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# Dietary nucleotides can directly stimulate the immunity of zebrafish independent of the intestinal microbiota

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ABSTRACT

In this study, we firstly tested the effects of dietary nucleotides on the disease resistance and innate immunity of zebrafish. Further, we investigated the role of intestinal microbiota in the nucleotides-induced immunostimulatory effect by using a germ-free zebrafish model and microbiota transfer technique. Fish were fed control or nucleotides (NT)-supplemented diets (at 0.05%,0.1%, 0.15% or 0.2%, *m/m*) for 4 weeks, followed by immersion challenge with *Aeromonas hydrophila* NJ-1. The results showed that 0.1% NT group enhanced the resistance of zebrafish against *A. hydrophila* infection. We further observed that the relative expressions of mucin, claudin16, occlusin1, hepcidin, defensin beta-like, myeloperoxidase (Mpo), and serum amyloid A (Saa) increased in the 0.1% NT group compared with control ($P < 0.05$), indicating that dietary nucleotides enhanced the physical barrier and mucosal immunity in the intestine of zebrafish. Moreover, ROS level in the head kidney was significantly increased in NT fed zebrafish versus control ($P < 0.05$), indicating enhanced systematic immunity. Furthermore, dietary NT significantly elevated the relative expressions of *mpo*, *saa* and the ROS activity in germ-free zebrafish, while germ-free zebrafish colonized with NT-altered microbiota had no significant difference in the relative expressions of *mpo*, *saa* and the ROS activity compared with the control microbiota-colonized fish, suggesting that the immunostimulatory effect of dietary NT is mediated by direct action of NT and does not involve the microbiota. Consistently, dietary NT can protect germ-free zebrafish from pathogenic infection, whereas germ-free zebrafish colonized with NT microbiota showed no difference in disease resistance compared with control microbiota colonized counterparts. Together, these results indicated that the immunostimulatory and disease protection effect of dietary nucleotides in zebrafish was mediated by direct action of the nucleotides, and does not involve the intestinal microbiota.

## 1. Introduction

Nucleotides (NT) are low molecular weight intracellular compounds which play major roles in almost all biological processes [1]. Nucleotides have traditionally been considered to be non-essential nutrients [2], because the animal can *de novo* synthesize nucleotides. However, the processes to produce nucleotides are thought to be energetically costly [3]. Additionally, under infection or during fast growth and development, *de novo* synthesis of nucleotides cannot satisfy the demand, and supplementation through the diet can improve both human and animal health performances [4].

In aquatic animals, nucleotides have been implicated as feed attractants for a long time [5]. Research on growth and health benefits of dietary nucleotides in aquaculture species started in the early 2000s [6]. Till now, dietary nucleotides have been reported to enhance growth performance [7], immune responses [8], disease resistance [9], and gastrointestinal physiology and morphology [10] in various aquatic species. In particular, research on fish has shown that exogenous nucleotides can influence both innate immunity and adaptive immunity of the host. Previous studies showed that the serum complements, lysozyme activity, phagocytosis as well as blood neutrophil oxidative radical production were significantly increased in various types of fish

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following administration of exogenous nucleotides [3,11]. Nucleotides also influence lymphocyte activity and immunoglobulin production. For example, dietary nucleotides enhanced antibody titer in hybrid striped bass [12], and expression of immunoglobulin M and recombinase activating gene in gill and spleen of turbot [13].

The intestinal tract of vertebrates contains a complex microbiota [14,15]. The intestinal microbiota plays crucial roles in various physiological functions of the host [16,17]. Research has also indicated that fish microbiota contributes to a wide range of biological processes including nutrient metabolism and innate immune responses [18–20]. Our previous study revealed the involvement of microbiota in the immune stimulation function of essential oils in tilapia, implying the importance to consider microbiota-mediated effect in elucidating the mechanism of benefits attained by dietary additives in fish [21]. However, to our knowledge, there are few studies on the role of dietary nucleotides on the intestinal microbiota. Singhal et al. reported that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants, and the intestinal microbiota was proposed as potential mediator of the benefits of nucleotides supplementation for immune function [22]. Xu et al. reported the nucleotides supplementation exerted a moderate influence on the intestinal microbiota of tilapia [23]. In our previous study, we also observed an influence of nucleotides supplementation on the gut microbial community of zebrafish. Moreover, our results revealed that the growth promotion effect of nucleotides is associated with the microbiota-mediated reduction in energy expenditure [24], which indicated an important role of the intestinal microbiota in nucleotides-induced effects. Nevertheless, the involvement of intestinal microbiota in the nucleotides-induced immunomodulation effect was not clear. In this study, we firstly tested the effect of dietary nucleotides on the immunity and disease resistance of zebrafish. Further, we investigated the role of intestinal microbiota in the nucleotides-induced effect by using a germ-free zebrafish model and microbiota transfer technique.

