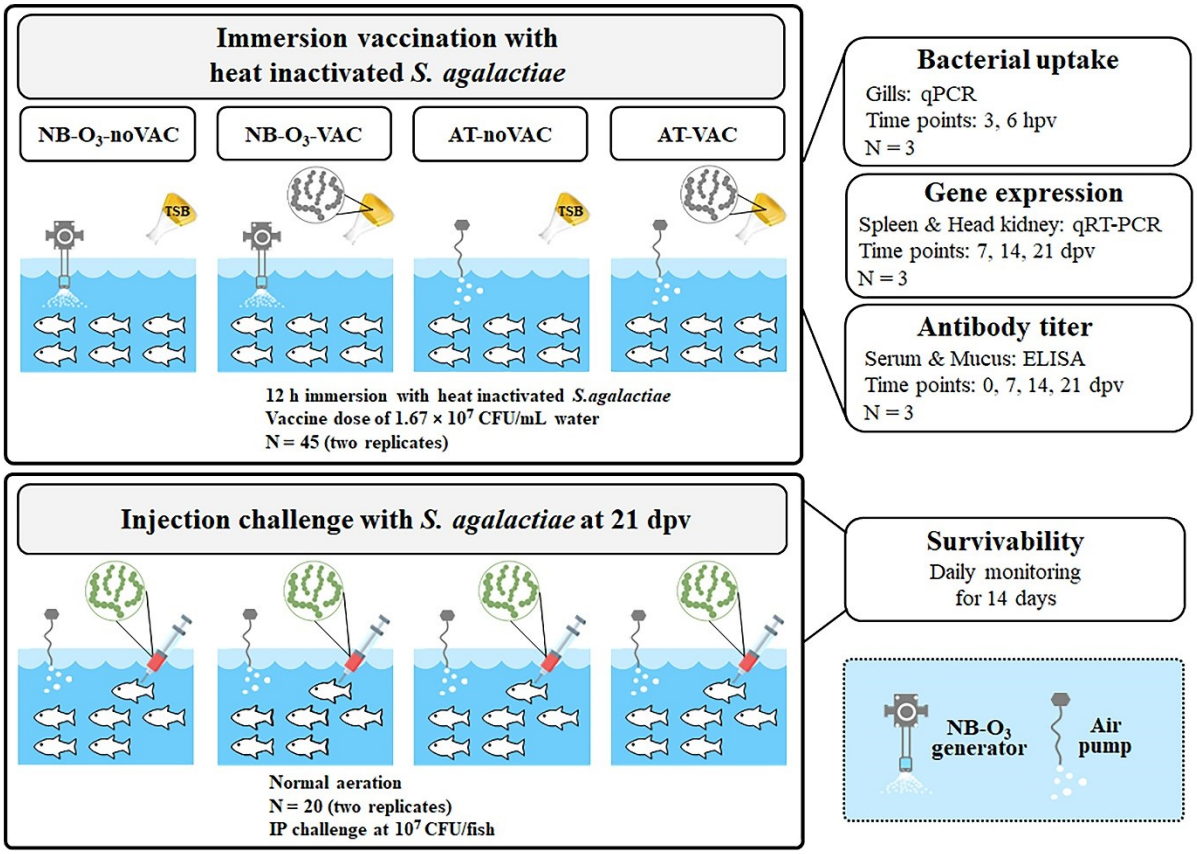


Fig. 1. Schematic diagram of experimental design illustrating before and after immersion vaccination, and Nile tilapia species used. AT-noVAC, air-stone with no vaccine; NB-O_3-noVAC, ozone nanobubbles without vaccine; AT-VAC, air-stone with vaccine; NB-O_3-VAC, ozone nanobubbles with vaccine. TSB: Tryptic Soy Broth media.

