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Research article

1-Deoxynojirimycin from *Bacillus subtilis* improves antioxidant and antibacterial activities of juvenile *Yoshitomi tilapia*

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ABSTRACT

Background: Juvenile *Yoshitomi tilapia* is often infected by pathogens and results in low-level survival rate. *Bacillus subtilis*, as a probiotic, may have beneficial effects on *Y. tilapia* with compound 1-deoxynojirimycin (DNJ), which has antibacterial activities. The effects of dietary probiotic supplementation on *Y. tilapias* were evaluated.
Results: Juvenile *Y. tilapia* was fed with *B. subtilis* for 56 d. *Y. tilapia* was infected by *Aeromonas hydrophila* and survival rate was compared. Dietary *B. subtilis* increased weight gain rate, specific growth, food conversion ratios and food intake rate of *Y. tilapia*. The diet improved the cumulative survival rate (CSR) of juvenile *Y. tilapia* when the concentration of *B. subtilis* was more than 2.05×10^{10} cfu/kg and CSR reached a maximum rate when the concentration of bacillus was 4.23×10^{10} ($P < 0.05$). Meanwhile, *B. subtilis* improved total antioxidant capacity (TAC), spleen index, the activities of serum lysozyme, alkaline phosphatase (ALP), superoxide dismutase (SOD) and catalase (CAT) ($P < 0.05$). In contrast, *B. subtilis* reduced serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA) and C3 complement ($P < 0.05$). DNJ was isolated from secondary metabolisms and proved to increase the levels of SOD, CAT and reduce the levels of AST, ALT and MDA at cell levels. After *A. hydrophila* infection, DNJ prevented the reduction in survival rate of *Y. tilapia* ($P < 0.05$).
Conclusions: 1-Deoxynojirimycin from *Bacillus subtilis* can be used to improve the growth performance of juvenile *Y. tilapia* by affecting its antioxidant and antibacterial activities.

1. Introduction

High-density intensive aquaculture has been developed, but the culture also causes fish diseases and pathogen infection. Various antibiotics have been widely used to control the occurrence of various diseases. However, wide use of antibiotics will kill the beneficial microbes in fish and destroy the balance between animals and microbes. Meanwhile, most antibiotic-resistant bacteria have been developed with the use of antibiotics [1,2]. *Yoshitomi tilapia*, as a kind of farm species, has been widely cultured in China and Southeast Asia [3] while many factors can affect the growth performance of the tilapia and yields. Bacterial infection is also a great challenge for tilapia culture [4]. Furthermore, toxic cyanobacterial cells contain microcystins, which can induce oxidative stress in liver, kidney and gill tissues in tilapia [5].

Bacillus subtilis, a kind of probiotics, has been found to secrete protease [6], lipase [7], amylase [8], cellulase [9] and other important digestive enzymes [10]. Meanwhile, *B. subtilis* can synthesize vitamin B, E, K and other nutrients, which promote the absorption of calcium and phosphorus and enhance growth performance of fish [11]. *B. subtilis* produces various antibiotics with specificity, such as sublancin, polyketide and non-ribosomally synthesized peptide [12]. Antibacterial activities of *B. subtilis* have been widely reported too [13,14]. *B. subtilis* metabolizes lactic acid and other organic acids, as well as acetic acid, propionic acid and other volatile fatty acids, maintains intestinal acidic environment and inhibits the growth of pathogenic bacteria [15]. On the other hand, 1-deoxynojirimycin (DNJ) is an efficient alpha-glucosidase inhibitor with potential applications as a therapeutic agent by controlling the overgrowth of bacteria [16]. DNJ can be produced from mulberry leaves [17] and *Streptomyces lawendulae* [18]. In this study, *B. subtilis* has been widely reported to produce DNJ at a high level [19,20]. However, the molecular mechanism for antibacterial function of DNJ remains widely unclear, but DNJ may show its anti-bacterial *via* anti-adherence of bacteria according to a previous report [16].

B. subtilis can affect phagocytic activity, and improve superoxide dismutase (SOD) level, lysozyme activity and serum complement

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activity [21]. Phagocytic activity [22], lysozyme [23] and serum complement [24] are associated with anti-inflammatory properties of human and animals while SOD can improve antioxidant properties [25]. The results suggest *B. subtilis* may improve anti-inflammatory and antioxidant activities of fish species. Previous studies showed that *B. subtilis* improves immune and antioxidant functions in shrimp [26], grass carp (*Ctenopharyngodon idellus*) [27], hybrid sturgeon (*Acipenser baeri* × *Acipenser schrenckii*) [28], *Procambarus clarkii* [29], Chinese mitten crab (*Eriocheir sinensis*) [30] and other fish [31]. Dietary *B. subtilis* has been reported to alter the autochthonous gut bacterial communities, increase the number of adhesive viable bacteria and the expression of cytokines (IL-1b, TGF-β and TNF-α), and reduce the expression of HSP70 in hybrid tilapia [32]. According to an early report, the probiotic strain, *Bacillus licheniformis* protected Asian catfish *Pangasius hypophthalmus* against infection by improving its immune and antioxidant activities [33]. Similarly, *B. subtilis*, as a kind of bacillus strain, may have the similar functions for protecting fish against pathogens infection. However, the effects of dietary *B. subtilis* on the survival rate and immune functions of *Y. tilapia* remain unclear. There is no any work for the effects of probiotics on *Y. tilapia*. Therefore, the effects of *B. subtilis* on *Y. tilapia* and related molecular mechanisms were explored here.

2. Materials and methods

2.1. Diet preparation for Y. tilapia

B. subtilis is Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA) [34] and presumed not to have an adverse effect [35]. Soybean food (5 g) was homogenized in 40 mL of 0.9% NaCl. Aliquots were spread on LB agar plate, and then colonies were developed and selected after 24-h culture at 37°C. A strain of *B. subtilis* was isolated from soybean and confirmed by using 16S rRNA sequencing. *B. subtilis* was cultured in Luria-Bertani (LB) broth shaken at 37°C until the cell reached a log phase.