## 2. Methods

### 2.1. Fish husbandry and experimental diets

All experimental and animal care procedures were approved by the Feed Research Institute of the Chinese Academy of Agricultural Sciences Animal Care Committee, under the auspices of the China Council for Animal Care (assurance 2013ZZGCC02). Larval zebrafish (N = 360, mean initial mass 50 mg) were randomly assigned into twenty 3-L tanks with 4 randomly assigned replicate tanks/treatment and 18 fish in each tank. Basal diet formulation and proximate composition analysis (AOAC, 1995) are shown in Table 1. In Experiment 1, NT mixture (Sigma Chemical) of inosine monophosphate (IMP), adenosine monophosphate (AMP), guanosine monophosphate (GMP), uridine monophosphate (UMP) and cytidine monphosphate (CMP) at ratio of 1:1:1:1:1 was added to the basal diet at concentrations of 0.05%, 0.1%, 0.15% and 0.2% NT (*m/m*). Experiment 2 was a follow-up based on the results of the Experiment 1 in which 0.1% NT diet was used for treatment diet. Basal diet without NT supplementation (NT-free) was used as control in both Experiments. Fish of two experiments were fed diets at level of equal weight all groups, six days per week, for 4 weeks in Expt.1 and for 2 weeks in Expt.2.

### 2.2. Challenge test

After the feeding trial, four fish were randomly selected from each tank and were put in a separate challenge tank. Fish were infused with *A. hydrophila* NJ-1 (a gift from Dr. Yongjie Liu of Nanjing Agricultural University, Nanjing, China) at a final density of $10^8$ cells/ml. Intestines of zebrafish were sampled after 24 h post immersion. In parallel, the rest fish in the original tanks were all fed with control diet for three days after the feeding trial. Then fish were randomly selected, challenged, and sampled as described above. All sample were stored at −80 °C for testing the activity of intestinal alkaline phosphatase (AKP). Briefly, intestinal samples of two fish from each tanks were pooled, weighed, homogenized, and incubated in *p*-nitrophenyl phosphate liquid substrate system (Sigma-Aldrich Trading Co.Ltd, Shanghai, China) for 30min. Afterwards, the absorbance was measured at 405 nm using a microtiter plate reader (Multiskan MK3; Thermo, Marietta, OH, USA). P-nitrophenol (PNP) was used as standard. The linear relation between alkaline phosphatase and $OD_{405}$ was calculated. The standard curve equation was $y = 2.883x - 0.123$ ($R^2 = 0.994$) and the examination range was between 0 and 2.70.

**Table 1**
Feed formulation and chemical composition of diets for 1month zebrafish (dry matter, g/kg).

| Ingredient | g/kg DM | | | | |
|---|---|---|---|---|---|
| | Control | 0.05% | 0.10% | 0.15% | 0.20% |
| Casein | 400.0 | 400.0 | 400.0 | 400.0 | 400.0 |
| Gelatin | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Dextrin | 350.0 | 350.0 | 350.0 | 350.0 | 350.0 |
| Soybean oil | 60.0 | 60.0 | 60.0 | 60.0 | 60.0 |
| Lysine | 3.3 | 3.3 | 3.3 | 3.3 | 3.3 |
| Vitamin premix[a] | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Mineral premix [b] | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Ascorbic phosphate ester | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Choline chloride | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Monocalcium phosphate | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| Sodium alginate | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| Nucleotide mixture | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 |
| Alpha-cellulose | 39.7 | 39.2 | 38.7 | 38.2 | 37.7 |
| *Proximate analysis (dry matter, g/kg)* | | | | | |
| Crude protein | 421.9 | 422.1 | 421.6 | 422.4 | 422.8 |
| Crude lipid | 60.9 | 60.5 | 60.8 | 61.0 | 60.2 |
| Crude fiber | 43.6 | 42.9 | 42.9 | 43.1 | 42.8 |
| Gross energy (kJ/g DM) | 18.55 | 18.53 | 18.49 | 18.51 | 18.53 |

[a] Containing the following (g/kg vitamin premix): thiamine, 0.438; riboflavin, 0.632; pyridoxine·HCl, 0.908; *d*-pantothenic acid, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B-12, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; retinyl acetate, 0.677; cholecalciferol, 0.116; *dl*-α-tocopherol-acetate, 12.632.

[b] Containing the following (g/kg mineral premix): $CoCl_2{\cdot}6H_2O$, 0.074; $CuSO_4{\cdot}5H_2O$, 2.5; $FeSO_4{\cdot}7H_2O$, 73.2; NaCl, 40.0; $MgSO_4{\cdot}7H_2O$, 284.0; $MnSO_4{\cdot}H_2O$, 6.50; KI, 0.68; $Na_2SeO_3$, 0.10; $ZnSO_4{\cdot}7H_2O$, 131.93; Cellulose, 501.09.

### 2.3. Respiratory burst activity in head kidney

The respiratory burst activity in head kidney was detected following the protocol described previously [25].

