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Dietary chitosan-selenium nanoparticle (CTS-SeNP) enhance immunity and disease resistance in zebrafish

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ABSTRACT

Selenium (Se) is an essential micronutrient for human and animals. It plays an important role in antioxidative stress, selenoenzymes regulation and immunomodulation. In this study, two common immunostimulants chitosan (CTS) and Se were used to synthesize nanoparticles (CTS-SeNP). Immunomodulation of CTS-SeNP were explored in wild-type zebrafish (*Danio rerio*). Dietary supplementation of CTS-SeNP enhanced lysozyme activity, phagocytic respiratory burst as well as splenocytes proliferation stimulated by LPS and ConA. CTS-SeNP showed immunomodulation effect from 5 to 20 μg/g but the best outcome was observed at 10 μg/g. Immunomodulation effect were rapidly induced after 3-9d and can sustain to 60. The zebrafish fed with 10 μg/g CTS-SeNP also showed 26.7% higher survival rate than the control after intraperitoneal injection of common bacterium *Aeromonas hydrophila*. Our results suggested that CTS-SeNP is an effective immunostimulant to fish and has potential application in aquaculture.

1. Introduction

Selenium (Se) is an essential micronutrient for human and animal health, and its biological functions are mainly exhibited through selenoproteins [1]. To date, 30 selenoproteins have been identified in humans, and many of them play an important role in redox balance and immunomodulation [2]. Interestingly, supranutritional Se levels are often associated with other health benefits such as reduced cancer risk and inflammatory diseases [1]. Immunomodulation of Se has been studied to assess potential health applications for both human and other animals [2,3]. For example, researchers found that Se supplementation in polio patients increased T-lymphocyte proliferation and produced more T-helper cells [4]. In studies of thyroid disease, Se supplementation directly increased the number of T-lymphocytes [5] and reduced pro-inflammatory gene expression [6].

Different chemical forms of Se have different bioavailability and toxicity [7]. Se nanoparticles (SeNP) exhibit higher bioavailability, lower toxicity and strong bioactivity in comparison with inorganic and organic Se [8,9]. Furthermore, combining a functional polysaccharide with SeNP often result in enhanced biological activities [10,11]. For example, SeNP surface stabilized with polysaccharide from *Polyporus rhinoceros* effectively induced apoptosis and cell cycle arrest in A549 human lung adenocarcinoma cells [12]. SeNP combined with polysaccharide from *Ulva lactuca* exhibited anti-inflammatory activity and attenuated colitis in mice [13]. Therefore, a combination of SeNP with immune-active polysaccharides such as chitosan (CTS) may result in stronger SeNP immunomodulation function. CTS is a linear polysaccharide, produced through deacetylation of the chitin shells of shrimp and other crustaceans. It is commonly used as an immunostimulant for agricultural and biomedical purposes [14,15]. Recently, chitosan based SeNP (CTS-SeNP) were successfully synthesized and its anticancer function and antioxidant capacities were studied [16,17]. However, research is required to study the immunomodulation function of CTS-SeNP.

This study explores the potential of CTS-SeNP as an immunostimulant for fish. CTS-SeNP were synthesized and mixed into a commercial fish feed at various concentrations. Zebrafish were fed with these feeds and their innate immune responses were studied at different timepoints. Finally, zebrafish were challenged with a fish pathogen *Aeromonas hydrophila* to understand the overall immune performance. *A. hydrophila* is a common pathogen in warm water aquaculture in Asia and is associated with many diseased widespread in carps [18].

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2. Materials and methods

2.1. Fish maintenance

Adult zebrafish (*Danio rerio*) (9–12 months) were maintained in 15-L tanks in a flow-through system at a temperature of 28 ± 1 °C and pH 7 under a 14:10 light-dark cycle. Zebrafish is a widely used model organism in biomedical and immunological studies. The immune system of zebrafish is highly similar to humans and other vertebrates [19]. Daily care and experiments were carried out under the approved animal care and maintenance protocols of the institutes. The fish were fed with commercial fish feed (Otohime B1, USA) three times per day and brine shrimp nauplii once per day.

2.2. Selenium nanoparticle synthesis and characterization

Chitosan stabilized selenium nanoparticles (CTS-SeNP) were synthesized using controllable reduction methods as described in Shi et al. [7]. Briefly, aqueous chitosan solution (0.25%) was mixed with freshly prepared ascorbic acid solution (100 mM) with magnetic stirring. Aqueous sodium selenite solution (25 mM) was dropwise added to the mixture in the dark. The mixture was reconstituted to 25 mL by MilliQ water and allowed to react at room temperature for around 12 h before extensive dialysis (Mw cut off: 8000). The characteristics of CTS-SeNP were analyzed by transmission electron microscopy (TEM; JEOL 2010 + Horiba EX-250, USA) and NanoSight NS300 (Malvern Instruments Limited, USA) for particle size distribution. In TEM, elemental composition of the SeNP was characterized by Energy Dispersive X-ray Spectroscopy (EDX). Nanoparticle size distribution were measured by taking average of 3 measurements in NanoSight. ICP-MS (Agilent 7500) was used to determined total Se concentration of this CTS-SeNP stock.

2.3. CTS-SeNP diet preparation

Different amounts of CTS-SeNP were added to the base dry diet (Otohime B1, USA) to prepared low (5 μg/g), medium (10 μg/g) and high (20 μg/g) CTS-SeNP diets using the method described in Shi et al. [7]. Briefly, the appropriate amount of CTS-SeNP was diluted with MilliQ water (Millipore, USA) to make a 10 mL solution. The suspension was thoroughly mixed with 10 g of dry food in a Petri dish to ensure the liquid was evenly distributed and well incorporated. The mixture was then freeze-dried overnight and then broken apart gently by passing through a 100 μm sieve to ensure the particle size was suitable for zebrafish. In addition, one control diet (with the addition of just MilliQ water) and two ingredient control diets were made using the same protocol. CTS ingredient diet (containing 0.025 μg/g chitosan only) was prepared using the same chitosan solution (0.25%) as in nanoparticle synthesis. Sodium selenite ingredient diet (containing 10 μg/g Se only) was prepared using sodium selenite solution (25 mM) as in nanoparticle synthesis. All diets were stored in 50 mL centrifuge tubes at 4 °C until the experiment. CTS-SeNP diets were also analyzed by TEM and EDX. Total Se concentration of the base diet and all experimental diets were determined by ICP-MS.