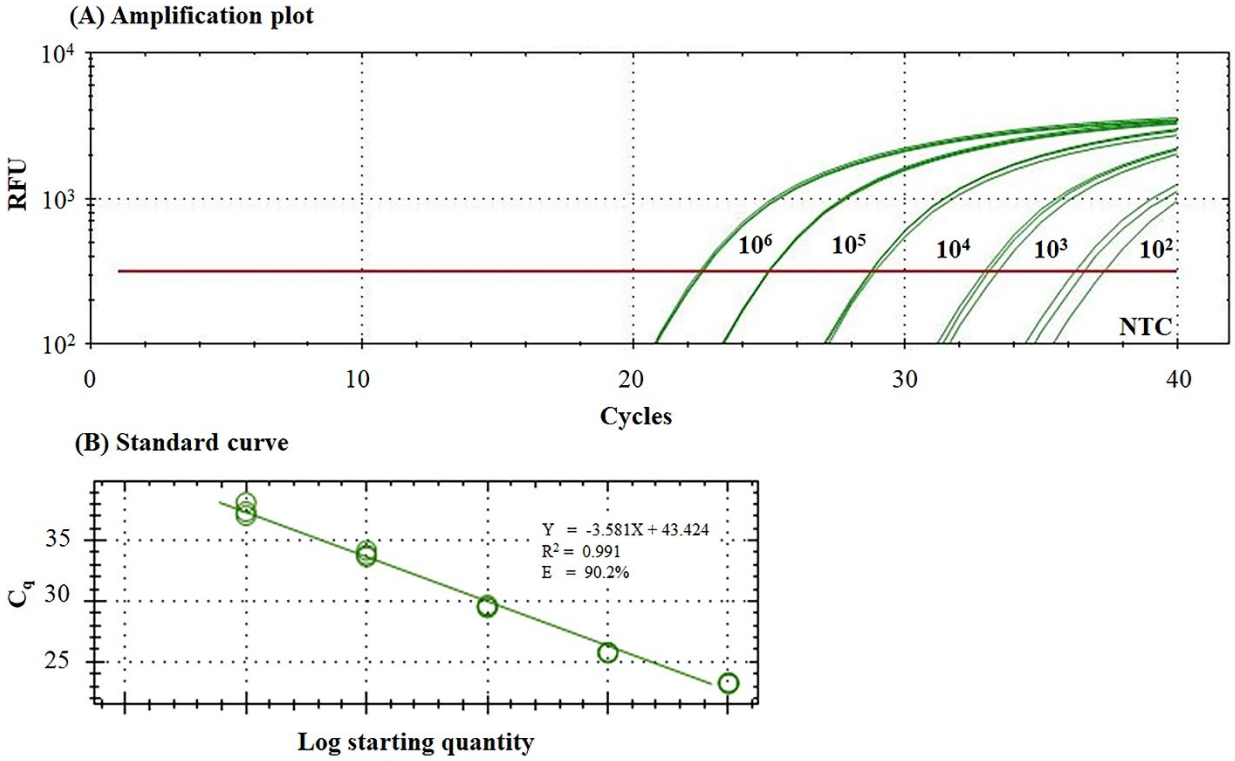