### 2.4. qRT-PCR analysis

The intestinal expression of the lysozyme, hepcidin, defensin beta-like, mucin, claudin, occludin, serum amyloid A protein (Saa), and myeloperoxidase (Mpo) was determined by qRT-PCR as described previously [24]. The primer sequences used for PCR are shown in Table 2. The data were analyzed by the ΔΔCt method and were normalized to Rps11 expression level as an internal control.

### 2.5. Generation and rearing of germ-free (GF) zebrafish

Germ-free zebrafish were prepared as described previously [18,24]They were incubated in an illuminated incubators, where temperature was maintained at 28.5 °C ± 1 °C and photoperiod at 14 h light/10 h dark.

**Table 2**
Primers used to analyze gene expression in this study.

| Gene | Forward primer sequence | Reverse primer sequence | Accession # |
|---|---|---|---|
| *rps 11* | ACAGAAATGCCCCTTCACTG | GCCTCTTCTCAAAACGGTTG | NM213377.1 |
| *lyz* | GATTTGAGGGATTCTCCATTGG | CCGTAGTCCTTCCCCGTATCA | NM139180.1 |
| *hepcidin* | CACAGCCGTTCCCTTCATAC | AGTATCCGCAGCCTTTATTG | NM205583.2 |
| *defbl1* | AGGATGCAGCCTCATTCTCTTT | TGAAGCCCCAGAGCATATTTATC | BC150348.1 |
| *muc* | AATATGCCTTGCGGAACAAC | GTGCTGAGGTTGCAGAATGA | XM_009303625 |
| *claudin 12* | TGCTCCATCACAAAGCTACG | TGTGTCTGTGTGAGTTTGAGTGT | NM131773.2 |
| *claudin 15* | CACCACATCGACCCTGTATG | TACCGGCTATTCTGCCTTTG | BC153629 |
| *claudin-c* | GTACCCTCCGCAAAGTCGTA | CTTTCAAGGAAAGACTGACAGC | NM131764.1 |
| *occludin* | CAAAATCAGGCAAAGGCTTC | AACAATAGTGGCGATGAGCA | BC0952386 |
| *saa* | CGAGCGCA TCAAGCAACA | TCGCTCGTTTTTCATCGTAATCT | NM001005599.1 |
| *mpo* | GCAGATGACGGTTATGGTGTTTC | CCGTCTCAGGACTGGAGAACTT | NM001351837.1 |

### 2.6. Direct effect of NT on GF zebrafish

To determine the direct effect of NT on GF zebrafish, the microparticulate diet of zebrafish larvae was formulated as described previously, and the NT was added to the microparticulate diet at concentrations of 0.1% NT (*m/m*) diet [24]. The manufacture of microparticulate diet was according to the method described by Chepkirui-Boit et al. [26]. In brief, dietary ingredients were ground and passed through a 0.04-mm mesh sieve and homogenized for 10 min in a blender. Soybean oil, phospholipid, cod liver oil and premixes were gradually added, with warm water added during mixing. Sodium alginate was added as binders, and the wet mixture was pelletized by pelleting machine. The 200-μm pellets obtained were air-dried and sealed in 2.5 ml EP tube. Before feeding, the microparticulate diet was sterilized by irradiating with gamma ray 20 kGy in a center of atomic energy (Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences).

Zebrafish larvae hatch from their chorions at 3 days postfertiliztion (dpf). At 5 dpf, the yolk is largely absorbed and the GF zebrafish started to feed the sterile diets (control diet or 0.1% NT diet). At 8 dpf, 6 replicates of each treatment were sampled for the total RNA extraction. *q*RT-PCR analysis was conducted to evaluate the gene expression of Mpo and Saa. Meanwhile, the production of ROS was measured in GF zebrafish fed control or NT diet for three days. Conditions for detection of ROS by fluorescence measurements were detected as described as above [25], except that all solutions were prepared in egg water. As a parallel experiment, germ free zebrafish larvae fed control or NT sterile diets were challenged with $10^7$ CFU/mL *A. veronii* Hm091 by immersion at 8 dpf. Mortality was observed for 36 h.

### 2.7. Transfer of gut microbiota of zebrafish (Exp. 2) to GF zebrafish

The transfer of gut microbiota of zebrafish (Exp. 2) to GF zebrafish were according to the method described by Rawls et al. [27]. The digestive tract contents were pooled from ten zebrafish fed control diet or diet supplemented with 0.1% NT for 4 weeks, and suspended in 1 mL PBS. Microbial density was detected as described previously [24].The bacterial suspension was added to gnotobiotic zebrafish medium (GZM) containing 3 dpf GF zebrafish at a concentration of $10^6$ CFU/mL. At 6 dpf, 6 replicates of each treatment were sampled for the total RNA extraction, and *q*RT-PCR analysis was conducted to evaluate the gene expression of Mpo and Saa as in that of intestine. The production of ROS was detected in GF zebrafish colonized with control or NT-altered microbiota, as described above. In parallel, the control or NT microbiota-recipient zebrafish were challenged with $10^7$ CFU/mL *A. veronii* Hm091 by immersion at 6 dpf. Mortality was observed for 36 h.