According to the nutritional needs of tilapia [36], the fish feed was prepared as Table 1 showed. The colony forming units (CFU) of *B. subtilis* were measured by plate counting. Different doses of *B. subtilis* were used as 0, 0.03%, 0.06%, 0.09%, 0.12%, 0.15% and 0.18% for seven groups (from G1 to G7), corresponding to actually viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively. The different doses of *B. subtilis* were incorporated into the diet with a blender, and the feed was prepared by a meat grinder and made of $2 \times 2 \times 2$ mm^3 sticks, and dried in 60°C incubator for 5 h. Ten-milligram samples were homogenized by using a tissue power homogenizer and dissolved in one-milliliter 0.9% NaCl solution. Ten-microliter solution was spread on the LB plate and cultured for 24 h at 37°C. The cell viability was confirmed by using plate counts before feeding.

Table 1
The ingredients of basal diet.

Ingredient	Amount (%, w/w)
Nile tilapia fishbone meal	32.4
Soybean	22.3
Oat flour	32.1
Choline	0.5
Phagostimulant betaine	0.03
Vitamins premix	0.5
Bean oil	1.1
Feed binder	2.3
Dextrin	1.57
Water	8.4
% dry matter	
Crude protein	33.5
Crude lipid	8.2
Ash	11.2
Fiber	12.6
Energy (kcal/g dry matter)	19.2
Protein	34.8
Lipid	8.5
Carbohydrate	36.7

2.2. Feeding management

A total of 1050 pieces of fish, with an average body length 2.80 ± 0.03 cm and weight 0.43 ± 0.02 g, were provided by the Guangxi Institute of Fisheries (Nanning, China). Fish health was confirmed according to an earlier report [37] and maintained in re-circulating dechlorinated water. All fish were domesticated for 7 d and randomly assigned into 7 groups (150 pieces/group). There were three tanks (each tank 1.5 m × 1.5 m × 1 m) for each group and the whole testing period was 56 d. Tap dechlorinated water was aerated to maintain oxygenation whole day. At 9:00 and 17:00 daily, the feed was provided with an amount of 5 to 7% of the fish weight and to make sure that all foods were eaten up. The feeding duration was 2 h each time. The average water temperature was maintained at 27.6 ± 0.8°C, water dissolved oxygen (DO) was 7.10 ± 0.19 mg/L, pH was 6.9 ± 0.1 and NH_3-N was 0.04 ± 0.01 mg/L.

2.3. Biochemical analysis

At the end of experiment, all fish were weighed, measured and weight gain was calculated. Specific growth rate and feed conversion ratio were measured too. In each pond, 10 fish were randomly selected and dissected. The spleen was completely separated, weighed, and spleen index was calculated. Ten fish were randomly selected from each group, and 0.5-mL blood was obtained rapidly from each fish and placed stand in a refrigerator at 4°C for 2 ~ 4 h until serum coagulation formed and centrifuged (3000 r/min) at 4°C for 15 min. Serum was separated and placed in −40°C refrigerator. Serum lysozyme, SOD, catalase (CAT), total antioxidant capacity (TAC) and malondialdehyde (MDA) were measured by using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Automatic biochemical analyzer was used to measure the activities and or levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), C3 complement, total protein (TP), albumin (ALB) and globulin (GLB).

2.4. 1-Deoxynojirimycin (DNJ) separation from B. subtilis

Secondary metabolites of *B. subtilis* may play an important role in the function of the species. Thus, main bioactive compound was separated from the bacteria. Bioactive compounds were isolated by using semi-preparative HPLC (Beckman, Brea, CA, USA; Ultrasphere™ C18 column, 5 μm, 10 × 250 mm). For the mobile phase, methanol and acetonitrile was used to separate secondary metabolisms. Methanol is a polar proton solvent, and acetonitrile is a polar aprotic solvent, which may explain why acetonitrile is more capable of isolating the sample. It is well known that acids and bases can improve the HPLC peak shape. After comparing the different mobile phase ratios, 70% acetonitrile was used. One-liter fermentation liquid was lyophilized to 10-gram solids and dissolved in 20-mL water and the injection volume was set to 2 mL, the flow rate was set at 0.8 mL/min, and the column temperature was maintained at 30°C.

Ten-milligram DNJ was weighted and dissolved in ddH_2O to prepare 1.0 mg/mL solution. The stock solution was stored in the refrigerator at 4°C. The stockpile standard solution was then continuously diluted with water to prepare standard calibration solutions with different concentrations. Each time 2 mL of sample was injected into HPLC-ELSD. The analysis was performed on a Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA) in which the Waters 2424 ELSD was coupled to the Waters system. The chromatographic data was collected and processed by Empower Chromatography Station (Waters).

2.5. Bacterial infection parameters

The pathogens *Aeromonas hydrophila* were purchased from Guangdong Microbial Culture Collection Center (GIMCC) (Guangzhou, China). *A. hydrophila* were inoculated in Fresh Water fish Agar (FWA) medium (g/L, peptone 5, beef extract 2.5, yeast extract 2.5, glucose 1, NaCl 15, $MgSO_4$ 0.05, K_2HPO_4 0.2, agar 15, pH 7.2 ~ 7.4) at 30°C for 36 h. The cell count was performed by using hemocytometers. Lethal dose test (LD) for juvenile fish was carried out. The LD often used is LD50, a dose in which 50% of fish die. But considering the ethical panel from several countries advice, a lower LD, LD10 and LD30 are often considered. The results showed that the concentration of LD10 was 1×10^8 cfu/mL. According to the dose, the cells were diluted with 0.85% sterile saline solution and adjusted to the concentration of 1×10^8 cfu/mL. After 56 d, 10 fish were randomly selected from each tank and injected with 0.3 mL *A. hydrophila* at the pectoral fin. For each dietary group infected with pathogen agent, a fish group injected with saline solution and without the pathogen was used as control group. Different concentrations of DNJ were incorporated into the diet with a blender, and the feed was prepared by a meat grinder and made of $2 \times 2 \times 2$ mm^3 sticks, and dried in 60°C incubator for 5 h. The survival rates were calculated at 1, 24, 48, 72, 96, 120, 144 and 168 h, respectively.

