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Antibiotic growth promoter olaquindox increases pathogen susceptibility in fish by inducing gut microbiota dysbiosis

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Low dose antibiotics have been used as growth promoters in livestock and fish. The use of antibiotics has been associated with reduced pathogen infections in livestock. In contrast, antibiotic growth promoter has been suspected of leading to disease outbreaks in aquaculture. However, this phenomenon is circumstantial and has not been confirmed in experimental conditions. In this study, we showed that antibiotic olaquindox increased the susceptibility of zebrafish to *A. hydrophila* infection. Olaquindox led to profound alterations in the intestinal microbiota of zebrafish, with a drastic bloom of *Enterobacter* and diminishing of *Cetobacterium*. Moreover, the innate immune responses of zebrafish were compromised by olaquindox ($P<0.05$). Transfer of microbiota to GF zebrafish indicated that while the immuo-suppression effect of olaquindox is a combined effect mediated by both OLA-altered microbiota and direct action of the antibiotic ($P<0.05$), the increased pathogen susceptibility was driven by the OLA-altered microbiota and was not dependent on direct antibiotic effect. Taken together, these data indicate that low level of OLA induced gut microbiota dysbiosis in zebrafish, which led to increased pathogen susceptibility.

antibiotic growth promoter, pathogen susceptability, gut microbiota, fish, olaquindox

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INTRODUCTION

For more than 60 years, low doses antibiotics have been used as growth promoters (AGP) in livestock (Castanon, 2007; Khadem et al., 2014; Xiong et al., 2015). In aquaculture, dietary supplementation with antibiotics has also been shown to improve growth and feed efficiency of fish (Castanon, 2007; Li et al., 2014). Due to the concerns on antibiotic resistance and human health, the European Union and USA has banned the use of antibiotic growth promoters (AGP) in animal feed (Casewell et al., 2003). However, AGP are still being used in aquaculture in many other countries. Also, due to the low cost and reproducible effect on growth, the illegal use of AGP has never completely disappeared worldwide, especially in some small aquafeed companies (Oliveri Conti et al., 2015). China has the largest aquaculture industry in the world, accounting for 61% of the global fish production (Zhang et al., 2015b). Since 2002, China has been the largest exporter of fish and fish products, followed by the United States, Japan and South Korea (Bellmann et al., 2016). In China, AGP used in aquafeed have been banned in 2013, but illegal use still exists. The annual antibiotics abuse in aquaculture exceeds 10,000 ton/year in China (Lillicrap et al., 2015), which to some extent reflects the existence of illegal AGP usage and implicates potential food safety issues.

The vertebrate gastrointestinal (GI) tract contains a composite microbial ecosystem. The composition of the microbiota affects immune responses and susceptibility of

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the host to infection by intestinal pathogens (Fečkaninová et al., 2017; Hai, 2015; Ubeda and Pamer, 2012). Full (therapeutic) dose antibiotic treatment perturbs the intestinal microbiota and renders the host more susceptible to infection by pathogens (Pamer, 2016; Ubeda and Pamer, 2012). Although less substantial relative to the case of full dose administration, sub-therapeutic dose of antibiotics also induces shifts in the composition of intestinal microbiota, as observed in pigs (Looft et al., 2012) and mice (Cho et al., 2012; Cox et al., 2014). However, the impact of sub-therapeutic antibiotic induced microbiota change on pathogen susceptibility of host has been less investigated. The use of low doses antibiotics in livestock has been associated with reduced clinical and subclinical infections under less favourable hygiene conditions, which was suggested as one of the mechanisms for growth promotion (Brüssow, 2015). In line with this, the ban of AGP in Europe led to increased infections and therapeutic antibiotic use in livestock (Casewell et al., 2003; Castanon, 2007). In contrast to livestock, dietary supplementation of AGP was often associated with disease outbreaks and higher mortality in aquaculture practice (Defoirdt et al., 2007). However, the association between sub-therapeutic antibiotic supplementation and increased pathogen susceptibility of fish is circumstantial and has never been investigated under controlled experimental conditions, and the involvement of antibiotic-altered microbiota is not clear.

Olaquindox, a quinoxaline 1,4-dioxide derivative, is known as a potent synthetic antibacterial agent against several gram-positive and gram-negative species (Halling-Sørensen, 2001). Olaquindox was used as a feed additive to improve growth rate and feed conversion in aquaculture (Li et al., 2014). In this study, by using olaquindox as the representative antibiotic, we developed a zebrafish model to demonstrate that antibiotic growth promoter can increase the susceptibility of fish to infections by aquatic pathogen. Furthermore, we used gnotobiotic zebrafish model to evaluate the underlying mechanisms, especially the contribution of olaquindox-altered microbiota to the increased pathogen susceptibility of fish.

RESULTS

Olaquindox supplementation increased pathogen susceptibility of zebrafish at the doses for growth promotion

The body weight of zebrafish was higher in the groups supplemented with olaquindox at 150 and 200 mg kg^{-1} compared to control after four weeks of feeding (P<0.05) (Figure 1A). However, olaquindox supplementation at 150 and 200 mg kg^{-1} led to a trend of higher mortality in zebrafish after *A. hydrophila* challenge (P=0.07) (Figure 1B).