### 2.8. Statistical analysis

Statistical analyses were conducted with GraphPad Prism 5. Data from Expt. 1 were analyzed by ANOVA with Tukey's post hoc test. The remaining data were analyzed by Student's *t*-test. The variance homogeneity of the data was examined with Levene's test. Differences were considered significant when $P$ values $< 0.05$.

## 3. Results

### 3.1. Dietary nucleotides enhanced the disease resistance of zebrafish

The intestinal alkaline phosphatase (AKP) activity was positively correlated with mortality of fish after challenge by *A. hydrophila*, and protection of the fish against pathogen can be reflected by lower intestinal AKP activity after challenge [28]. In this study, zebrafish fed diet supplemented with 0.1% NT had significantly lower AKP activity versus control group after challenge with *A. hydrophila* NJ-1 ($P < 0.05$), indicating enhanced resistance against infection (Fig. 1A). The intestinal AKP activity of zebrafish fed with 0.05%, 0.15%, and 0.20% NT diets was numerically reduced compared with control, but with no statistical significance ($P > 0.05$) (Fig. 1A). In addition, the protective effects of the 0.1% NT were sustained after feeding with control diet for three days, while 0.2% NT also showed significant protection of zebrafish after the control diet feeding (Fig. 1B). Overall, the dose of 0.1% NT showed the optimal protection of zebrafish against infection. Therefore, we selected this dose for further investigation.

### 3.2. Dietary nucleotides enhanced the physical barrier and mucosal immunity in the intestine of zebrafish

The relative expression of *mucin* of fish fed diet with NT was markedly up-regulated compared with those of the control group ($P < 0.05$) (Fig. 2A). On the other hand, expression of the tight junction genes *claudin15* and *occludin* was up-regulated in the NT groups ($P < 0.01$); there was no significant difference in the relative expressions of *claudin12* and *claudin-c* between the NT and control groups ($P > 0.05$) (Fig. 2B). The relative expressions of *hepcidin* and *defbl1* were increased in NT group of fish versus control ($P < 0.05$), while no significant difference was observed in the relative expressions of *lyz* between the NT and control groups ($P > 0.05$) (Fig. 2A). Moreover, the relative expression of two innate immune response marker genes, *mpo* and *saa*, elevated significantly in the NT groups compared with control ($P < 0.05$) (Fig. 2C).

### 3.3. Dietary nucleotides enhanced systematic immunity of zebrafish

ROS in the kidney was used to evaluate the systematic immunity of zebrafish. The fluorescence intensity of ROS in the zebrafish kidney is shown in Fig. 3A. ROS levels in the NT group was significantly increased than that of control group ($P < 0.05$). Furthermore, the expression of *lyz* and *hepcidin* was significantly improved in the head kidney of zebrafish fed 0.1% NT supplemented diet versus control (Fig. 3B), supporting an enhanced systematic immunity.

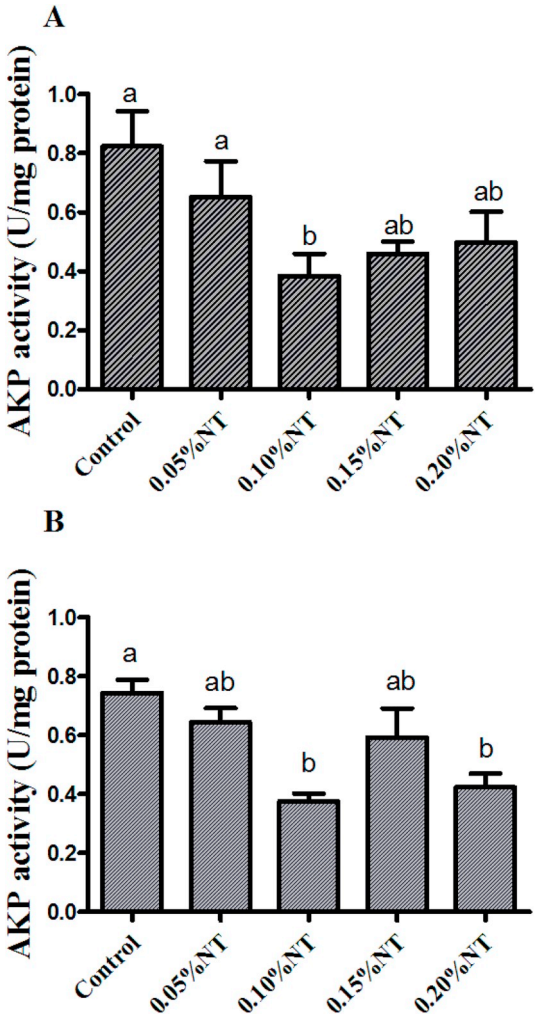


**Fig. 1.** (A) The intestinal alkaline phosphatase (AKP) activity of fish after immersion challenge by *A. hydrophila* NJ-1 for 24 h. (B) The intestinal AKP activity of fish that were fed control diet for 3 days after the feeding trial and then challenged by *A. hydrophila* NJ-1 for 24 h.