2.6. Growth performance test

The experimental parameters were determined by the following formula:

$$\text{Weight gain rate (WGR)} = (W_t - W_0)/W_0 \times 100\% \quad (1)$$

$$\text{Specific growth rate (SGR)} = (\ln W_t - \ln W_0)/t \times 100\% \quad (2)$$

$$\text{Feed conversion ratio (FCR)} = (W_{tt} - W_{0t} + W_{dt})/TI \times 100\% \quad (3)$$

$$\text{Feed intake rate (FIR)} = (Feed_{0t} - Feed_{tt})/(W_{tt} - W_{0t} \cdot d) \times 100\% \quad (4)$$

$$\text{Spleen index} = W_s/W_t \times 100\% \quad (5)$$

$$\text{Cumulative survival rate (CSR)} = X_t/X_0 \quad (6)$$

B. subtilis potency was measured by calculating the relative percent survival (RPS) according to Amend [38]: RPS = 1 - (% *B. subtilis* / % control mortality) 100%.

W_0 stands for fish average weight (g) at the start of the experiment. W_t stands for the average fish weight (g) at the end of the experiment. W_{0t} stands for the total fish weight (g) at the start of the experiment. W_{tt} stands for the total fish weight (g) at the end of the experiment. $Feed_{0t}$ stands for the total weight of feed at the start of the experiment daily. $Feed_{tt}$ stands for the total weight of feed at the end of the experiment daily. W_{dt} stands for the total weight of dead fish at the end of experiment; W_s stands for the mean spleen mass (g) at the end of the experiment. TI is the total feed intake. "t" is feeding time (d). X_0 stands the number of survival fish before pathogen infection. X_t stands for the number of survival fish at the end of pathogen infection.

Probiotic consumption is a combination of dietary probiotic content and feed consumption rate. Thus, VFI was used for evaluating the effects of the dietary treatment. Fish were fed daily and left feed were collected daily over 56 d. VFI was calculated in g of dry feed/fish in one day (g·dm/d).

2.7. The effects of DNJ on the antioxidant activity of spleen cells

The effects of DNJ on the antioxidant activity were measured in the spleen cells. Spleen cells were separated according to an earlier report [39]. Spleen cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μg/mL penicillin and streptomycin, respectively. The cells reached log phase and were adjusted to 1×10^6 cells/mL and randomly assigned into control group (saline solution) and DNJ treated group (5 mg/L). The cells were further cultured for 72 h. The antioxidant activities were measured by using above methods.

2.8. Statistical analysis

All data were presented as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) by using SPSS 15.0. There are significantly statistical differences if $P < 0.05$.

3. Results and discussion

3.1. B. subtilis improves growth performance of juvenile Y. tilapia

There was no significant difference among three tanks in each group. Thus, there was no tank effect on present experiment ($P > 0.05$). Table 2 showed there was no significant difference between *B. subtilis*-treated groups and the control group if the concentrations of *B. subtilis* were too low ($P > 0.05$). The results showed that dietary *B. subtilis* increased WGR, SGR, FCR and FIR of *Y. tilapia* when the concentration of *B. subtilis* was more than 3.55×10^{10}, 4.23×10^{10}, 2.05×10^{10} and 2.05×10^{10}, respectively ($P < 0.05$). The diet improved the cumulative survival rate (CSR) of juvenile *Y. tilapia* when the concentration of *B. subtilis* was more than 2.05×10^{10} cfu/kg and CSR reached a maximum rate when the concentration of bacillus was more than 4.23×10^{10} ($P < 0.05$).

Table 2
The effects of dietary *B. subtilis* on growth performance of juvenile *Y. tilapia*.

	Initial weight W_0/g	Final weight W_t/g	Weight gain rate /%	Specific growth /%	Feed conversion ratio /%	Feed intake rate /%	Cumulative survival rate /%
G1	0.43 ± 0.02	45.74 ± 3.20[f,g]	10,536.09 ± 319.62[g]	8.33 ± 0.12[g]	1.04 ± 0.01[d,e,f,g]	0.84 ± 0.01[d,e,f,g]	89.12 ± 2.35%[c,e,f,g]
G2	0.42 ± 0.01	45.08 ± 2.28[f,g]	10,634.12 ± 320.61[g]	8.45 ± 0.15[g]	1.07 ± 0.02[d,e,f,g]	0.85 ± 0.01[d,e,f,g]	88.26 ± 2.75%[c,e,f,g]
G3	0.43 ± 0.02	45.91 ± 2.51[f,g]	10,576.75 ± 504.73[g]	8.58 ± 0.08[g]	1.05 ± 0.05[d,e,f,g]	0.82 ± 0.02[d,e,f,g]	93.42 ± 3.04%[a,b,e,f,g]
G4	0.44 ± 0.01	46.04 ± 1.08[g]	10,691.98 ± 105.89[g]	8.74 ± 0.12[g]	1.17 ± 0.05[a,b,c,g]	0.93 ± 0.03[a,b,c,g]	95.38 ± 3.89%[a,b]
G5	0.44 ± 0.04	47.20 ± 3.16	10,728.19 ± 618.46[g]	8.85 ± 0.11[g]	1.26 ± 0.04[a,b,c,g]	1.04 ± 0.04[a,b,c,g]	96.76 ± 2.13%[a,b,c]
G6	0.43 ± 0.01	48.35 ± 3.73[a,b,c]	10,808.12 ± 558.45[g]	8.96 ± 0.09[g]	1.27 ± 0.07[a,b,c,g]	1.08 ± 0.05[a,b,c,g]	97.33 ± 2.01%[a,b,c]
G7	0.42 ± 0.02	49.92 ± 4.84[a,b,c,d]	11,751.67 ± 851.87[a,b,c,d,e,f]	9.78 ± 0.14[a,b,c,d,e,f]	1.38 ± 0.09[a,b,c,d,e,f]	1.21 ± 0.06[a,b,c,d,e,f]	96.45 ± 2.92%[a,b,c]

Note: Different doses of *B. subtilis* were for seven groups (from G1 to G7), corresponding to viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively.