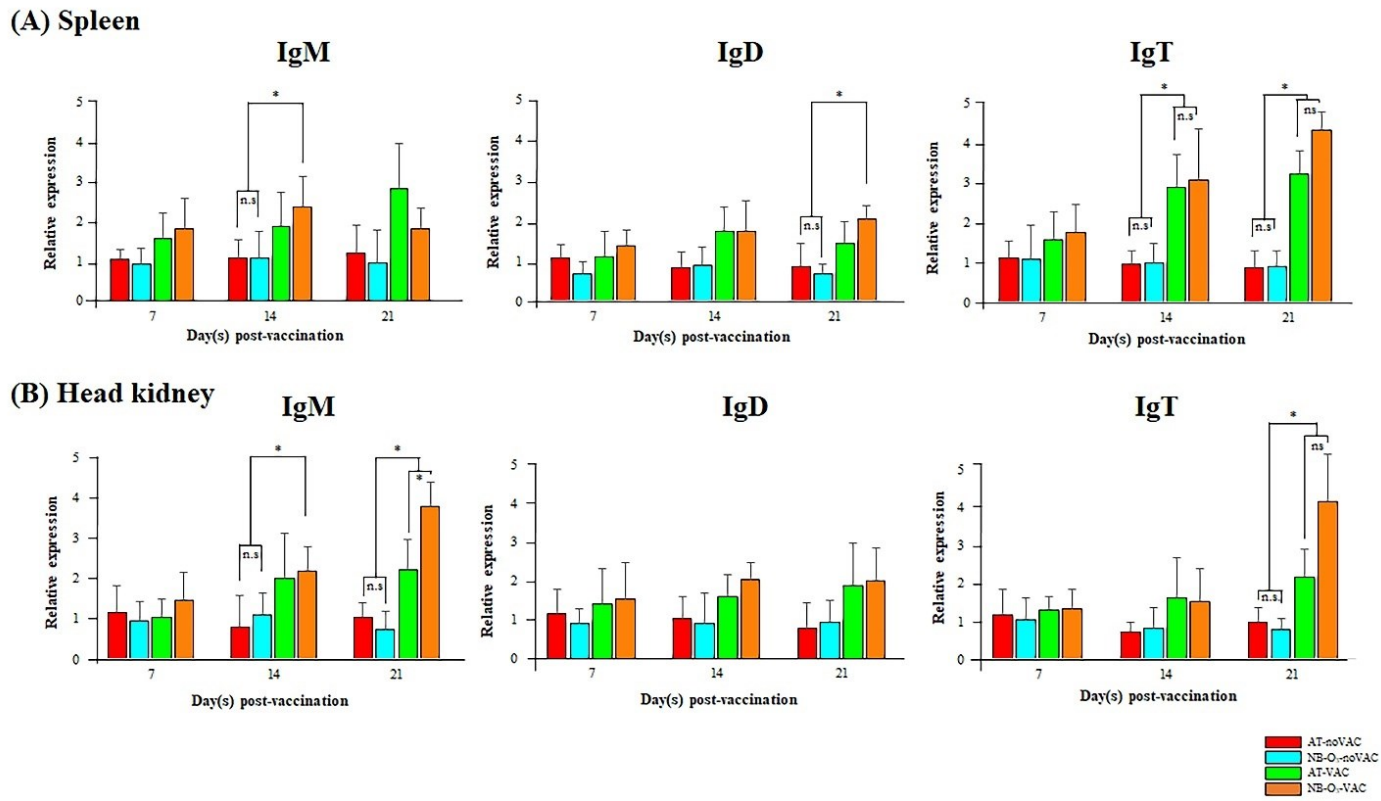