In the following experiments, we chose the olaquindox dose at 150 mg kg^{-1} and a two-week feeding regime. Consistent with the results above, olaquindox supplementation at 150 mg kg^{-1} promoted growth (P<0.05) (Figure 1C) but meanwhile increased the pathogen susceptibility of zebrafish (P<0.01) (Figure 1D) after two weeks of feeding.

The binding and proliferation of *A. hydrophila* ZJB-1 on the intestinal inner surface of fish in the olaquindox group were higher than those in the control group (P<0.05) (Figure 2A and B). Higher pathogen numbers were detected in the

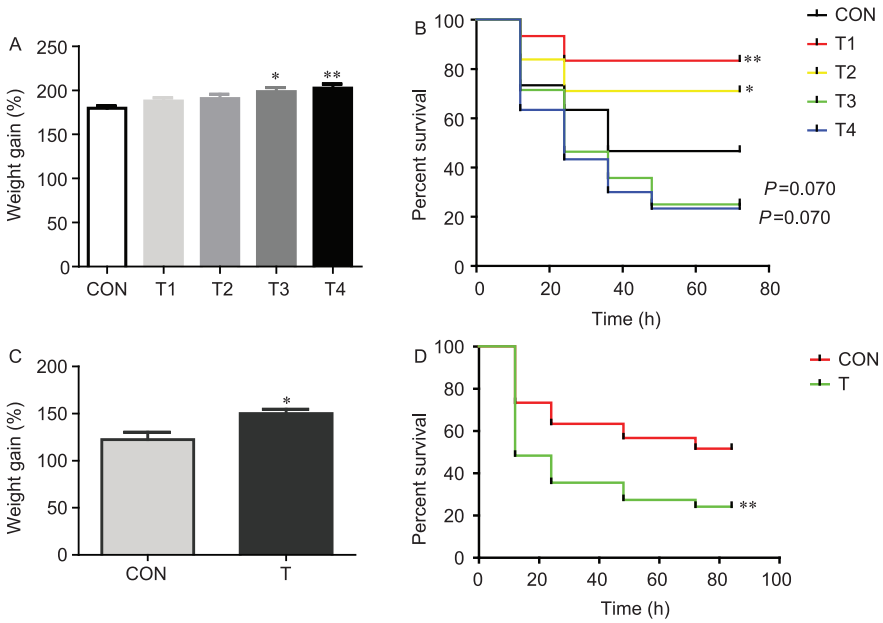

Figure 1 Effects of OLA on weight gain and pathogen challenge survival of zebrafish. A, Weight gain results of zebrafish feed dose gradients of OLA for 28 days. B, Challenge survival results of zebrafish feed dose gradients of OLA for 28 days. C, Weight gain results of zebrafish feed 150 mg kg^{-1} OLA for 14 days. D, Pathogen challenge survival results of zebrafish feed 150 mg kg^{-1} OLA for 14 days. An asterisk indicates a significant difference (*, P<0.05; **, P<0.01) between the control and antibiotic-treated group.