[a] $P < 0.05$ *vs.* G1.
[b] $P < 0.05$ *vs.* G2.
[c] $P < 0.05$ *vs.* G3.
[d] $P < 0.05$ *vs.* G4.
[e] $P < 0.05$ *vs.* G5.
[f] $P < 0.05$ *vs.* G6.
[g] $P < 0.05$ *vs.* G7.

Table 3
The effects of dietary *B. subtilis* on immune organs and immune indices of juvenile *Y. tilapia*.

Diet groups	G1	G2	G3	G4	G5	G6	G7
Spleen index (%)	$0.15 \pm 0.01^{b,c,d,e,f,g}$	$0.17 \pm 0.03^{a,d,e,g}$	$0.18 \pm 0.01^{a,d}$	$0.2 \pm 0.02^{a,b,c,f}$	$0.19 \pm 0.02^{a,b}$	$0.18 \pm 0.01^{a,d}$	$0.19 \pm 0.02^{a,b}$
Lysozyme (U/mL)	$401.84 \pm 12.04^{b,c,d,e,f,g}$	$438.21 \pm 18.98^{a,e,f}$	468.38 ± 5.153^{a}	459.07 ± 26.41^{a}	$482.23 \pm 20.42^{a,b}$	$486.89 \pm 10.56^{a,b}$	464.95 ± 10.95^{a}
Complement C3 (mg/L)	$0.65 \pm 0.11^{c,d,e,f,g}$	$0.69 \pm 0.12^{c,d,e,f,g}$	$0.83 \pm 0.13^{a,b,d,e,f,g}$	$1.14 \pm 0.18^{a,b,c,g}$	$1.16 \pm 0.15^{a,b,c,g}$	$1.05 \pm 0.17^{a,b,c,g}$	$1.36 \pm 0.19^{a,b,c,d,e,f}$
Alkaline phosphatase (U/mL)	$29.13 \pm 1.53^{c,d,e,f,g}$	$31.27 \pm 0.58^{d,e,f,g}$	$32.89 \pm 1.15^{a,f,g}$	$34.62 \pm 2.89^{a,b}$	$35.18 \pm 2.52^{a,b}$	$37.21 \pm 1.73^{a,b,c}$	$36.57 \pm 1.53^{a,b,c}$
Aspartate aminotransferase (U/mL)	$92.94 \pm 6.03^{b,c,d,e,f,g}$	$75.82 \pm 5.03^{a,c,d,e,f,g}$	$60.34 \pm 2.52^{a,b,f}$	$63.41 \pm 1.15^{a,b,e}$	$54.32 \pm 6.03^{a,b,d,f,g}$	$62.18 \pm 3.21^{a,b,e}$	$59.23 \pm 5.03^{a,b,e}$
Alanine aminotransferase (U/mL)	$26.35 \pm 2.31^{b,c,d,e,f,g}$	$20.44 \pm 1.46^{b,e,f,g}$	$22.46 \pm 2.08^{a,d,e,f,g}$	$16.37 \pm 0.29^{a,b,c,e,f,g}$	$13.24 \pm 2.65^{a,b,c,d}$	$13.18 \pm 0.55^{a,b,c,d}$	$13.54 \pm 1.52^{a,b,c,d}$
total protein (mg/mL)	26.43 ± 1.28	27.11 ± 0.75	26.67 ± 0.81	26.43 ± 0.38	26.34 ± 0.44	27.24 ± 1.35	27.49 ± 0.53
albuminuria (mg/L)	10.04 ± 0.36	10.63 ± 0.34	10.10 ± 0.42	10.17 ± 0.31	10.23 ± 0.61	10.22 ± 1.05	10.56 ± 0.71
globulin (mg/L)	16.43 ± 0.81	16.12 ± 0.45	16.57 ± 1.17	16.38 ± 0.29	17.07 ± 0.31	17.23 ± 0.32	16.83 ± 0.35

Note: Different doses of *B. subtilis* were used (from G1 to G7), corresponding to viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively.

[a] $P < 0.05$ *vs.* G1.
[b] $P < 0.05$ *vs.* G2.
[c] $P < 0.05$ *vs.* G3.
[d] $P < 0.05$ *vs.* G4.
[e] $P < 0.05$ *vs.* G5.
[f] $P < 0.05$ *vs.* G6.
[g] $P < 0.05$ *vs.* G7.

VFI was affected by the addition of the amounts of *B. subtilis* in fish feed. Feed intake increased linearly with the increase of *B. subtilis*. The feed intake [g DM/(fish·d)] was increased from 5.8 (control group) to 6.5 (2.05×10^{10} cfu/kg *B. subtilis*). The results suggest that *B. subtilis* increases VFI of juvenile *Y. tilapia*.

3.2. B. subtilis regulates immune of juvenile Y. tilapia

Table 3 showed that the fish spleen index, lysozyme and ALP activities were increased with the increase in the concentration of *B. subtilis*. The spleen index reached the highest level in G4 when compared with G1, G2 and G3 ($P < 0.05$), but there was no significant difference among G4, G5, G6 and G7 groups ($P > 0.05$). Lysozyme and ALP activities reach the highest level in G6 and G7 with 486.89 U/mL and 475.12 U/mL, respectively, which were significantly higher than G1 with 37 U/mL ($P < 0.05$). C3 complement content was also increased with the increase in the amount of *B. subtilis*, but it was reduced in G6, and then reached the highest level in G7 ($P < 0.05$). The serum AST and ALT activities were decreased with the increase in the concentrations of *B. subtilis* and reached the lowest level in G5, G6 and G7 groups ($P > 0.05$). On the other hand, there was no significantly statistical difference for serum TP, ALB and GLB of *Y. tilapia* when dietary *B. subtilis* was increased ($P > 0.05$).