2.4. Exposure experiments

2.4.1. Effective dose of CTS-SeNP

Zebrafish were fed with different dosage of CTS-SeNP diet (5, 10 and 20 μg/g Se) at a ration of 2% body weight with a feeding frequency of three time per day for 9d. For each concentration there were 3 replicates of 9 fish. Effects from the two CTS-SeNP ingredients were tested simultaneously using CTS diet and sodium selenite diet. The serum, kidney and spleen from 9 individual zebrafish were collected and pooled separately as an experimental sample and triplicate measurements were carried out for all of the endpoints mentioned Section 2.5.

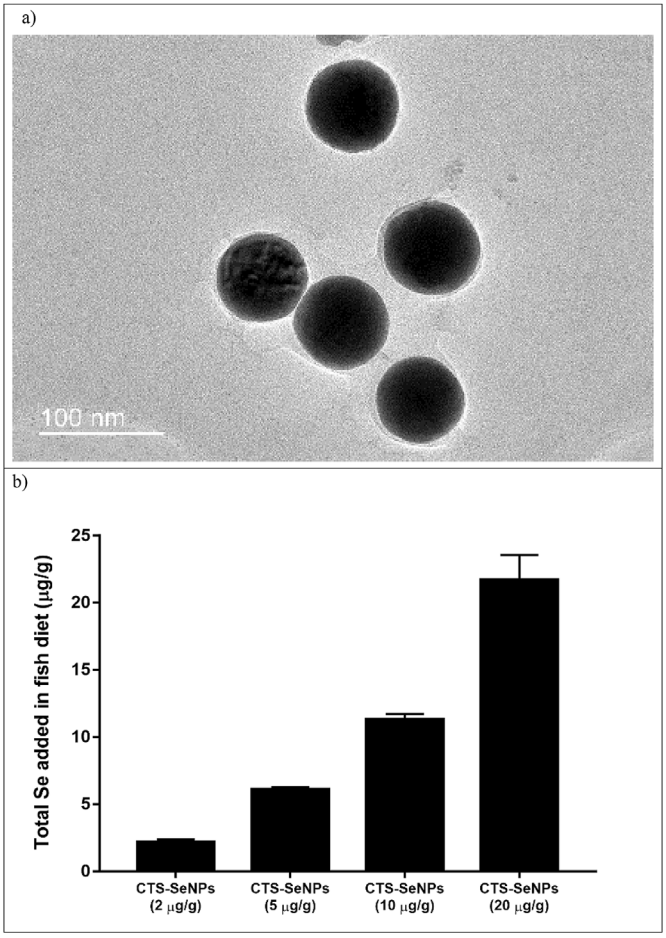

Fig. 1. Chitosan stabilized selenium nanoparticles (CTS-SeNP): a) representative TEM image; b) measured concentration versus nominal concentration of CTS-SeNP fish diets as determined by ICP-MS.

2.4.2. Effects of CTS-SeNP over time

To understand the effects of CTS-SeNP over time, the most effective dose of CTS-SeNP identified in the experiment in Section 2.4.1 was fed to zebrafish at the same ration and frequency as previously described. Immune responses from innate immune parameters were studied at d3, d6, d9, d12 and d60. For each time point, there were 27 fish used. The serum, kidney and spleen from 9 individual zebrafish were collected and pooled separately as an experimental sample and triplicate measurements were carried out for all of the endpoints mentioned Section 2.5.

2.5. Immune response analyses

2.5.1. Zebrafish organs collection and immune cell isolation

The collection of zebrafish immune organs and recovery of immune cells followed protocols published in previous studies [20,21]. Briefly, zebrafish were euthanized by MS-222 (ethyl 3-aminobenzoate methane sulfonate, Sigma, USA) 1.0 mg/mL for 2 min. Zebrafish blood was collected using a gentle centrifugation approach [22]. Firstly, the caudal peduncle of zebrafish was severed with a sharp scalpel (Swann Morton, UK). With the wound pointing down, fish were individually put into a 0.5 mL micro-centrifuge tube with a small hole at the end. The 0.5 micro-centrifuge tube was then placed inside a 1.5 mL micro-centrifuge tube (Eppendorf, Germany) and the assembly was centrifuged at 700g for 5 min at 11 °C. Blood from 9 individuals was pooled into a 1.5 mL centrifuge tube and allowed to clot on ice for 30 min. Serum was separated with the cells by centrifugation (1200g, 10 min, 4 °C) and

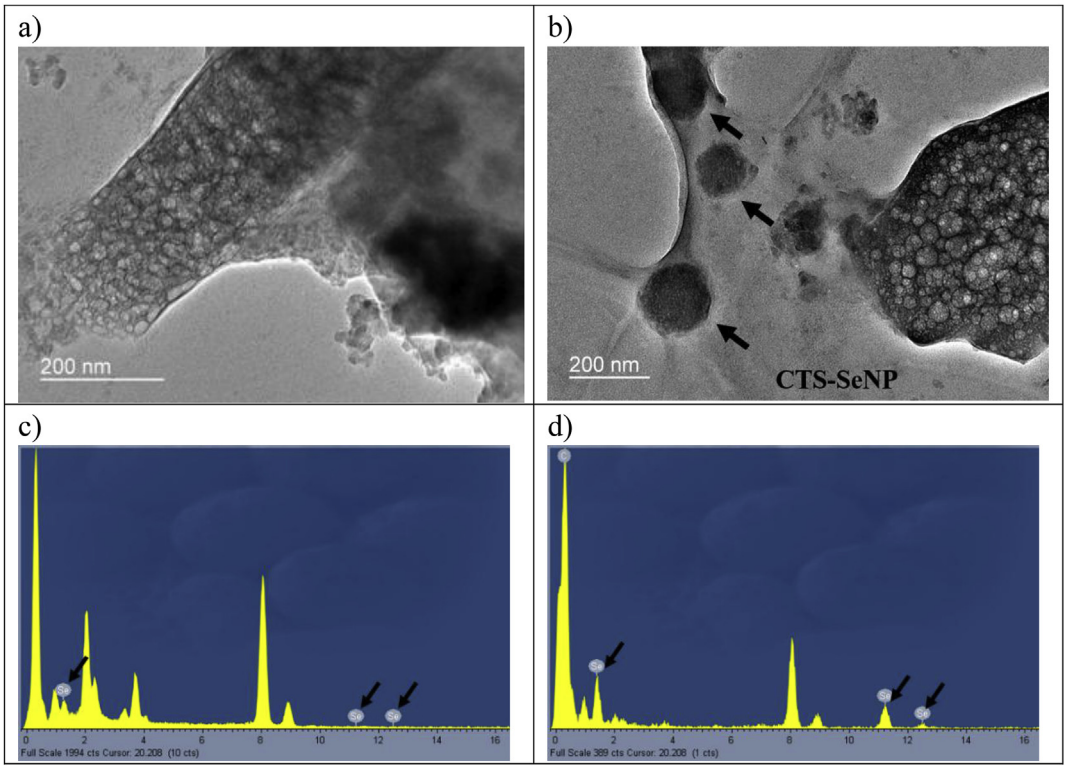

Fig. 2. Representative TEM images of zebrafish pellet diet a) control; b) after addition of CTS-SeNP (black arrows); c) corresponding EDX spectrum of control diet; d) corresponding EDX spectrum of CTS-SeNP diet (arrows indicate spectra for Se element).