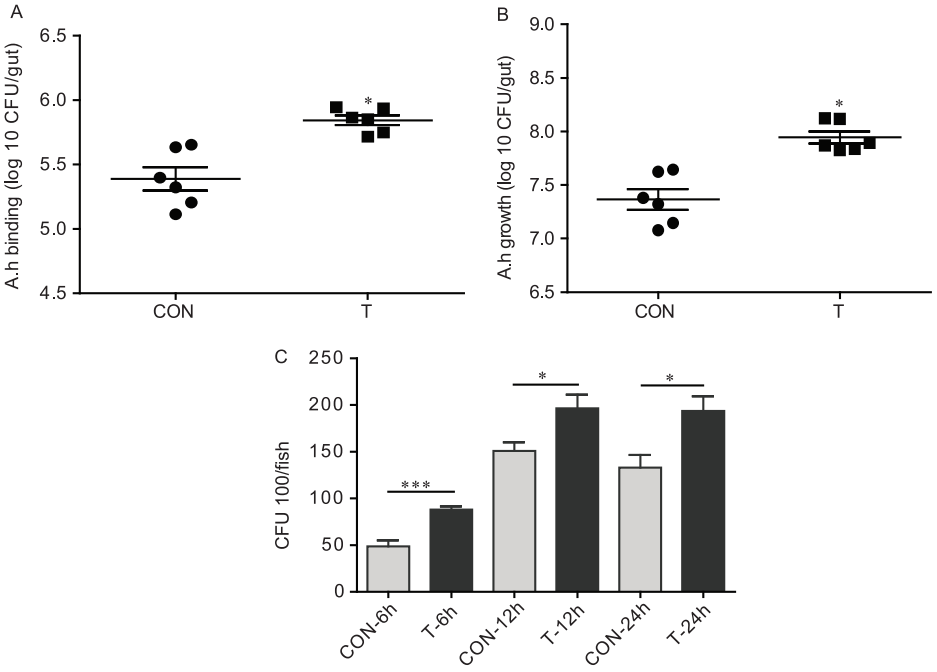

Figure 2 The amount of *A. hydrophila* ZJB-1 in tissues of zebrafish after pathogen challenge to antibiotics treated fish. A, *A. hydrophila* ZJB-1 binding on zebrafish intestinal tissue after OLA feeding for14 days. B, *A. hydrophila* ZJB-1 proliferation on zebrafish intestinal tissue after OLA feeding for 14 days. After antibiotics treatment, zebrafish intestines were opened to expose the inner surface. *A. hydrophila* ZJB-1 cells were mounted on the inner surface for binding and growth measurement. C, The amount of *A. hydrophila* in the kidney after pathogen challenge sampled at 6, 12 and 24 h. An asterisk indicates a significant difference (*, $P<0.05$) between the CON and antibiotic-treated group.

kidney of olaquindox treated fish than those in the control group at 6, 12 and 24 h post challenge ($P<0.05$) (Figure 2C) .

Effect of olaquindox on reactive oxygen species (ROS) production in zebrafish kidney

The fluorescence intensity of ROS in the zebrafish kidney is shown in Figure 3. ROS levels in the fish treated with olaquindox were significantly lower than the control ($P<0.05$) (Figure 3A). The mRNA expressions of two ROS related genes (*Ndi* and *iNos*) in zebrafish kidney were significantly down-regulated in olaquindox-treated group versus control ($P<0.05$) (Figure 3B and C).

Olaquinodx affects gut mucosa immunity

Olaquinodx supplementation suppressed the expression of *Il1β* and *Il10* in the gut mucosa of fish compared with control ($P<0.05$) (Figure 4A and C). The expression level of *Tnfα* and *Hsp70* was not affected by olaquindox ($P>0.05$) (Figure 4B and D).

Olaquindox alters bacterial community composition and diversity

The total bacterial count was lower in the olaquindox group of fish compared to control ($P<0.05$) (Figure 5A). Also, the Shannon index of gut microbiota was lower in the antibiotic treated group than control ($P<0.05$) (Figure 5E). The relative abundance of 10 genera was significantly different between the olaquindox group and control ($P<0.05$) (Figure 5B). The dominant phylum changed from Fusobacteria in control to Proteobacteria in antibiotic group (Figure 5C). In particular, *Cetobacterium* was the only genus in Fusobacteria, and the bloom of Proteobacteria post OLA treatment was mainly attributed to *Enterobacter* (Figure 5D).

Microbiota-mediated and direct antibiotic effect on the immunity of zebrafish

To investigate microbiota-mediated effects, we transferred microbiotas from the control (CON) or OLA group of fish after two weeks feeding to GF zebrafish and compared the responses of the recipients. The results showed that the olaquindox microbiota led to attenuated induction of ROS in the recipient GF zebrafish compared with control microbiota at the 10^6 CFU mL^{-1} microbiota-inoculation concentration ($P<0.05$) (Figure 6A). Consistent with the ROS result, zebrafish colonized with OLA microbiota showed attenuated induction of *Saa* (an acute-phase protein), *Il1β*, *Il10*, *Il8* (a chemokine) and *Cclc25ab* compared with those colonized with CON microbiota ($P<0.05$) (Figure S1 in Supporting Information). The induction levels of *Mpo* (a granulate-specific biomarker) and *Cfb* were not significantly different in zebrafish colonized with the two microbiotas ($P>0.05$) (Figure S2 in Supporting Information).

To investigate the direct effect of the antibiotic, GF zebrafish larvae were incubated with olaquindox for 3 days. We

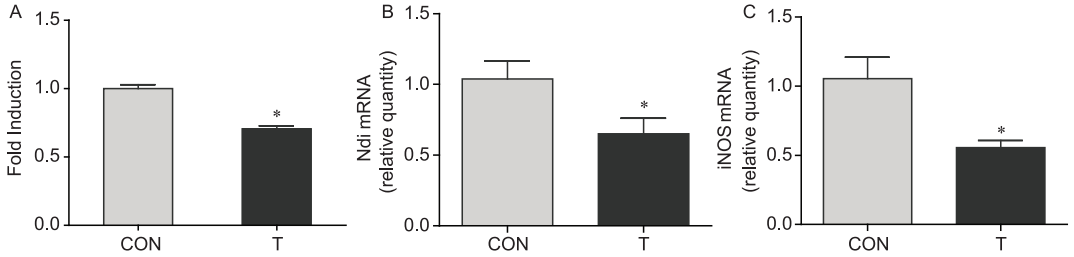

Figure 3 ROS activities and expression of genes related to the regulation of ROS production in the kidney of zebrafish treated with OLA for 14 days. A, Fold-induction of ROS of kidney treated by a OLA compared to the level of CON. B, NADH dehydrogenase subunit (*Ndi*) transcription level of kidney. C, The inducible form of nitric oxide synthase (*iNos*) transcription level of kidney. Each bar represents the average from eight individuals. An asterisk indicates a significant difference (*, P<0.05) between the control and OLA-treated group.