3.3. Effect of B. subtilis on the antioxidant activities of Y. tilapia

The levels of SOD, CAT, T-AOC were positively correlated with the concentrations of dietary *B. subtilis* (Table 4). Serum SOD, CAT and TAC activities reached the highest level with 82.93 ± 2.09, 8.84 ± 0.21 and 7.15 ± 0.3 U/mL in G7 group ($P < 0.05$), respectively. In contrast, the serum MAD level reached the lowest level in G7 group with 5.01 ± 0.34 nmol/mL. Serum SOD and CAT activities were higher in from G4 to G7 group than in other experimental groups ($P < 0.05$). Serum TAC activities were higher in from G5 to G7 group than in other experimental groups ($P < 0.05$). *B. subtilis* decreased serum MDA levels when compared with control group G1 ($P < 0.05$). The serum MDA levels were lower in from G4 to G7 when compared with other groups ($P < 0.05$).

3.4. Effect of B. subtilis on the CSR of juvenile Y. tilapia

The fish readily consumed the diets without a learning curve. Fin and tail rot could be observed when the fish was infected with the pathogens and without *B. subtilis* treatment. Meanwhile, the fish lost its vigor. The CSR of juvenile *Y. tilapia* was greatly reduced after 168-h infection with *A. hydrophila*. In contrast, the CSR was higher in the groups treated with *B. subtilis* when compared with G0 (without addition of *B. subtilis*) and G1 ($P < 0.05$, Table 5). The results following the *A. hydrophila* infection of *Y. tilapia* were similar between G0 and G1 groups. At the same time, the fish increase its vigor. After twenty-four-hour infection, the lowest CSR was 73.33% in a control group, and the CSR was 96.67% in group G7; after 48 h, the CSR dropped to 33.33% in a control group, and was lower than in other groups ($P < 0.05$); After 96 h, the CSR was 26.67% in the control group, and the CSR was highest in group G7. After 168 h, CSR was stabilized in each group. On the other hand, there was no statistical significance of differences among all groups when the fish was not infected by pathogens ($P > 0.05$) (Table 6). The results suggested that *B. subtilis* reduced pathogens infection of juvenile fish.

3.5. Quantification of DNJ

The content of DNJ in the fermentation broth of *B. subtilis* was quantified according to the established HPLC method. The results show that the species has DNJ and are rich in DNJ (Fig. 1B) when compared with a standard sample (Fig. 1A). The contents of DNJ in the

Table 4
The effects of dietary *B. subtilis* on anti-oxidation activities of juvenile *Y. tilapia*.

Diet groups	Superoxide dismutase /(U/mL)	Catalase /(U/mL)	Total antioxidant capacity /(U/mL)	Malondialdehyde /(nmol/mL)
G1	$63.27 \pm 5.98^{c,d,e,f,g}$	$6.34 \pm 0.17^{b,c,d,e,f,g}$	$6.15 \pm 0.56^{e,f,g}$	$7.63 \pm 0.51^{b,c,d,e,f,g}$
G2	$64.98 \pm 4.53^{c,d,e,f,g}$	$7.67 \pm 0.38^{d,e,f,g}$	$6.25 \pm 0.47^{e,f,g}$	$6.04 \pm 0.35^{a,d,e,g}$
G3	$72.93 \pm 7.19^{a,b,d,e,f,g}$	$7.71 \pm 0.73^{a,d,e,f,g}$	$5.96 \pm 0.42^{e,f,g}$	$5.87 \pm 0.54^{a,d,e,g}$
G4	$81.31 \pm 7.01^{a,b,c}$	$8.62 \pm 0.42^{a,b,c}$	$6.19 \pm 0.21^{e,f,g}$	$5.31 \pm 0.39^{a,b,c}$
G5	$80.07 \pm 1.48^{a,b,c}$	$8.59 \pm 0.61^{a,b,c}$	$6.91 \pm 0.15^{a,b,c,d}$	$5.27 \pm 0.43^{a,b,c}$
G6	$81.22 \pm 2.47^{a,b,c}$	$8.51 \pm 0.44^{a,b,c}$	$7.06 \pm 0.12^{a,b,c,d}$	$5.49 \pm 0.22^{a,g}$
G7	$82.93 \pm 2.09^{a,b,c}$	$8.84 \pm 0.21^{a,b,c}$	$7.15 \pm 0.37^{a,b,c,d}$	$5.01 \pm 0.34^{a,b,c,f}$

Note: Different doses of *B. subtilis* were used for seven groups (from G1 to G7), corresponding to viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively.
[a] $P < 0.05$ *vs.* G1.
[b] $P < 0.05$ *vs.* G2.
[c] $P < 0.05$ *vs.* G3.
[d] $P < 0.05$ *vs.* G4.
[e] $P < 0.05$ *vs.* G5.
[f] $P < 0.05$ *vs.* G6.
[g] $P < 0.05$ *vs.* G7.

fermentation of *B. subtilis* were quantified according to the established HPLC method. The results showed that all of the samples were rich in DNJ. The average content of DNJ was 1 g/L. These data provide an important reference for the quality of fermentation liquid of the species for the treatment of bacterial infection or as a material to obtain the DNJ for use as an α-glucosidase inhibitor in functional food.

3.6. The effects of DNJ on the RPS of juvenile Y. tilapia infected with A. hydrophila

DNJ treatment reduced *A. hydrophila* infection in *Y. tilapia*. There was statistical significance of differences in RPS among different groups ($P < 0.05$) (Fig. 2). DNJ consumption resulted in 69%, 73%, 74%, 78%, 80% and 94% RPS of *Y. tilapia* in six groups at 24 h post-challenge, respectively. Even at 168 h post-challenge, DNJ consumption resulted in 12%, 14%, 23%, 33%, 41% and 49% RPS of *Y. tilapia* in six groups, respectively (Fig. 2). These results demonstrate that DNJ from *B. subtilis* have beneficial effects on *Y. tilapia* by improving its survival rates with the increase of the concentrations of probiotics.