stored at −80 °C.

Kidneys and spleens were removed and pooled from 9 individuals and placed separately in 1 mL of phosphate buffered saline (PBS). Single cell suspension were recovered from pooled tissues by using Micro-pestle homogenizer (Sigma-Aldrich, USA). To remove red blood cells and tissue debris, these homogenates were then passed through a sterile syringe loosely packed with glass wool. The cell suspensions were centrifuged at 700g for 5 min at 4 °C. The kidney cells were re-suspended in PBS for phagocytic respiratory burst assays. The cell pellet from spleen was resuspended in L-15 media (Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin/streptomycin (Sigma, USA), 0.5% L-glutamine (Sigma, USA), and 15 mM HEPES buffer (Sigma, USA) for splenocytes proliferation assay. Cell numbers/viability was determined by hemocytometer and trypan blue.

2.5.2. Lysozyme activity assay

Lysozyme is an antimicrobial enzyme abundant in host's serum and its activity is an important part of the humoral immunity and the innate immune system [23]. Serum lysozyme activity was determined using the EnzChek Lysozyme Assay Kit (Molecular Probes, USA). DQ lysozyme substrate (fluorescein labeled *Micrococcus lysodeikticus*) stock suspension (1.0 mg/mL) and 1000 U/mL lysozyme stock solution were prepared according to the manufacturer's instruction. 10 µL serum was diluted with 40 µL reaction buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5) and incubated with 50 µL DQ lysozyme substrate for 30 min at 28 °C in the dark. The fluorescence was measured in a fluorescence microplate reader using absorption wavelengths of 494 nm and fluorescence emission wavelengths of 518 nm. Background fluorescence was corrected by subtracting the value from no-enzyme control. Serum lysozyme activity was calculated from a standard curve prepared with lysozyme from chicken egg white.

2.5.3. Phagocytic respiratory burst assay

Intracellular and extracellular respiratory burst activity are important for phagocytosis and digestion of foreign pathogens. Superoxide production in kidney cells was measured with and without addition of a stimulating agent, phorbol 12-myristate 13-acetate (PMA, Sigma, USA). Intracellular and extracellular superoxide production were measured as a change in absorbance resulting from reduction of Cytochrome C and oxidation of nitroblue tetrazolium (NBT) as described from previous studies [24]. For the extracellular superoxide production, 5×10^5 kidney cells in 50 µL supplemented L-15 medium and 100 µL cytochrome C (4 mg/mL were added to wells of a 96 wells plate. In half of the wells, 0.5 µg/mL PMA was added. Superoxide production burst was measured with and without addition of 37.5 µg/mL superoxide dismutase (SOD). The plate was incubated at 28 °C for 60 min then was measure spectrophotometrically at 550 nm.

For intracellular superoxide production, 5×10^5 kidney cells were allowed to attach in wells of 96-well plate for 90 min. After attachment of cells, medium was removed, and the removed medium should contain 0.25×10^6 cell/mL. 1 mg/mL NBT (Sigma, USA) with or without SOD and PMA were added into four different wells. The plate was then incubated in 28 °C for 60 min. The formazan produced by NBT reduction in attached kidney cells was dissolved by DMSO and 2 M KOH (Sigma, USA) and measured at a wavelength of 620 nm. To calculated both PMA-stimulated and non-PMA-stimulated superoxide production in both assay, the absorbance of wells with SOD were subtract the one without SOD. Total nmol of superoxide anion were calculated by multiplying final OD values by 15.87 [25]. The final extracellular and intracellular superoxide production were expressed as nmoles of $\cdot O_2^-$ produced/5×10^5 cells/60 min.

2.5.4. Splenocytes proliferation assay

For cellular immune responses, splenocyte-responses towards lipopolysaccharide (LPS) or concanavalin A (ConA) was studied according to previous lymphocyte proliferation assay [26–28]. Proliferation of