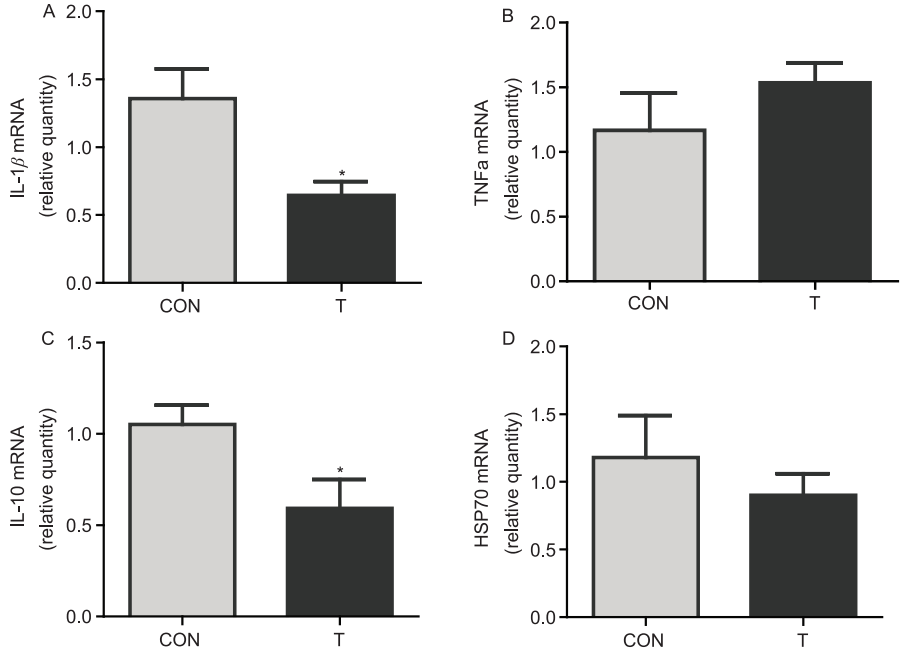

Figure 4 Quantitative PCR analysis of the expression of cytokine genes associated with gut mucosal immunity in zebrafish. A, *Il1β*; B, *Tnfα*; C, *Il10*; D, *Hsp70*.

observed that olaquindox treatment decreased the ROS level in GF zebrafish at 1, 10 and 100 μg mL^{-1} compared with the control (P<0.05) (Figure 6B).

Microbiota-mediated and direct antibiotic effect on the pathogen susceptibility of zebrafish

To investigate the contribution of OLA-altered microbiota to the increased pathogen susceptibility of zebrafish upon OLA feeding, we transferred the OLA and CON microbiota to GF zebrafish and challenged the recipients with *A. hydrophila*. GF recipients colonized with OLA microbiota showed higher mortality compared with those colonized with CON microbiota at 10^6 CFU mL^{-1} microbiota-inoculation concentration (P<0.05) (Figure 7A and B).

Similarly, we also tested the direct antibiotic effect by incubating the GF zebrafish with olaquindox for 3 days and challenging them with *A. hydrophila*. No difference in mortality was detected between the olaquindox-treated groups and control (P>0.05) (Figure 7C and D).

Differential ROS induction by marker species from the CON and OLA microbiota

Several component species of the microbiota were individually inoculated to GF zebrafish at 10^6 CFU mL^{-1} and the ROS activity of the recipients were tested. There was a higher ROS activity in zebrafish colonized with *Cetobacterium somerae* and *Aeromonas veronii* (marker species of CON microbiota) (P<0.05). In comparison, *Enterobacter asburiae* and *Enterobacter* sp., which bloomed in the OLA microbiota, induced lower ROS activity in the GF recipients (P<0.05) (Figure 8A). Accordingly, supplementation of the marker species of CON microbiota to OLA-altered microbiota leads to ROS induction in GF zebrafish comparable to that by CON microbiota (Figure 8B).