3.7. DNJ improves the antioxidant activities

During three-day culture, the levels of SOD (Fig. 3A), CAT (Fig. 3B), AST (Fig. 3C), ALT (Fig. 3D) and MDA (Fig. 3E) were stable in spleen cells from day 0 to day 3 ($P > 0.05$) when no DNJ was added. Compared with control group, DNJ treatment increased the levels of SOD (Fig. 3A) and CAT (Fig. 3B), and reduced the levels of AST (Fig. 3C), ALT (Fig. 3D) and MDA (Fig. 3E). SOD and CAT reached the highest level on the third day and AST, ALT and MDA reached the lowest level on the third day of culture ($P < 0.05$). The results suggest that DNJ treatment improves the antioxidant activities.

In this study, *B. subtilis* improves the growth performance of juvenile *Y. tilapia* and weight gain and feed conversion ratio were significantly higher in G7 when compared with control group ($P < 0.05$). Present findings suggest *B. subtilis* is beneficial for the aquaculture of juvenile *Y. tilapia*.

WGR [40], SGR [41], FCR [42], FIR [43] and CSR [44] are important parameters for evaluating the growth performance of fish and can be used in the assessment of the effects of *B. subtilis* on juvenile *Y. tilapia*. These parameters altogether enable to reflect the growth performance results. The presented findings demonstrated that dietary *B. subtilis* increased WGR, SGR, FCR and FIR of *Y. tilapia* when the concentration of *B. subtilis* was more than 3.55×10^{10}, 4.23×10^{10}, 2.05×10^{10} and 2.05×10^{10} cfu/kg were used, respectively (Table 2, $P < 0.05$). The diet improved the CSR of juvenile *Y. tilapia* when the concentration of *B. subtilis* was more than 2.05×10^{10} cfu/kg, and CSR reached maximum rate when the concentration of bacillus was 4.23×10^{10} (Table 2, $P < 0.05$). All these results suggest the addition of *B. subtilis* in fish feed can improve the growth performance of *Y. tilapia*.

Present results showed that the addition of *B. subtilis* increased lysozyme activities, which were accordant with previous reports in

Table 5
Cumulative survival rate (CSR) of juvenile *Y. tilapia* injected with *A. hydrophila* (%).

	Time (h)				
	1	24	48	96	168
G0	100	$74.26 \pm 6.52^{d,e,f,g}$	$37.68 \pm 5.79^{b,c,d,e,f,g}$	$26.75 \pm 4.13^{b,c,d,e,f,g}$	$24.18 \pm 3.06^{b,c,d,e,f,g}$
G1	100	$73.51 \pm 5.70^{d,e,f,g}$	$38.36 \pm 5.24^{b,c,d,e,f,g}$	$28.34 \pm 3.25^{b,c,d,e,f,g}$	$26.67 \pm 2.41^{b,c,d,e,f,g}$
G2	100	$76.54 \pm 5.48^{e,f,g}$	$60.24 \pm 6.32^{a,c,d,e,f,g}$	$40.01 \pm 10.26^{a,d,e,f,g}$	$31.46 \pm 3.17^{a,c,d,e,f,g}$
G3	100	$77.31 \pm 5.09^{e,f,g}$	$73.19 \pm 6.75^{a,b,e,g}$	$43.19 \pm 10.25^{a,b,e,f,g}$	$36.27 \pm 4.26^{a,b,d,e,f,g}$
G4	100	$82.36 \pm 5.21^{a,b,f,g}$	$75.48 \pm 8.24^{a,b,g}$	$46.92 \pm 5.34^{a,b,c,e,f,g}$	$43.28 \pm 5.13^{a,b,c,f,g}$
G5	100	$86.27 \pm 6.29^{a,b,c,g}$	$80.34 \pm 7.56^{a,b}$	$63.24 \pm 11.55^{a,b,c,d,f}$	$50.64 \pm 6.54^{a,b,c,d,g}$
G6	100	$90.18 \pm 8.23^{a,b,c,d}$	$78.67 \pm 8.25^{a,b}$	$57.34 \pm 6.38^{a,b,c,d,e,g}$	$53.71 \pm 7.20^{a,b,c,d,g}$
G7	100	$96.45 \pm 9.15^{a,b,c,d,e}$	$86.59 \pm 8.37^{a,b,c,d}$	$65.27 \pm 7.12^{a,b,c,d,f}$	$60.87 \pm 10.18^{a,b,c,d,e,f}$

Note: G0, no *B. subtilis* in the group. Different doses of *B. subtilis* were used for seven groups (from G1 to G7), corresponding to viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively.
[a] $P < 0.05$ *vs.* G1.
[b] $P < 0.05$ *vs.* G2.
[c] $P < 0.05$ *vs.* G3.
[d] $P < 0.05$ *vs.* G4.
[e] $P < 0.05$ *vs.* G5.
[f] $P < 0.05$ *vs.* G6.
[g] $P < 0.05$ *vs.* G7.

Table 6
Cumulative survival rate (CSR) of juvenile *Y. tilapia* without pathogen infection (%).

	Time (h)				
	0	24	48	96	168
G1	100	98.36 ± 1.35	97.88 ± 2.18	97.31 ± 2.54	97.01 ± 2.80
G2	100	98.12 ± 1.78	97.91 ± 1.89	97.68 ± 2.21	97.58 ± 2.32
G3	100	97.08 ± 2.48	97.89 ± 1.37	97.02 ± 2.73	97.00 ± 2.35
G4	100	98.54 ± 1.46	97.25 ± 2.45	97.44 ± 2.15	97.18 ± 2.02
G5	100	99.33 ± 0.53	98.14 ± 1.68	97.98 ± 2.07	97.34 ± 2.55
G6	100	98.25 ± 1.06	97.91 ± 1.34	97.34 ± 1.89	97.11 ± 2.06
G7	100	98.58 ± 1.42	98.12 ± 1.64	97.61 ± 2.13	97.21 ± 2.547
P values	1	0.92	0.89	0.85	0.80

Note: Different doses of *B. subtilis* were used for seven groups (from G1 to G7), corresponding to viable cells: 0.2×10^2, 0.69×10^{10}, 1.38×10^{10}, 2.05×10^{10}, 2.82×10^{10}, 3.55×10^{10} and 4.23×10^{10} cfu/kg, respectively. The cell concentration was adjusted by the same volume of saline solution. There was no statistical significance of difference if $P > 0.05$.

other fish. The results were similar with those from rainbow trout (*O. mykiss*) [45,46], hybrid sturgeon (*A. baeri* × *A. schrenkii*) [47] and silver carp (*C. auratus*) [48]. In 2007, Newaj et al. found that dietary *B. subtilis* significantly improved serum lysozyme activity of rainbow trout [49]. Lysozyme hydrolyzes mucopolysaccharides, which are basic components of bacterial cell wall and kill pathogens [50]. Some studies showed that fish lysozyme activity is stronger in fish than high-grade vertebrates and plays an important role in the defense system [51,52]. Our results also showed that *B. subtilis* increased lysozyme activity of *Y. tilapia* ($P < 0.05$).