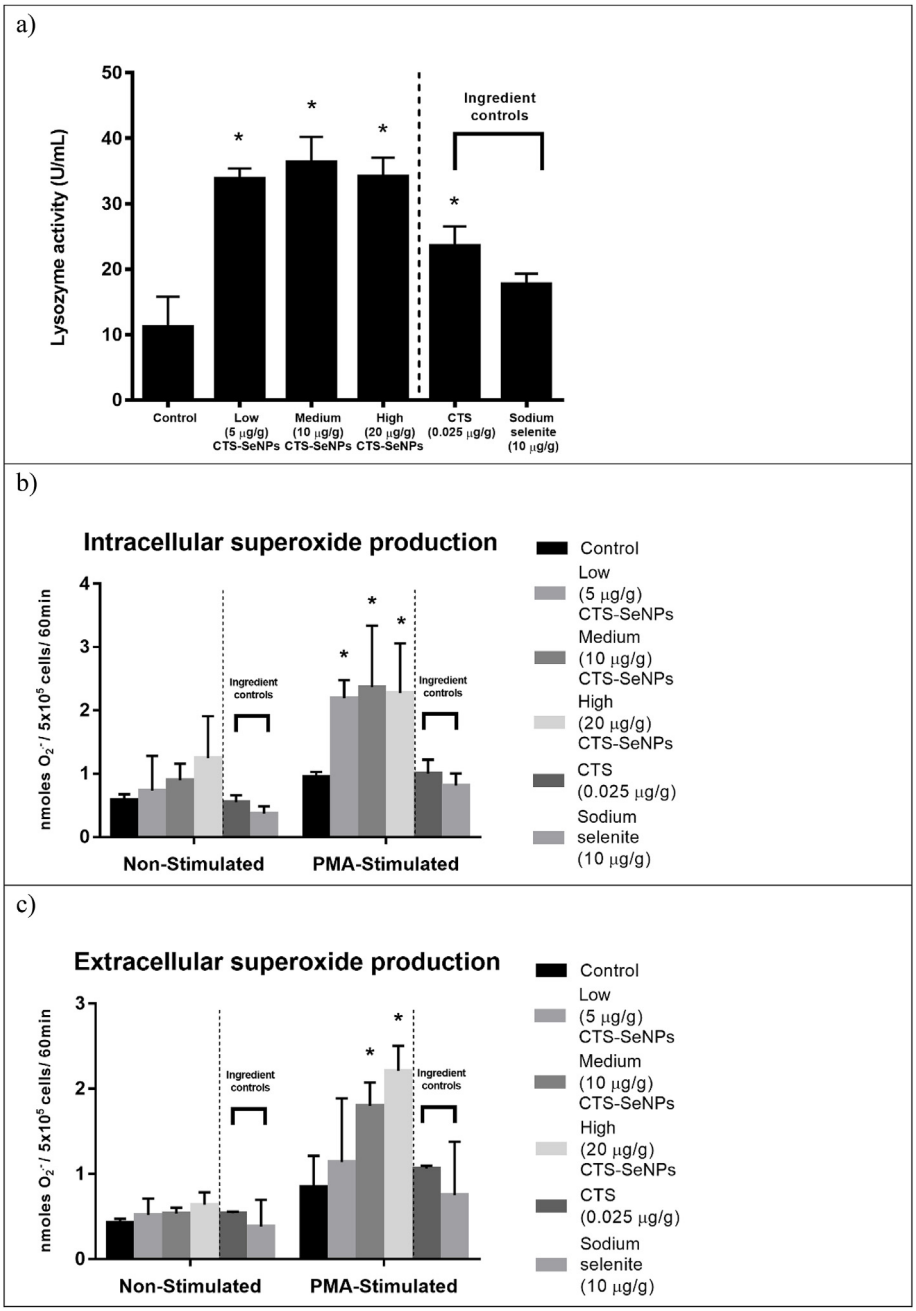

Fig. 3. Innate immune responses in zebrafish after 9d exposure to different concentration of CTS-SeNP and ingredient controls: a) lysozyme activity in zebrafish serum; b) intracellular respiratory burst activity; c) extracellular respiratory burst activity. Statistical significant difference from control was represented by asterisks.

splenocytes in zebrafish was determined by a microtiter assay with modification by using a Vybrant MTT Cell Proliferation Assay Kit (invitrogen, USA). Briefly, splenocytes (5×10^6 cells/well) in 100 μL L-15 medium were added wells of a 96 wells plate. Proliferation of splenocytes was determined in response to 100 μg/well of LPS (Sigma, USA) and 100 μg/well of ConA (Sigma, USA) respectively. After 96-h incubation at 28 °C, 10 μL 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) was added to each well for another 4-h incubation at 28 °C. The reaction was ceased by adding 100 μL sodium dodecyl sulphate (SDS, Sigma, USA) solution (1 mg SDS in 10 mL of 0.01 M HCl) to each well. After incubation at room temperate for 10 min, the mixture containing soluble formazan crystals were read absorbance at 570 nm by using CLARIOstar Microplate Reader (BMG Labtech, Germany). Proliferation was calculated as the change in absorbance at 570 nm.

2.6. Bacterial culture and challenge

Overall immunity of zebrafish by CTS-SeNP was studied under bacterial challenge. After feeding on 10 μg/g CTS-SeNP, 0.025 μg/g CTS or 10 μg/g sodium selenite for 9 days, zebrafish were challenged by a common freshwater fish bacterium, *Aeromonas hydrophila* (ATCC 7699, USA) for 72 h. Using the optimal treatment time and the most effective concentration of CTS-SeNP diet determined as described in section 2.4, a total 120 zebrafish were used in a bacterial challenge study. After 9 days of feeding, zebrafish on control and CTS-SeNP treatment diets were intraperitoneal (*ip*) injected with 2.5×10^6 cfu of *A. hydrophila* in 10 μL PBS. This bacterial concentration for zebrafish infection was well established in our laboratory and can induce mortality of ~80% in 72 h. A negative control was conducted with another 30 zebrafish individuals by *ip* injection of 10 μL PBS only. All zebrafish were