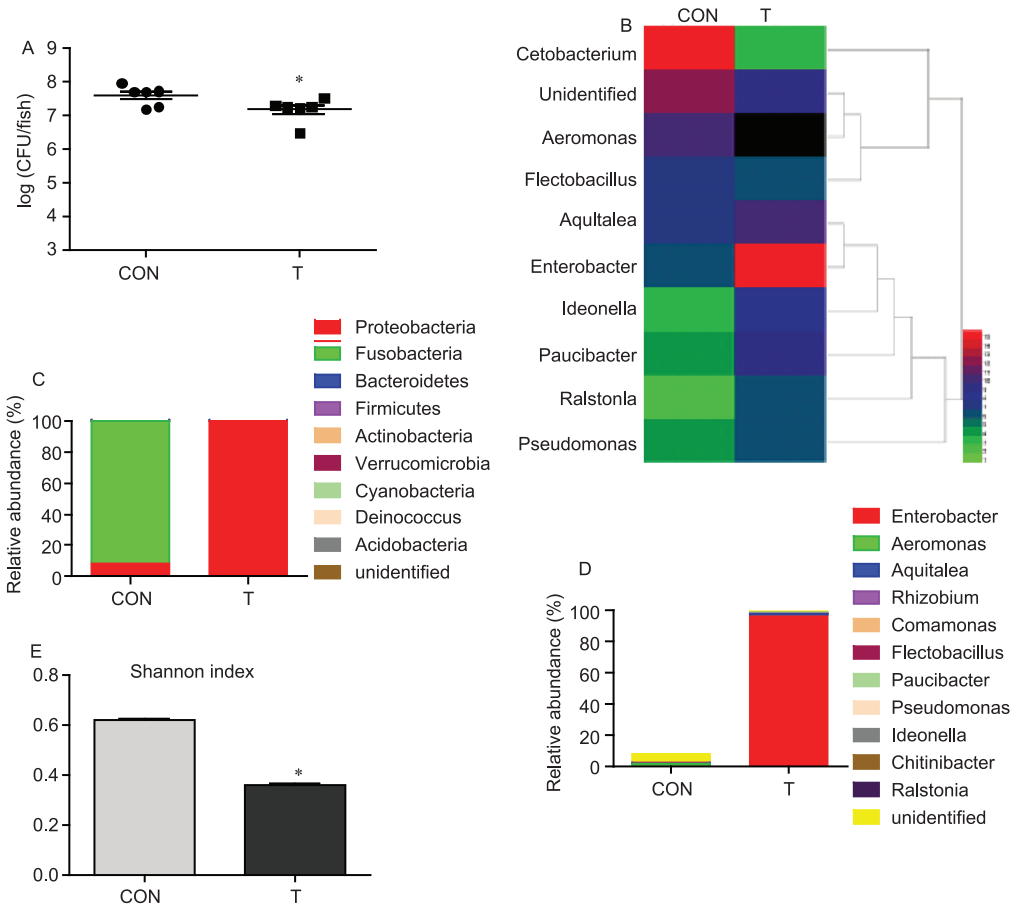

Figure 5 Microbiota composition changes in the gut digesta in response to OLA exposure for 14 days. A, Relative abundance of total bacteria in the gut digesta. B, Heat map of specimens showing the relative abundance of main bacteria identified at the family level. C, Stacked bar chart showing the composition of gut digesta bacterial taxa CON and OAL groups. D, Taxa relative of Proteobacteria abundances in CON and OAL groups. E, Shannon index of gut microbiota of CON and OAL groups.

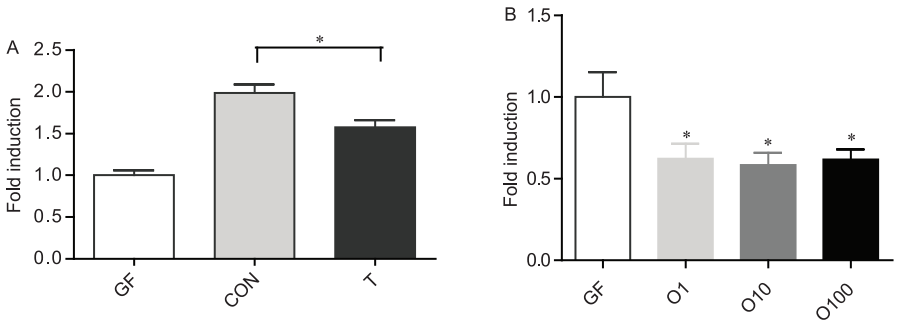

Figure 6 Fold-induction of ROS of larvae. Each bar represents the average from eight individual larvae. Data are representative of three independent experiments. A, GF larvae were treated with gut microbiota from CON or OLA treated zebrafish at 10^6 CFU mL^{-1} for 3 days. B, GF larvae were treated with 1, 10 and 100 μg mL^{-1} OLA for 3 days. An asterisk indicates a significant difference (*, P<0.05) between the control and antibiotic-treated group.

DISCUSSION

It is well appreciated that therapeutic antibiotic treatment may increase host susceptibility to a range of bacterial infections (Faber et al., 2016; Rivera-Chávez et al., 2016). The impact of growth-promotion dose of antibiotics on pathogen susceptibility of host has been less investigated. However, antibiotic growth promoters has been associated with reduced clinical or subclinical infections in livestock (Brüssow, 2015). In this study, we show that antibiotic growth promoter can lead to increased pathogen susceptibility of fish in a zebrafish model. This suggests that fish are more vulnerable to low dose antibiotic influence, which may account for the disease outbreaks and higher mortality that often accompanies antibiotic growth