The spleen is one of major immune organs of fishes [53] and the spleen index is often used to reflect immune function [54]. Present findings showed that spleen index was increased with the increase in the concentration of *B. subtilis* ($P < 0.05$). This result suggests that *B. subtilis* can stimulate immune activity of *Y. tilapia* by affecting relative weight of spleen since the organ plays an important role in immune responses. Comparatively, low dose of *B. subtilis* had no significant

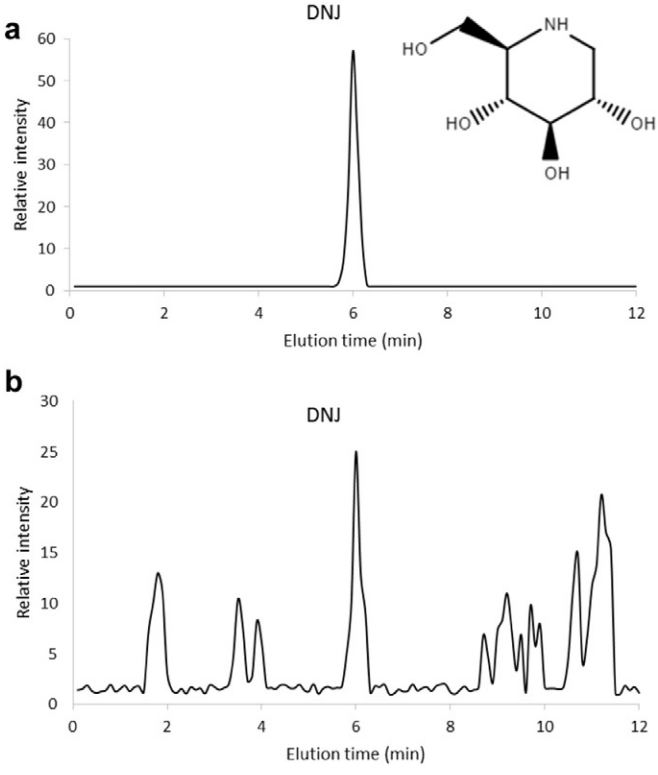

Fig. 1. HPLC analysis of secondary metabolisms of *Bacillus subtilis*. (a) the standard sample DNJ was eluted at 6 min. (b) there was a peak at 6 min from the secondary metabolisms of *Bacillus subtilis*.

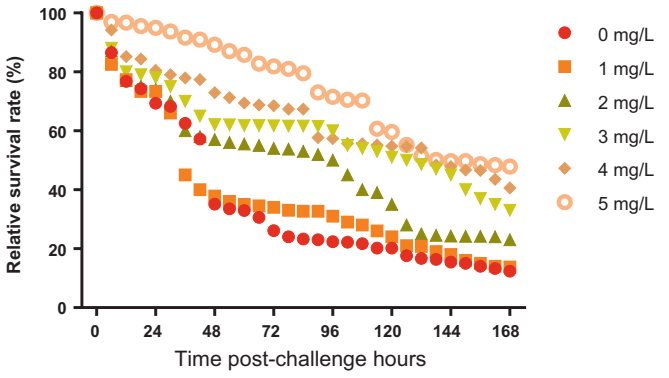

Fig. 2. Evaluation of DNJ on the RPS of juvenile *Y. tilapia* infected with *A. hydrophila*. Different doses of DNJ were used as 0, 1, 2, 3, 4 and 5 mg/L for six groups, respectively. There was no statistical significance of differences for the replicate groups. RPS, relative percent survival.

effect on the relative weight of spleen ($P > 0.05$). One important thing should be paid, the results did not approve that the probiotics can be used in other fish species. For instance, *B. subtilis* consumption in rainbow trout (*Oncorhynchys mykiss*) and brown trout (*Salmo trutta*) widened intestinal lamina propria and submucosa, and increased presence of inflammatory cells. This inflammatory process caused the damage in villi and enterocytes. The results suggest *B. subtilis* has no beneficial effects in trout species [55].

ALP is an important hydrolase enzyme in the body and involved in the transfer and metabolism of phosphate groups. ALP has been demonstrated to have general anti-inflammatory functions as it dephosphorylate potentially deleterious molecules including nucleotide phosphates, the pathogenic endotoxin lipopolysaccharide, and the contact clotting pathway activator polyphosphate, and thus reduces inflammation and coagulopathy [56]. Present findings showed that ALP activity was gradually increased when *B. subtilis* levels were increased and reached the highest level in G6 and G7 ($P < 0.05$). *B. subtilis* increases ALP activity and has been widely reported [57,58].

AST and ALT are sensitive indicators of liver damage [59,60]. *B. subtilis* can significantly reduce serum AST and ALT activity [61]. Present results also showed that that the activities of serum AST and ALT were decreased in *Y. tilapia* with the increase in the concentration of *B. subtilis* ($P < 0.05$). The results suggest that *B. subtilis* has a protective effect on the liver by reducing the activity of serum AST and ALT. The reason may be that *B. subtilis* enters fish intestine and suppresses the propagation of enteric pathogens. *B. subtilis* can remove ammonia, nitrite and other harmful substances [62,63], reduce the burden of liver detoxification, and plays a protective role on the liver [35].