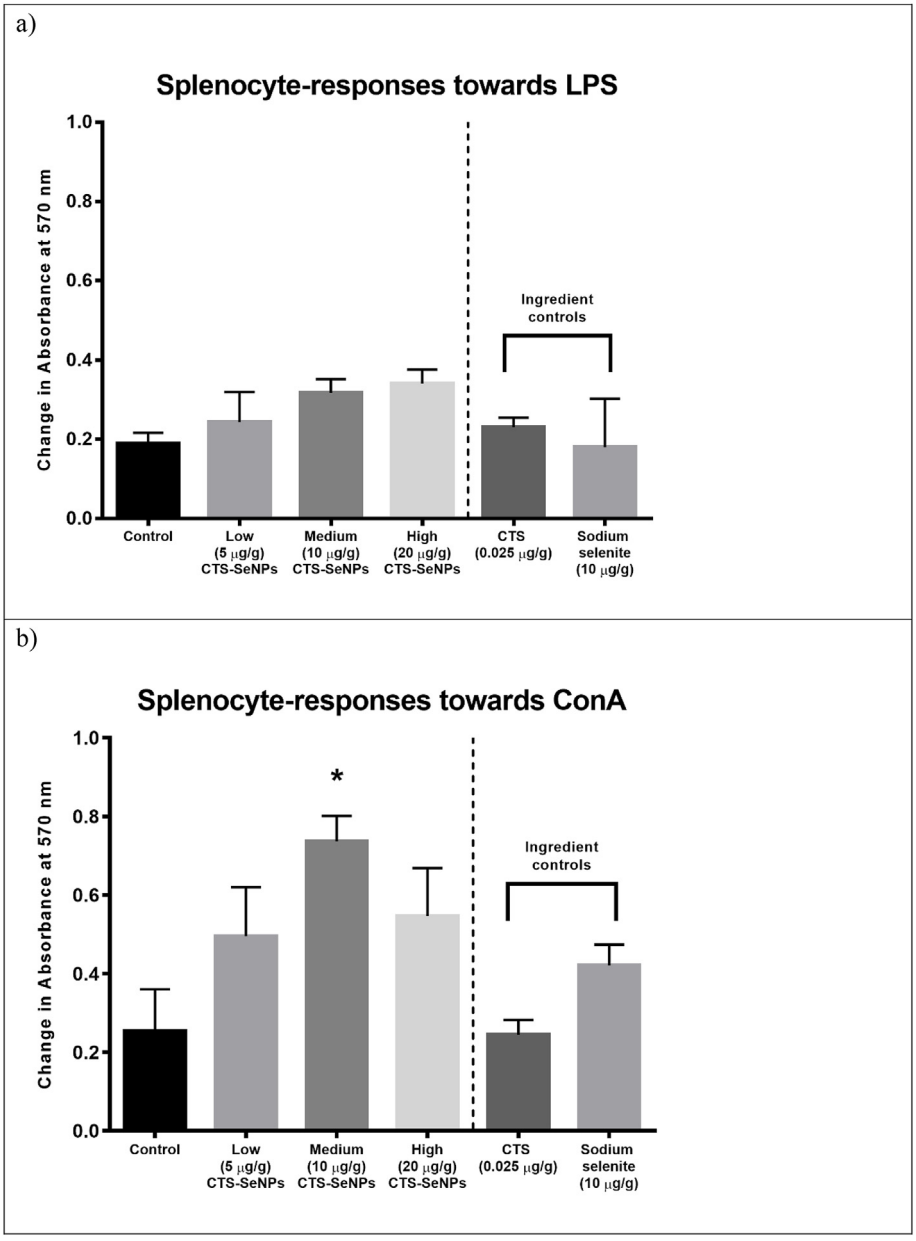

Fig. 4. Lymphocyte proliferation assay in zebrafish splenocytes after 9d at different concentrations of CTS-SeNP and ingredient controls: a) splenocyte-responses towards LPS and b) splenocyte-responses towards ConA. Statistical significant difference from control was represented by asterisks.

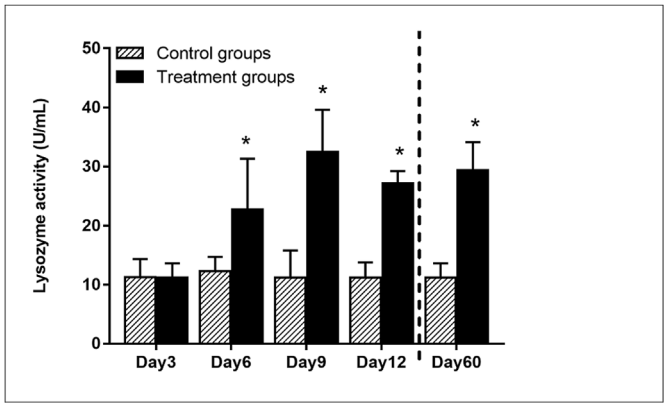

Fig. 5. Lysozyme activity in zebrafish serum at different time points after dietary exposure to 10 μg/g CTS-SeNP. Statistical significant difference from control was represented by asterisks.

maintained at 28 ± 1 °C after injection and was observed for 72 h. Survival rate of fish in each group was monitored every 8 h.

2.7. Statistical analysis

Data were presented as mean ± SD and considered to be significantly different at $P \leq 0.05$ level. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism ver 6.00 (GraphPad Software, USA). Survival rate data of the bacteria challenge experiment were compared using Kaplan-Meier analysis coupled with log-rank test (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests in SPSS (ver 15.0, IBM SPSS Statistics, USA).

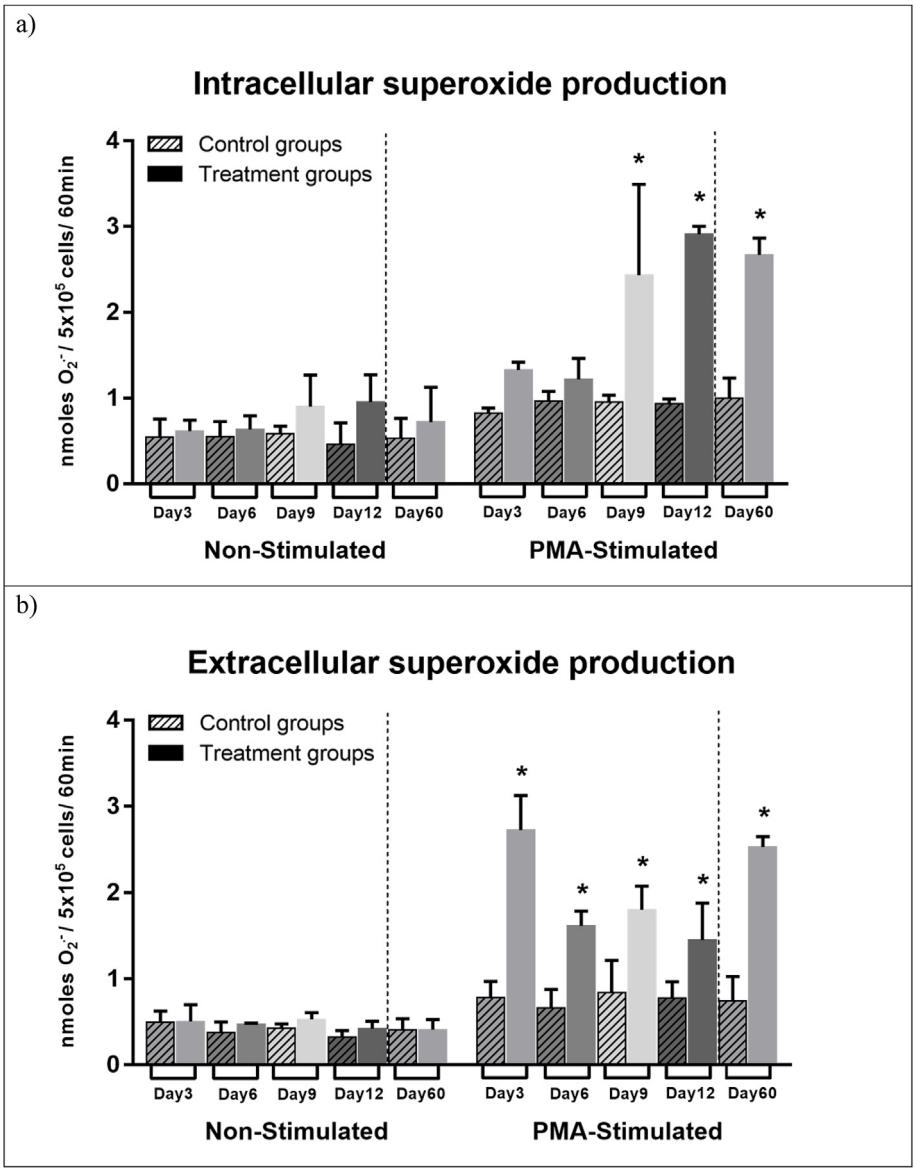

Fig. 6. Phagocytic respiratory burst assay in zebrafish kidney cell at different time points after dietary exposure to 10 μg/g CTS-SeNP: a) intracellular respiratory burst activity and b) extracellular respiratory burst activity. Statistical significant difference from control was represented by asterisks.