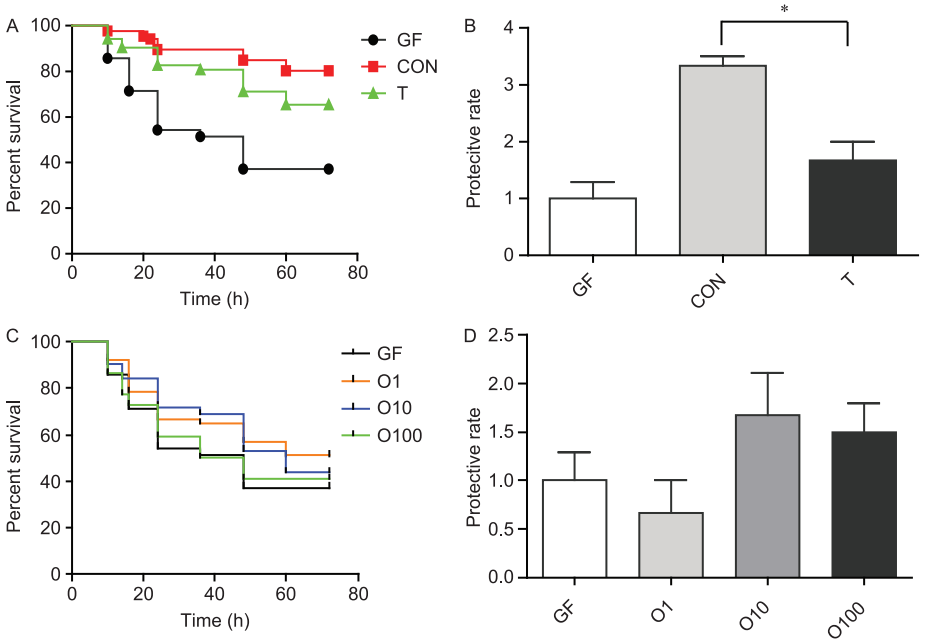

Figure 7 Survival rate of larvae after *A. hydrophila* ZJB-1 bath-infection. A, The survival rate of GF larvae after *A. hydrophila* ZJB-1 bath-infection which were pretreated with gut microbiota from CON treatment or OLA treatment at dose 10^6 CFU mL^{-1} for 3 days. B, The final survival rate of GF larvae after *A. hydrophila* ZJB-1 bath-infection which were pretreated with gut microbiota from CON treatment or OLA treatment at dose 10^6 CFU mL^{-1} for 3 days. C, The survival rate of GF larvae after *A. hydrophila* ZJB-1 bath-infection which were pretreated with 1, 10 and 100 μg mL^{-1} OLA for 3 days. D, The final survival rate of GF larvae after *A. hydrophila* ZJB-1 bath-infection which were pretreated with 1, 10 and 100 μg mL^{-1} OLA for 3 days. An asterisk indicates a significant difference (*, $P<0.05$) between the CON and OLA-treated group.

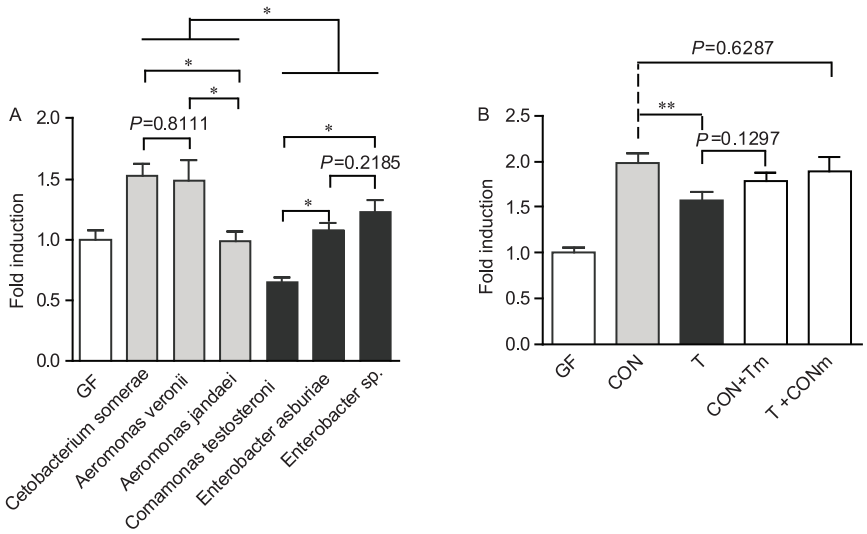

Figure 8 ROS activity of GF larvae colonized with individual bacterial species. Each bar represents the average from eight individual larvae. Data are representative of three independent experiments. A, Fold-induction of ROS activity in GF fish with isolated strains. B, Fold-induction of ROS activity in GF fish with gut digesta microbiota transplant from CON or OLA treatment and the isolated discriminatory bacterial strains. CON, gut digesta microbiota from the CON feeding for 14 days; T, gut digesta microbiota from the OLA feeding for 14 days; CONm, contain 10^6 CFU mL^{-1} *Cetobacterium somerae*, *Aeromonas veronii* and *Aeromonas jandaei* mixed strain isolated from the CON feeding; Tm, contain 10^6 CFU mL^{-1} *Enterobacter asburiae*, *Enterobacter* sp. and *Comamonas testosteroni* mixed strain isolated from the antibiotics feeding. An asterisk indicates a significant difference (*, $P<0.05$; **, $P<0.01$) between the control and antibiotic-treated group.

promoters usage in aquaculture practice (Gao et al., 2012).