### 3.4. The contribution of microbiota-mediated effect to the immunostimulating effect of NT

Dietary NT altered the intestinal microbiota of zebrafish (Results published in our previous study, Reference 19). To investigate microbiota-mediated effects, the gut microbiotas from the control and 0.1% NT groups were transferred to germ-free zebrafish at $10^6$ CFU/mL. There was no significant difference in the relative expressions of *mpo* and *saa* between the nucleotide microbiota-colonized zebrafish and the control microbiota-colonized fish ($P > 0.05$) (Fig. 4A). Similarly, the ROS activities of whole body of nucleotide microbiota-colonized zebrafish and control microbiota-colonized fish were not significantly different ($P > 0.05$) (Fig. 4B).

### 3.5. Direct effect of NT on the immunity of zebrafish

To investigate the direct effect of NT, germ-free zebrafish were fed with sterilized diets. After feeding for 3 days, the relative expressions of *mpo* and *saa* were significantly up-regulated in germ-free zebrafish fed the NT diet ($P < 0.05$) (Fig. 5A). Moreover, the ROS activities of whole body of germ-free zebrafish in the NT group were higher than those of control group ($P < 0.05$) (Fig. 5B).

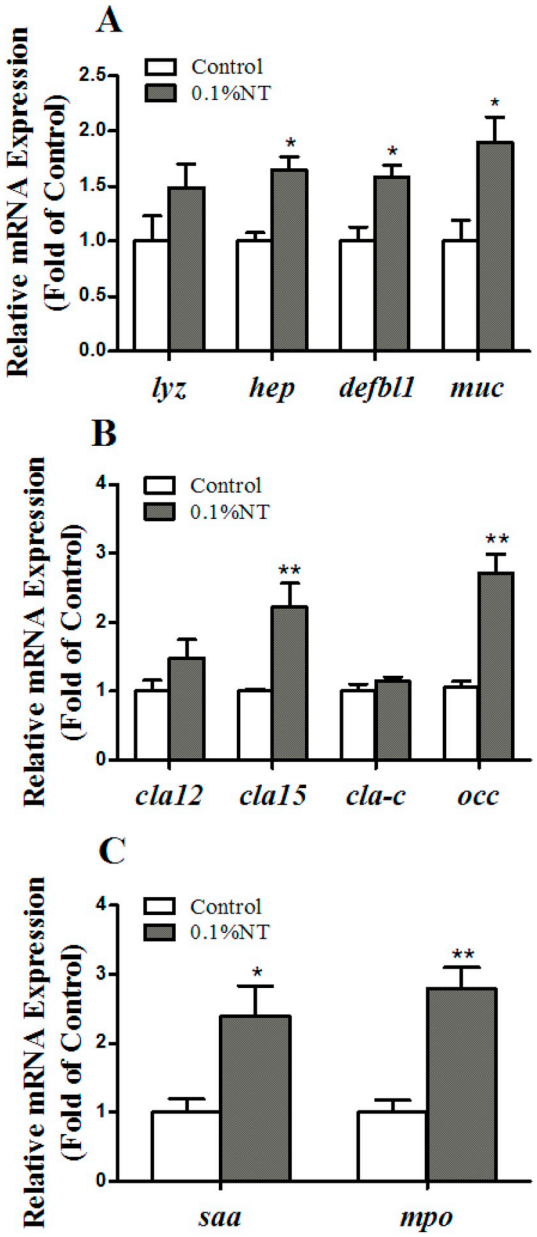


**Fig. 2.** Relative mRNA expressions of antimicrobial genes and mucin (A), tight junction genes (B) and innate immune response marker genes (C) in intestine of zebrafish fed control or 0.1%NT diets for 2 weeks. Values are means ± SEMs, n = 4 (2fish/replicate). Asterisks indicate significant differences compared to control,*$P < 0.05$,**$P < 0.01$. *lys*, lysozyme; *hep*, hepcidin; *def*, defensin beta-like; *muc*, mucin; *cla 12*, claudin 12;*cla 15*, claudin 15;*cla-c*, claudin-c; *occ*, occludin; *saa*, serum amyloid A; *mpo*, myeloperoxidase.