3. Results

3.1. Characterization of CTS-SeNP and CTS-SeNP diets

As shown in Fig. 1.a, CTS-SeNP were largely spherical with a homogeneous structure. The average diameter was 72.1 nm (SD = 2.27) under TEM. NanoSight showed average particle diameter of CTS-SeNP to be 117 nm (SD = 32 nm). TEM image showed that the distance between the two layers of Se atoms in the NP was 0.31 nm (SD = 0.06 nm) with a regular crystalline structure. Using EDX, CTS-SeNP was shown to contain 82.7% Se and some carbon (17.3%), which was likely from the CTS surface stabilizer.

Added Se concentrations in CTS-SeNP fish feeds were determined by using ICP-MS (Fig. 1b) and the measured concentration well matched the nominal concentration. The original Se concentration in base fish feed is 3.6 μg/g (sodium selenite), which is similar to the value reported by earlier studies using the same base fish feed [7,29]. CTS-SeNP was attached to fish feed particulates and these NP-like structures were not observed in the control diet (black arrows, Fig. 2a and b). Elemental composition analysis by EDX indicated that Se concentration in CTS-SeNP diet was higher than control diet (Fig. 2c and d).

3.2. Effective concentration of CTS-SeNP

Lysozyme activity increased by approximately 3-fold compared to the control for all concentrations of CTS-SeNP (Fig. 3a). CTS also led to significant increase in lysozyme activity but sodium selenite diet had no significant effect. Intracellular and extracellular respiratory burst also improved under multiple concentrations of CTS-SeNP. Without PMA, both intracellular and extracellular superoxide production were slightly increased at the 20 μg/g but the change was not statistically significant (Fig. 3b and c). Intracellular respiratory burst after PMA stimulation showed more pronounced increase in all three concentrations while only 10 and 20 μg/g have significant effect on extracellular respiratory burst. Ingredient controls had no significant effect on both extracellular and intracellular superoxide production.

For cellular immunity, splenocyte-responses towards LPS and ConA again responded differently under the three CTS-SeNP concentrations (Fig. 4). Splenocyte-responses towards LPS were not impacted significantly by CTS-SeNP while splenocyte-responses towards ConA were

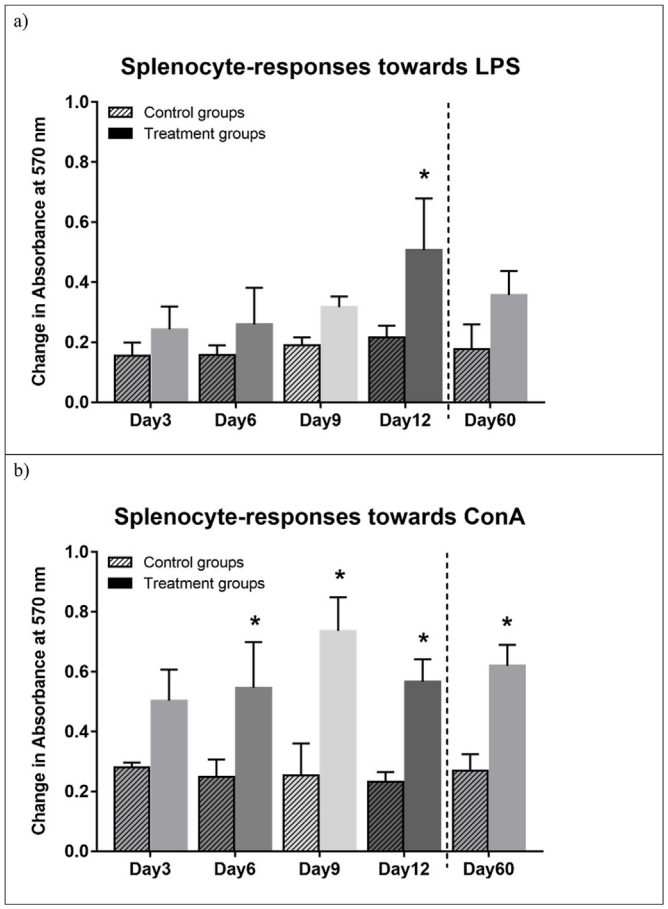

Fig. 7. Lymphocyte proliferation assay in zebrafish splenocytes after at different time points after dietary exposure to 10 μg/g CTS-SeNP: a) splenocyte-responses towards LPS and b) splenocyte-responses towards ConA. Statistical significant difference from control was represented by asterisks.

significantly increased only at 10 μg/g. Ingredient controls had no significant effect in both cases. In summary, 10 μg/g was the optimal dose of CTS-SeNP and this dose was used to study the effects of CTS-SeNP over time.

3.3. Effects of CTS-SeNP over time

Lysozyme activity was found to significantly increase after 6 days feeding on CTS-SeNP and reached a peak of 32 U/mL at d9 (Fig. 5). Lysozyme activity remained high even at d60 at 29 U/mL.

Without PMA stimulation, there was no difference between CTS-SeNP and control for both intracellular and extracellular responses. When PMA was present, both control and treatment groups were able to respond by producing superoxide anion intracellularly (Fig. 6a) and extracellularly (Fig. 6b). CTS-SeNP treated groups produced significantly higher response in extracellular superoxide production just after 3d of CTS-SeNP treatment and the increase was sustained even after 60d. For intracellular oxidative burst, a significant increase in response in the CTS-SeNP treatment was observed after 9d and again the increase was sustained even after 60d.