Sub-therapeutic olaquindox resulted in profound change in the gut microbiota of zebrafish. The OLA-induced microbiota alteration was characterized by a drastic bloom of *Enterobacter* and diminishing of *Cetobacterium*, which accompanied a significant reduction in diversity and bacterial load. Full dose antibiotics may cause microbiota dysbiosis in human and mice, which is commonly characterized by a reduced diversity of Firmicutes and Bacteroidetes, and often accompanied by an overgrowth of the family Enterobacteriaceae

(Lange et al., 2016). In this regard, the OLA-induced microbiota change resembles the post-antibiotic dysbiosis observed in mammals, both showing reduced abundance of the dominant phylum and a bloom of Enterobacteriaceae. Sub-therapeutic dose of antibiotics induced significant shift in the intestinal microbiota of mice (Cho et al., 2012; Cox et al., 2014) and piglets (Looft et al., 2012), with broad changes observed in the abundance of different taxa. However, the overall alteration of the microbiota was moderate. In one study, the low dose penicillin treatment even increased the phylogenetic diversity of the microbiota (Cox et al., 2014). In comparison, the results in our study suggest that zebrafish microbiota is more vulnerable to antibiotic influence, and the microbiota alteration induced by low dose olaquindox is comparable to the dysbiosis post therapeutic antibiotic administration in mammals (Jeong et al., 2009).

Many studies have reported that depletion of commensal microbes and changing the microbiota composition by therapeutic dose of antibiotics may affect the intestinal immune defenses (Ubeda and Pamer, 2012). In most cases, an immuno-suppression effect was observed. A suppression effect on immune responses has been reported. Low dose of penicillin reduced expression of genes related to intestinal immune responses in mice (Cox et al., 2014). Growth-promotion dose of cyclines decreased the serum amyloid A (SAA) protein level in pigs (Soler et al., 2016) and plasma α_1-acid glycoprotein in broilers (Khadem et al., 2014), both pointing to a systematic anti-inflammatory effect. In the latter case, a local anti-inflammatory effect was also observed, as indicated by the down-regulated jejunal expression of *Il1β*, *Il10* and *iNos* (Khadem et al., 2014). Respiratory burst of phagocytes can be used as a reliable measure of the innate immune response of host (Hermann et al., 2004). After the feeding trial, olaquindox reduced ROS and iNos expression level in the kidney of zebrafish, indicating a suppression effect on the systematic innate immune responses. Also, a local anti-inflammatory effect was indicated by down-regulated intestinal expression of *Il1β* and *Il10*, but not *Tnfα* and *Hsp70*. Other study also showed that antibiotics could down-regulate the expression of a subset of inflammatory cytokines. Ciprofloxacin reduced the total amount of IL-1α and Tnfα produced by LPS-stimulated human monocytes but not the total amount of IL-6, IL-10 and IL-12 (Araujo et al., 2002). The differential effects on the inflammatory cytokines might be attributed to different mechanisms of antibiotics action. All these results suggest that sub-therapeutic dose of olaquindox compromised the innate immune responses of zebrafish, which is consistent with previous reports regarding the effect of low dose antibiotics on host immunity. The microbiota transfer experiment indicated that OLA-altered microbiota contributed to the reduced ROS activity (Figure 6). ROS are produced by macrophages and neutrophils and have been widely used to evaluate the host immune status (Hermann et al., 2004). Notably, the observed difference of CON and OLA microbiota in ROS induction could only be attributed to the microbial structure, as the inoculation concentration was the same. However, OLA administration reduced the bacterial load in the fish intestine. The depletion of microbial populations by antibiotics may lead to depression of immunity (Morgun et al., 2015; Xu et al., 2014). Therefore, the scale of ROS-reduction effect of the original OLA-altered microbiota should be larger. Interestingly, the incubation of GF zebrafish larvae with olaquindox also reduced ROS activity, suggesting that olaquindox may directly interact with the host tissue and suppress the immunity. Together, the results suggest that the immuo-suppression effect of sub-therapeutic olaquindox is a combined effect mediated by both OLA-altered microbiota and the direct action of the antibiotic, which deserves further investigation.

The microbiota transfer experiment indicated that the increased pathogen susceptibility of olaquindox treated zebrafish was driven by the OLA-altered microbiota and was not dependent on direct antibiotic effect on the host. The intact microbiota can exclude invading bacteria, a function termed as "colonization resistance". Microbiota may directly inhibit pathogens by outcompeting nutrients and space (Hornef, 2015) or by antagonistic activity (Coyne et al., 2016). Also, microbiota may indirectly confer colonization resistance through interaction with the host, such as by inducing host innate immune responses (Round and Mazmanian, 2009; Thaiss et al., 2016), or by producing metabolites that improve the epithelial barrier (Kim et al., 2016). The mechanisms underlying colonization resistance are complex and remain incompletely defined (Pamer, 2016). In this study, we are not able to conclude the exact mechanism of colonization resistance by the zebrafish microbiota and which part of the underlying mechanism was impaired by the OLA-mediated alteration. However, the reduced immunity induction of OLA-microbiota should be an important contributing factor to the higher pathogen susceptibility of fish. Notably, although olaquindox may directly reduce ROS activity, GF zebrafish incubated with olaquindox did not show increased pathogen susceptibility. This could be due to that the magnitude of olaquindox-induced ROS reduction was not big enough to affect host susceptibility or it was time dependent.