### 3.6. The disease protection effect of NT was mediated by direct effect of NT

To investigate the relative contribution of microbiota-mediated effect to the disease protection effect of NT in zebrafish, germ-free zebrafish colonized with the control or 0.1% NT-altered microbiota were challenged with *A. veronii* Hm091. The result showed that no difference in mortality was detected between the NT microbiota-colonized groups and the control ($P > 0.05$), indicating that the anti-disease effect of NT doesn't involve the microbiota (Fig. 6A). We further tested the direct effect of NT by challenging germ-free zebrafish that have been fed with control or NT diet for 3 days. We observed that the morality rate of the NT diet group was markedly decreased compared with the control ($P < 0.05$), supporting that NT can directly protect zebrafish from the

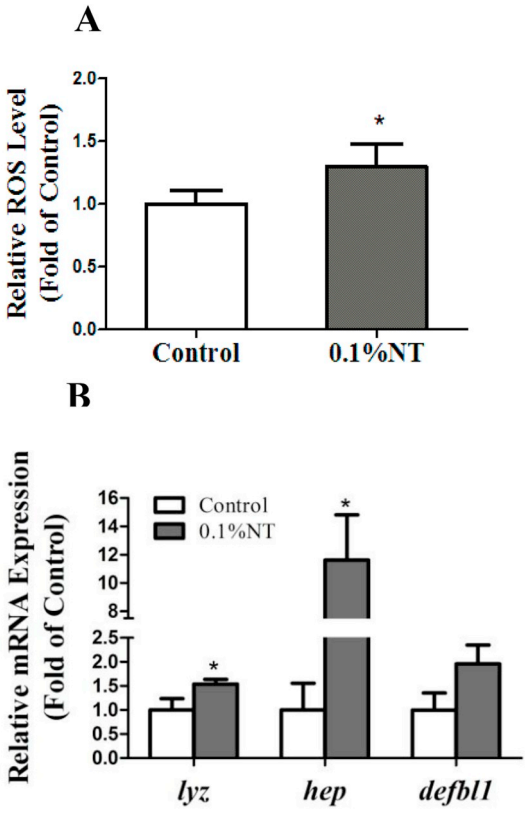


**Fig. 3.** Effect of dietary 0.1% NT supplementation on the respiratory burst activity (A) and expression of antimicrobial genes (B) in head kidney of zebrafish. Values are means ± SEMs, n = 6 (2 fish/replicate). Asterisks indicate significant differences compared to control,*$P < 0.05$.

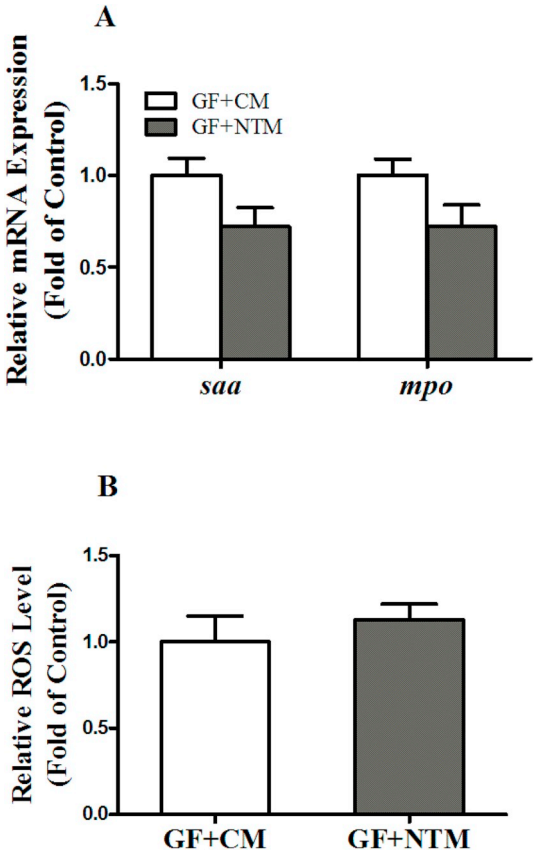


**Fig. 4.** Relative mRNA expressions of innate immune response marker genes (A) and respiratory burst activity (B) in germ-free zebrafish colonized with control or NT-altered microbiota. Values are means ± SEMs, n = 6 (15fish/replicate). GF + CM: germ-free zebrafish colonized with control microbiota; GF + NTM: germ-free zebrafish colonized with NT-altered microbiota.

pathogenic infection independent of the intestinal microbiota (Fig. 6B).

## 4. Discussion

In this study, we investigated the immunostimulatory effect of dietary nucleotides in zebrafish. The dose of 0.1% NT was selected as optimal one based on the challenge result. Interestingly, in our previous study on the growth promotion effect of dietary nucleotides, 0.1% NT was also the optimal dose for growth performance [24]. Therefore, the dose of NT showed some consistency for phenotypes on growth and immune responses, which together reflect good health of fish.

The intestinal AKP activity 24 h after challenge was used to evaluate resistance of zebrafish against *A. hydrophila*. Intestinal AKP is important in detoxifying the lipopolysaccharides (LPS) by dephosphorylation [29]. It can be induced by LPS from Gram negative bacteria, and has been suggested as a biomarker for host response to $G^-$ pathogens [29]. Accordingly, we previously observed that the intestinal AKP activity was positively correlated with the number of *A. hydrophila* cells in the intestine and mortality of fish after challenge [28]. In this study, the 0.1% NT group of fish showed lower intestinal AKP activity after challenge, indicating improved resistance against pathogen. Consistent with our results in zebrafish, dietary nucleotides have been reported to enhance resistance to various pathogenic bacteria in several fish species, including salmonids [10], common carp [11], hybrid striped bass [3] and Nile Tilapia [30]. Moreover, Hossain et al. reported that dietary nucleotides administration at 1.0–1.5 g $kg^{-1}$ can elevate the resistance of juvenile red sea bream [31].