LPS was used to stimulate splenocytes proliferation. There was a general trend of increased proliferation in the CTS-SeNP treatment group and reached the highest at 12d with a significant 2.6-fold increase but there was no significant effect after 60d (Fig. 7a). On the contrary, splenocyte-responses towards ConA was significantly increased just after 6d and the increase was sustained through 60d (Fig. 7b). Splenocyte-responses towards ConA reacted highest at 9d with a 2.9-fold increase comparing to control group. 10 μg/g CTS-SeNP have led to significant increase in lysozyme activity, phagocytosis and splenocyte-responses towards ConA proliferation after a minimum treatment time of 3-9d.

3.4. Survival rate of zebrafish after bacterial challenge

There was no mortality in the PBS injected fish throughout the experiment. Zebrafish injected with bacteria developed phenotype of hemorrhagic septicemia and abdominal swelling (Fig. 8a), which are typical symptoms of *A. hydrophila* infection [30,31]. In our study, infected zebrafish from control diet group were the first to display symptoms and mortality was recorded after just 4 h. After 24 h, survival rate of the group dropped to 26.7%, significantly lower than infected fish of the CTS-SeNP diet group which has a survival rate of 66.7% (Fig. 8b). After 72 h, survival rate in control diet group (20%) were still significantly lower than the CTS-SeNP diet group (46.7%). Meanwhile, CTS diet group had effects similar to the control diet group. Survival rate in sodium selenite diet group (16.7%) was even lower than the control.

4. Discussion

In this study, we explored immunomodulation effect of a nanoparticle which was produced from two known immunostimulants, namely Se and CTS. Through our experiments, we observed that CTS-SeNP have stimulatory effect on four of the five immune biomarker responses. This was beneficial to the immune system and led to significant protection effect of CTS-SeNP against bacterial infection from *A. hydrophila*. CTS and sodium selenite were inferior to CTS-SeNP in stimulating immune responses and protecting against bacterial infection. The immunomodulation effects observed for CTS-SeNP were more comprehensive than other immunostimulants or its primary ingredients inorganic selenite and CTS (Table 1). In general, polysaccharide immunostimulant only provide a boost to innate immune responses. Inorganic Se and organic Se mainly enhance the immune responses in cellular immune responses such as phagocytosis.

It is difficult to compare efficacy of CTS-SeNP with other traditional immunostimulants as this nanoparticle is a combined material from two immunostimulants. Effective dose of CTS-SeNP with Se (10 μg/g) was slightly higher than organic and inorganic Se (Table 1), where doses from 0.2 to 4 mg/kg were reported to have immunomodulation effects. Rainbow trout supplemented with inorganic Se (sodium selenite) or organic Se (selenium yeast) did not have effect on lysozyme activity [41,42]. However, the stock CTS-SeNP contained at most 0.25% of the chitosan when produced (see section 2.2). Therefore, at the effective dose of CTS-SeNP, there was only 0.025 μg/g of CTS. This concentration of CTS was 3-6 order of magnitude less than previous reports for polysaccharide immunostimulants to be effective (Table 1). Stimulation of lysozyme activity by CTS were similar to previously reported (Table 1). If we assume that stimulation of lysozyme activities by CTS-SeNP was driven by the CTS on the NP, then there must be a new mechanism helping CTS to be effective as a much lower concentration. A previous study showed that CTS uptake in the gastrointestinal tract was through ionic interaction between the positively charged amino groups of CTS and the negatively charged sialic acid residues in mucus [45]. This is a passive process and is concentration gradient dependent. On the contrary, SeNP were found to be actively taken up by cells through endocytosis [46] and this process can be increased by positive surface charge [47]. Therefore CTS-SeNP could be actively taken up by the fish's gastrointestinal tract cells as a whole, thus delivering the CTS into cells directly and leading to a much lower effective dose.

In terms of effects of CTS-SeNP over time, CTS-SeNP compares favorably with other immunostimulants. Our observation found that CTS-SeNP could stimulate immune response in zebrafish starting from d3 and the similar effects can be observed even after d60. Other studies reported that similar rapid effect of immunostimulants. Kelp grouper

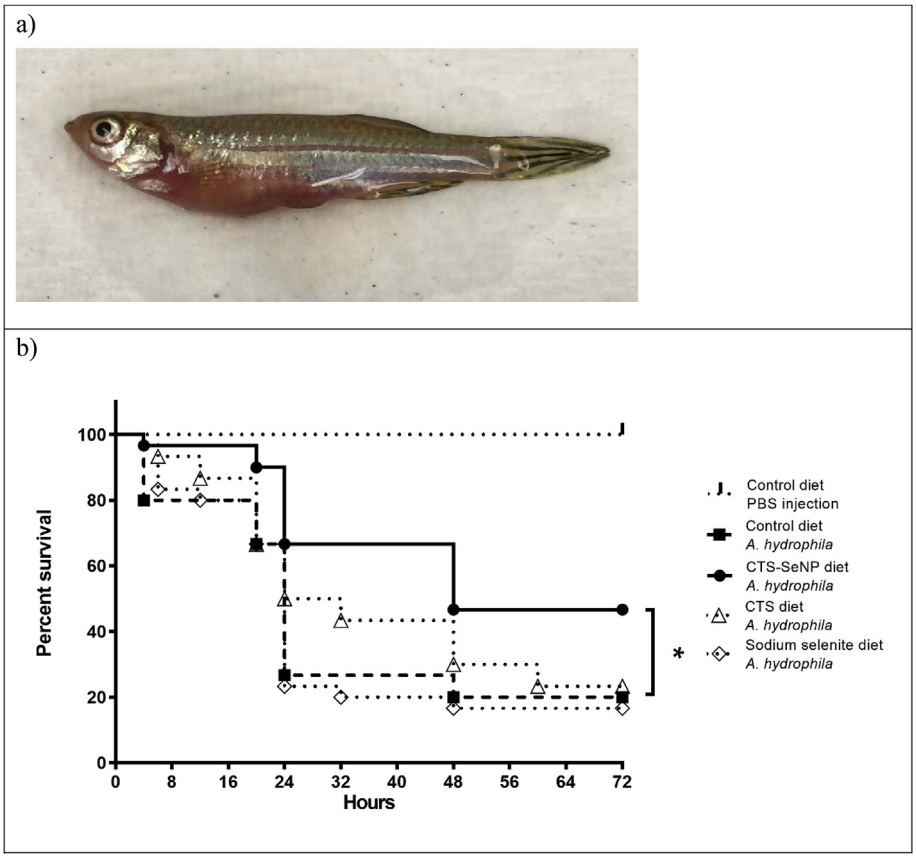

Fig. 8. Bacterial challenge in zebrafish: a) zebrafish after *ip* injection of *A. hydrophila* showing typical symptoms of infection including hemorrhagic septicemia and abdominal swelling; b) 72 h survival rate of zebrafish injection of *A. hydrophila*. Statistical significance from infected control fish was represented by asterisks.

supplemented with CTS showed increased lysozyme activity and improved hematological indices as quickly as just 6d [40]. When fingerlings of channel catfish *Ictalurus punctatus* were supplemented by sodium selenite, selenomethionine or selenoyeast for 9 weeks, they showed improved antibody production and macrophage chemotactic activity [41]. A previous study has also reported SeNP immunomodulation effects in fish [48]. Juvenile mahseer (*Tor putitora*) fish supplemented with SeNP in diet showed increased red blood cell amount, total protein content, lysozyme activity and glutathione peroxidase activity in a 70d feeding trial [48].