TAC has been used to evaluate the antioxidant ability of animal and shows the antioxidant response against the free radicals produced in a certain situation [64]. SOD has been proved to have radioprotective functions [65] and catalyzes H_2O_2, thereby clearing the reactive oxygen species and preventing cells from damaging [66]. *B. subtilis* increases the levels of exogenous SOD, which will significantly improve cell survival rate after gamma radiation [67]. In this experiment, *B. subtilis* increased the activities of TAC and SOD of *Y. tilapia*. MDA is an important product of oxygen free radicals and attacks the biofilm polyunsaturated fatty acids produced by the cells, and thus it may reflect the extent of cell damage and lipid peroxidation [68]. Therefore, SOD and MDA were generally antagonistic for antioxidant activities. Present findings indicated that *B. subtilis* significantly reduced the serum levels of MDA in *Y. tilapia*. All these results suggest that *B. subtilis* improves scavenging capacity for free oxygen radicals and improves the immune capacity of *Y. tilapia* by affecting TAC, SOD and MDA.

Peptidoglycans [69,70], lipopolysaccharides [71] and dextran [72,73] can stimulate the immune response of animals and improve their

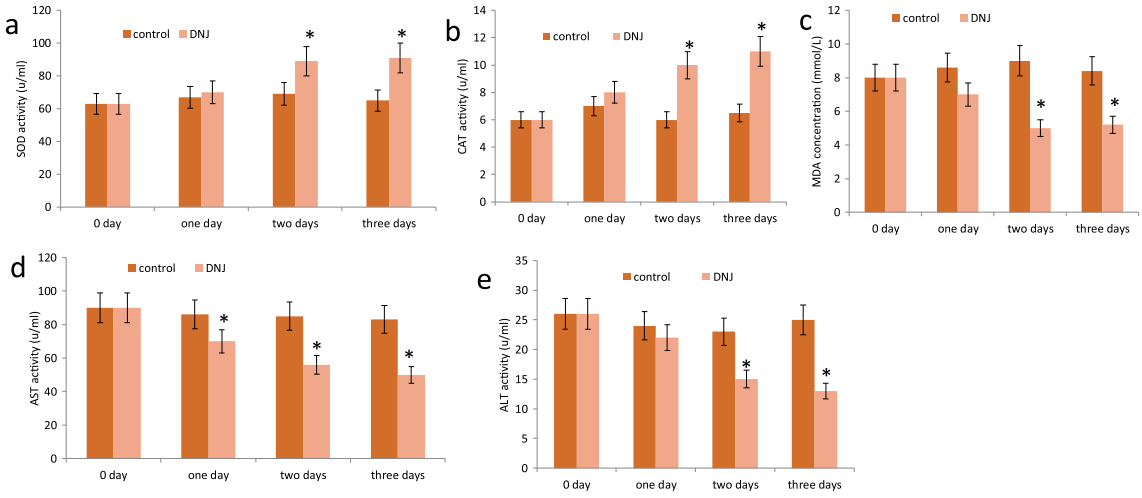

Fig. 3. The effects of DNJ on antioxidant activities of spleen cells. (a), the effects of DNJ on SOD activities. (b) the effects of DNJ on CAT activities. (c) the effects of DNJ on MDA concentration. (d) the effects of DNJ on AST activities. (e) the effects of DNJ on ALT activities. $^{*}P < 0.05$ *vs.* a control group.

immune system. In 1993, Himanen et al. found that LTA (Lipoteichoic Acid) and PG-TA (Peptidoglycan-teichoic Acid Complex, one important component of gram-positive cell wall) from *B. subtilis* was a good immune adjuvant [74]. In this study, the CSR of *Y. tilapia* infected with *Aeromonas hydrophila* was increased after consumption of DNJ when compared with control group without DNJ ($P < 0.05$). The results suggest that dietary DNJ may increase the resistance of *Y. tilapia* for pathogens *via* PG-TA.

As a major active ingredient in *B. subtilis*, DNJ has caused great interest for most scientists because of its effective and specific inhibition of different carbohydrate degrading enzymes involving extensive and important biological processes [75], including glycogenolysis, lysosomal glycoconjugates for catabolism, sugar digestion of sugar digestion and sugar chain maturation [76]. It is well known that DNJ is an α-glucosidase inhibitor that acts as an anti-hyperglycemic agent by slowing the rate of carbohydrate degradation to monosaccharides and can delay glucose uptake [77]. The structure of 1-deoxynojirimycin is similar to that of sugar (Fig. 1A). DNJ has been reported to have anti-virus functions and prevents virus infection [78]. Here we find that the compound shows antibacterial properties for bacterial infection. Further work is still needed to confirm the related molecular mechanisms.

One important question should be considered here. We want to prove that DNJ can improve antioxidant and antibacterial activities of juvenile *Y. tilapia*, but the DNJ concentration of broth is 1 mg/mL and the DNJ concentration of aquatic water in the strain treatment might be lower. *B. subtilis* were incorporated into the diet with a blender, and the feed was prepared by a meat grinder and made of $2 \times 2 \times 2$ mm^3 sticks, and dried. The fish was feed by the sticks, which could be used up in short time. There would be some loss for DNJ in water, but most can be contained in the sticks. Actually, the *B. subtilis* can still live and improve the intestinal microflora of juvenile *Y. tilapia*. The bacteria still can produce DNJ in intestinal environment. On the other hand, even for human beings, only 5 mg of DNJ per dose can be used for some patients [79].

There are some limitations for present work: 1) the components of *B. subtilis* fermentation are complex. The effects of other components on *Y. tilapia* were not measured here. 2) The work was not performed either since DNJ has been reported to have anti-virus functions and prevents virus infection. Virus infection of *Y. tilapia* may be another important issue. Actually, DNJ can affect virus infection by preventing the glycosylation of viral envelope proteins [80]. To confirm the results, further work is highly needed to be done in the future.

In this study, dietary *B. subtilis* improved the growth performance, antiinflammatory and antioxidant properties of juvenile *Y. tilapia* after pathogen infection. For *Y. tilapia* juveniles, the recommended dosage of *B. subtilis* was 0.12%, *i.e.*, 2.5×10^{10} cfu/kg.

Conflict of interest

The authors declare no conflict of interest.

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