In fish, the intestine barrier serves as the first line of host defense against pathogen infection, which is associated with its structural integrity and mucosal immune components [32–35]. Mucins are the major components of mucus, and play important roles in mucosal barrier against enteric pathogens. In the previous study, it was found that *Vibrio anguillarum* could increase the host susceptibility by inhibiting mucin-2 gene expression in intestinal epithelial cells [36]. Claudin and occludin are tight junctions proteins aiding in the sealing of epithelial barriers [37]. In this study, dietary NT up-regulated the mRNA expression of mucin, claudin16 and occludin1 in the intestine of zebrafish, indicating that NT can enhance the physical barrier of zebrafish intestine. Moreover, the relative expressions of hepcidin and defbl1 were increased by dietary NT. Hepcidin is a peptide hormone, which modulates iron absorption and iron delivery to erythrocytes by binding the iron transporter ferroportin. In zebrafish, hepcidin-2 can inhibit the growth of certain pathogenic bacteria by iron competition [38]. Defensin-like peptide is another antimicrobial peptide, which has been discovered in zebrafish. The identified fish defensin-like peptides mostly resemble beta-defensin family members of birds and mammals [39].Lysozyme is an important innate immune component in fish. In our study, the relative expression of lysozyme tended to increase with NT supplementation. Together, the expression patterns of hepcidin, defbl1 and lysozyme indicated an enhanced mucosal immune responses in the intestine of zebrafish fed NT. Lastly, we tested the expression of serum amyloid a (Saa) and myeloperoxidase (Mpo) in the intestine of zebrafish, Saa is acute phase protein [40,41], and Mpo is a marker of neutrophil lineage [42,43]. Both of them are innate immune response biomarkers [27]. Consistently, the intestinal expression of Saa and Mpo were up-regulated by NT, indicating an enhanced innate immunity in the intestine of zebrafish fed NT.

The respiratory burst response are produced by phagocytes in order to attack invasive pathogens during phagocytosis. The ROS production is initiated by cellular kinases, which are the key enzymes of respiratory

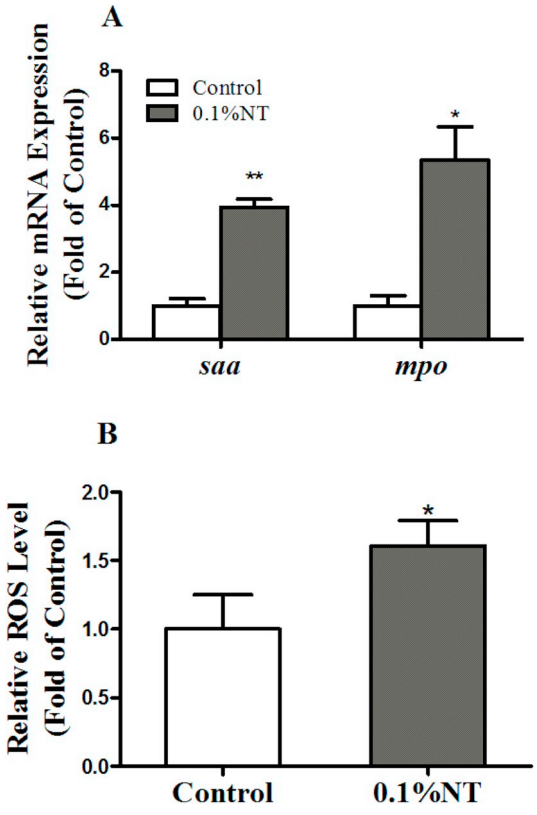


**Fig. 5.** Relative mRNA expressions of innate immune response marker genes (A) and respiratory burst activity (B) in 5-d postfertilization germ-free zebrafish fed the control or 0.1%NT sterile microparticulate diets for 3 days. Values are means ± SEMs, n = 6 (15fish/replicate). Asterisks indicate significant differences compared to control,*$P < 0.05$,**$P < 0.01$.