It is also worthy to highlighting that both extracellular and intracellular respiratory bursts were increased after supplementation of CTS-SeNP. Respiratory burst is a rapid immune response in activated phagocytes to release of reactive oxygen species (ROS) to eliminate foreign particles and bacteria to combat infections [49]. The mechanisms of extracellular and intracellular production of ROS in PMA-stimulated phagocytes are different [50]. Extracellular superoxide production mainly comes from NADPH oxidase from activated phagocytes to reduce molecular oxygen to superoxide anion radical [20]. Intracellular superoxide anion is mainly produced by the mitochondria during redox regulation processes. Previous study have reported that PMA-stimulated J774.4 macrophages released more superoxide after Se treatment, and Se deficient cells were partial perturbed the integrity of cellular membranes where the NADPH oxidase complex is assembled [51]. In addition, it was generally postulated that Se is involved in cellular activities as an antioxidant through improving production of Se-containing enzymes like glutathione peroxidase (GPx). Dietary SeNP can increase GPx activity in fish [52] and GPx plays a key role in removing hydrogen peroxide (H_2O_2). One possible mechanism of dietary SeNP improving extracellular and intracellular respiratory bursts was through increasing GPx scavenging activity to remove of hydrogen peroxide diffused from phagolysosomes [53]. The increased GPx activity could reduce the production of H_2O_2 and promote the circulation of NADPH oxidases during phagocytosis [54].

After exposure to CTS-SeNP, zebrafish splenocytes showed significantly better proliferation when stimulated with ConA and LPS. Previous studies suggested that these proliferated cells were T cells and B cells respectively [26–28]. It was reported that T-lymphocyte proliferation was highly associated with GPx1-dependent control of intracellular ROS accumulation [55]. Our CTS-SeNP diet might have increased GPx activity to affect intracellular ROS accumulation [56] and thus stimulating T-lymphocyte proliferation. Splenocyte-responses towards ConA can also be stimulated through increase in IL-2 production. Previous studies reported that IL-2 and IL-2 receptor were up-regulated at Se supplementation [56,57]. Therefore CTS-SeNP could improve splenocyte-responses towards ConA through these pathways. On the other hand, there was few studies showing that pathways regulating splenocyte-responses towards LPS are influenced by Se. Significant increase in splenocyte-responses towards LPS at 12d was more likely related to general improvement of redox status of the fish.

In the future, there is a need to perform more chronic studies to understand if CTS-SeNP can provide protection over the entire aquaculture production cycle. This will provide the opportunity to understand protection against infection under a more realistic scenario (i.e. lower bacteria dose but more chronic exposure) to examine if CTS-SeNP can delay or lower infection.

5. Conclusions

CTS-SeNP showed great potential as immunostimulant to fish to help boost host immunity before pathogen infection. Our findings collectively suggest that CTS-SeNP has strong immunomodulation effects

Table 1
Summary of immunomodulation effects of common immunostimulants.

Immunostimulants	Host species	Dosage and period	Innae Immunity			Adaptive Immunity		Pathogen Resistance	Ref.
			Lysozyme activity	Extracellular respiratory burst	Intracellular respiratory burst	Lymphocyte proliferation	Antibody production		
β-Glucans	*Oncorhynchus mykiss;* *Cyprinus carpio;* *Pseudosciaena crocea*	1.8% glucan for 21d; 0.5% glucan for 56d; 0.09% glucan for 56d	↑	↑	↑	–	NA	*Flavobacterium columnare;* *Aeromonas veronii;* *Vibrio harveyi;* *Edwardsiella tarda*	[32–34]
Lipopolysaccharide	*Oncorhynchus mykiss;* *Labeo bata*	0.15 mg/g for 14d; 100 mg/kg for 30d	↑	–	↑	–	NA	*Aeromonas hydrophila;* *Edwardsiella tarda*	[35,36]
Chitosan (CTS)	*Epinephelus bruneus;* *Oreochrmis niloticus;* *Cyprinus carpio*	2% CTS for 14d; 1% CTS for 21d; 1% CTS for 90d; 0.6% CTS for 56d; 0.2% CTS for 56d	↑	NA	↑	NA	NA	*Vibrio alginolyticus;* *Aeromonas hydrophila;* *Vibrio harveyi*	[33,37–40]
Inorganic Se	*Ictalurus punctatus;* *Oncorhynchus mykiss*	0.4 mg/kg (sodium selenite) for 9 or 10 weeks	–	–	↑	NA	↑	*Edwardsiella ictaluri*	[41–43]
Organic Se	*Ictalurus punctatus;* *Oncorhynchus mykiss*	0.2 mg/kg (selenomethionine; selenoyeast) for 9 weeks; 4 mg/kg (Sel-Plex) for 10 weeks	–	–	↑	NA	↑	*Edwardsiella ictaluri*	[41,42]
SeNP and vitamin C	*Tor putitora*	0.68 mg/kg (SeNP) and 100 mg/kg vitamin C for 60d	↑	NA	NA	NA	NA	NA	[44]
CTS-SeNP	*Danio rerio*	10 μg/g Se and 0.025 μg/g CTS for 9-60d	↑	↑	↑	↑	NA	*Aeromonas hydrophila*	this study

↑: increase the immune response.
-: no change the immune response.
NA: no available.

in host's innate immune system. Experiments supported that significant response was rapid (3-12d) and could be sustained to 60d. Overall survival rate also increased under assault of *A. hydrophila*. More studies are needed to under the understand mechanism and chronic effects in the future.

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