Fig. 2. Detection of *Streptococcus agalactiae* using quantitative polymerase chain reaction (qPCR). (A) Amplification plots of positive control plasmid pSNB1 serial dilutions from 10^6 to 10^2 copies with 200 ng spiked fish DNA in each reaction. Three technical duplicates are included for each dilution. (B) A standard curve is constructed by plotting C_q values versus $\log_{10}$ concentrations. Formula for calculating copy number, R^2, and E value are presented in the box.



509

510 **Fig. 3.** Comparative *IgM*, *IgD,* and *IgT* expression levels in the spleen and head kidney of the
511 control and treated fish (n = 6) on days 7, 14, and 21 after immunization. The expression levels of
512 three immunoglobulin genes were normalized by *β-actin*. Transcript levels of AT-noVAC groups
513 at day 7 were set as 1. Error bar indicates standard deviation; "*" indicates a statistical significance
514 ($P < 0.05$).

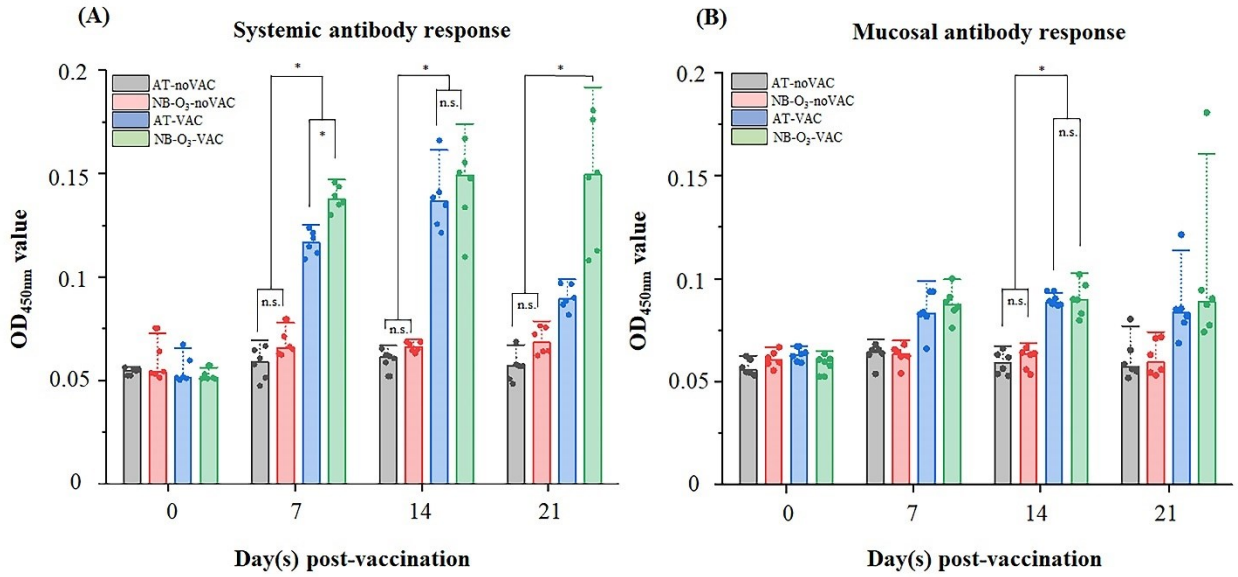
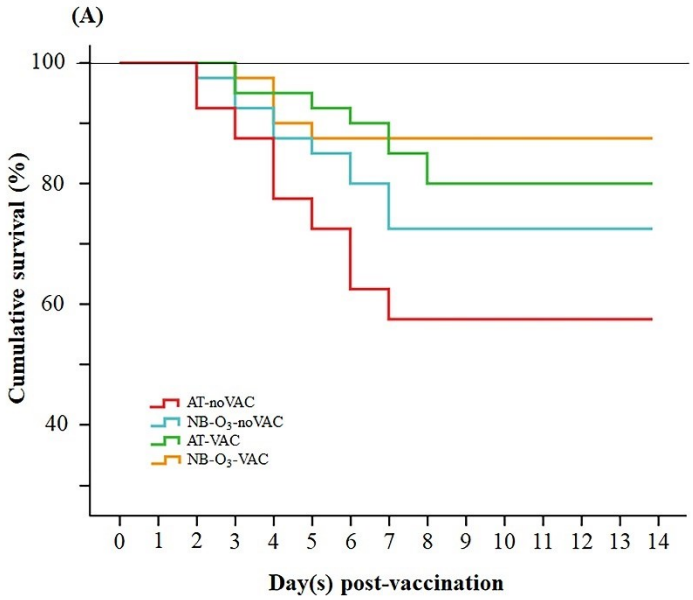


Fig. 4. Specific antibody titer in the serum (A) and mucus (B) of Nile tilapia on days 0, 7, 14, and 21 of the immersion vaccination trials determined by ELISA. Serum and mucus antibody titers were determined using 1:512 and 1:16 dilutions, respectively. The optical density (OD) values were determined at 450 nm. Data are shown in mean ± standard deviation (SD), with each dot representing one biological replicate (n = 6). Statistical significance was determined by Kruskal–Wallis test. "*" indicates a statistical significance ($P < 0.05$), whereas "ns" indicates non-significant.



(B)

Groups	Significance		
	NB-O_3-VAC	**NB-O_3-noVAC**	**AT-VAC**
NB-O_3-noVAC	0.106^{ns}		
AT-VAC	0.408^{ns}	0.383^{ns}	
AT-noVAC	0.003^{*}	0.141^{ns}	0.02^{*}

Fig. 5. Kaplan–Meier analysis of (**A**) the cumulative survival of Nile tilapia (n = 40) challenged with *S. agalactiae* at day 21 post-vaccination. AT-noVAC, air-stone with no vaccine; NB-O_3-noVAC, ozone nanobubbles without vaccine; AT-VAC, air-stone with vaccine; NB-O_3-VAC, ozone nanobubbles with vaccine. The average cumulative survival of two trials is shown in the data. (**B**) The log-rank test was used to assess differences between the study treatments. "*" indicates a statistical significance ($P < 0.05$), whereas "ns" indicates non-significant.