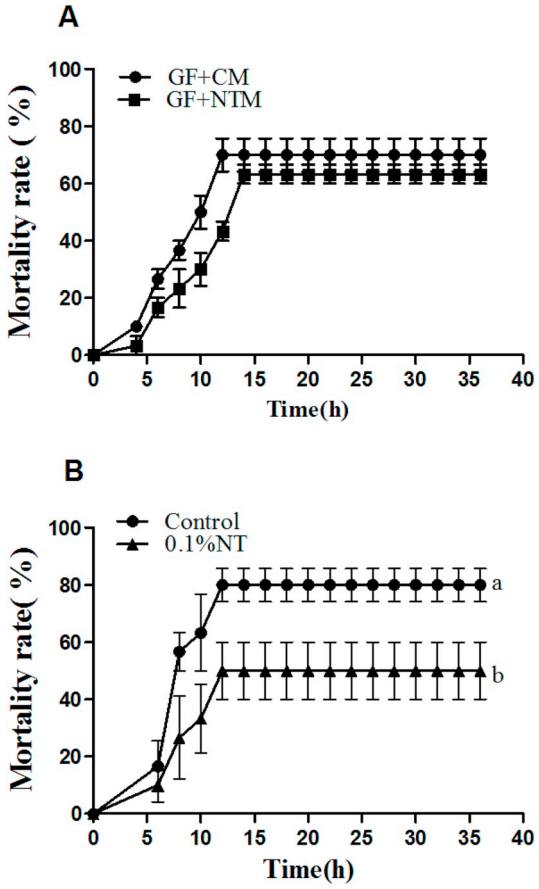


**Fig. 6.** The mortality of zebrafish challenged with *A veronii* Hm091. (A) Germ-free zebrafish colonized with control or NT-altered microbiota. (B) Five-days postfertilization germ-free zebrafish fed the control or 0.1%NT sterile microparticulate diets for 3 days. Fish were challenged with *A. veronii* Hm091 by immersion at $10^7$ CFU/mL. The mortality rate was recorded for 36 h. GF + CM: germ-free zebrafish colonized with control microbiota; GF + NTM: germ-free zebrafish colonized with NT-altered microbiota.

burst [44]. The current study found that the ROS level of head kidney was significantly increased with dietary NT supplementation. Similar results were reported in carp (*Cyprinus carpio* L.) [11], grouper [7] and sea cucumber [45]. In contrast, no enhancement of the respiratory burst activity was found in the head kidney macrophage of hybrid striped bass [3] and salmonids [4], suggesting that the effects of dietary NT on ROS may be species dependent. Nevertheless, the ROS result in this study indicated that dietary NT can improve systematic immunity in zebrafish, which was further supported by the expression pattern of antimicrobial genes in the head kidney.

A growing studies have ascertained that the gut microbiota has a tight and coordinated connection with host immunity [27,46]. In our previous study, we found dietary nucleotides supplementation altered the gut microbial community of zebrafish [24], suggesting that the intestinal microbiota of NT group fish might contribute to the immunomodulation. In this study, a germ-free zebrafish model and microbiota transfer technique were used to elucidate the contribution of a nucleotides-altered microbiota to the immunity of fish. The ROS level as well as the expressions of *saa* and *mpo* were tested in the zebrafish larvae colonized with different microbiotas to assess the innate immunity. Interestingly, zebrafish colonized with control or NT-altered microbiota showed no difference in both ROS and the expression of innate immunity biomarkers, suggesting that the intestinal microbiota-mediated effect does not contribute to the immunostimulatory function of dietary NT. Notably, our previous study showed that NT-altered microbiota contributed a major role to the growth promotion effect of NT [24]. This indicates that the NT-induced microbiota change influences energy metabolism but does not affect immunity stimulation, suggesting that the microbial determinants interacting with host metabolism and immunity are different, which awaits further investigation. Consistent with the microbiota-transfer results, dietary NT improved ROS level and expression of *saa* and *mpo* in germ-free zebrafish, indicating that NT can directly stimulate the innate immune responses of zebrafish, which does not rely on the intestinal microbiota. Similarly, previous study with essential oils indicated that the immunostimulatory effect of essential oils derived from direct effect of the essential oil compounds, while the intestinal microbiota-mediated action was an immune-relieving effect [21].

Challenge experiments with zebrafish larvae showed that feeding germ-free zebrafish nucleotides-supplemented diet reduced the morality rate to *A. veronii* infection, while the germ-free zebrafish colonized with nucleotides microbiota did not lead to significant difference in the morality rate. The results indicate that NT can protect zebrafish from the pathogenic infection in a direct way, which does not involve the intestinal microbiota. These data are consistent with the results on innate immunity, confirming an close correlation between innate immunity and disease resistance of fish. Notably, besides interacting with the host immune system, the microbiota can directly inhibit pathogens by outcompeting nutrients and space [47] or by antagonistic activity [48]. The microbiota-transfer results in our study can reflect both direct and indirect (by inducing hose immune responses) effects of the microbiota against pathogen invasion. Therefore, apart from immunostimulatory effect, our data also suggest that the NT-altered microbiota has no difference with the control in direct “colonization resistance” against the pathogen.

In conclusion, our study confirmed that dietary NT can enhance

both mucosal and systematic innate immune responses of fish. Furthermore, we investigated the relative contribution of direct effect and intestinal microbiota-mediated effect to the immunostimulatory function of dietary NT. The results showed that while NT can induce alteration in the microbiota, the NT-altered microbiota does not contribute a major role to the immunostimulatory effect of dietary NT. Rather, dietary NT can direct interact with host tissues and stimulate the innate immune responses. Lastly, the disease protection effect of NT in zebrafish was also attributable to direct effect, and does not involve the intestinal microbiota.

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