Consistent with the attenuated ROS induction of the OLA microbiota, the ROS induction of *Enterobacter* was significantly lower than that of *Cetoabacterium*. Also, as marker taxa showing less-scale abundance change, the ROS induction mediated by *Comamonas* was lower than *Aeromonas*, which also accords with the overall ROS induction result. Considering the dominance of *Cetobacterium* and *Enterobacter* in the respective microbiota, the OLA-induced replacement of *Cetobacterium* by *Enterobacter* might be the main contributor to the attenuated immunity induction of OLA mi-

crobiota. However, it is also possible that some low abundant taxa contributed to the reduced immune induction efficiency of OLA microbiota (Rolig et al., 2015). Moreover, although the inoculation concentration of the individual species was the same (10^6 CFU mL^{-1}), the actual colonization of each species in the GF zebrafish might differ, and how the immune induction activity of a bacterial strain correlates with the colonization level is unknown. Further detailed investigations focusing on individual component species of the microbiota including some less abundant ones are warranted to answer these questions. Live probiotics and bacterial ligands showed efficiency to supplement microbiota deficits and/or boost the immune tone after therapeutic antibiotics administration in mammals (Fečkaninová et al., 2017; Ubeda and Pamer, 2012). Based on the results in this study, *Cetobacterium* spp. is a good candidate as probiotics in fish. Also, more understanding of the molecular triggers that underlie the immune induction activity of *Cetobacterium* will provide new opportunities to boost immunity or counteract the negative effect of microbiota dysbiosis in fish.

In conclusion, our study demonstrates that growth-promotion dose of olaquindox may increase the pathogen susceptibility of zebrafish, which is attributable to an antibiotic-induced microbiota dysbiosis. Olaquindox and other antibiotics are still frequently used as growth promoters in aquaculture practice of China and many other countries. We hope the results from this study may confirm their drawbacks associated with disease induction and accelerate the abandonment of AGP usage in aquaculture practice. On the other hand, the proposed vulnerability of zebrafish microbiota to antibiotic influence in this study is intriguing. Considering the specificity of fish microbiota structure compared to mammals, the reason might be that fish microbiota is less resilient against external influences by itself. Also, this might involve host-microbiota interaction in fish, and implies a less stringent control of fish over its microbiota. The fact that fish harbour inefficient specific immunity compared with mammals (Zapata et al., 2006) may contribute to such a deficit. Further investigations in this subject will not only provide more insights in the host-microbiota interaction theoretically, also it will guide more reasonable usage of dietary ingredients and additives in the aquaculture practice, which hopefully takes the vulnerable microbiota of fish into account.

MATERIALS AND METHODS

Experimental animals and design

All experiments were done using AB wild type zebrafish. Experiments were approved of and conducted in accordance with the Chinese legislation associated with animal experimentation and the studies were approved by the Ethics Committee of the Feed Institute, Chinese Academy of Agricultural Sciences (2015-ZZG-ZF-011).

Experiment 1. Zebrafish at 1 month were allocated to five groups (CON, 0 mg kg^{-1} olaquindox; T1, 50 mg kg^{-1} olaquindox; T2, 100 mg kg^{-1} olaquindox; T3, 150 mg kg^{-1} olaquindox; T4, 200 mg kg^{-1} olaquindox. Table S1 in Supporting Information) with 18 fish per 10-L tank in a recirculating system with dechlorinated and aerated water. Each group included four replicate tanks (18 fish×4). The ingredients were ground, milled, weighed, mixed and pelleted with a noodle mincer through a 0.8 mm die. After drying, feed was added to a superfine grinder (Qijian, Jinhua) and ground to achieve a particle size that passed through a 1-mm^2 mesh and stored at −20°C.

Zebrafish in each group were fed corresponding diet twice a day at 9:30 and 15:30 (6% total weight daily and feeding amount adjusted every two days) for 28 days. During feeding, the tank water flow was stopped. The water in each tank was replaced 30% with freshwater every week. Body weight was measured on day 0, 14 and 28, pathogen challenge were detected at day 14 and 28. Standardized conditions were maintained: water was continuously mechanically and biologically filtered, aerated and kept at (28±1)°C, pH, 7.5–7.8; Unionized Ammonia, <0.50 mg N L^{-1}; Nitrite, <0.02 mg N L^{-1}, DO>5.0 mg O/L; photoperiod was kept at 14:10 (light:dark cycle), light spot (9 mm diameter, intensity of 20 μW cm^{-2}) .

Experiment 2. Based on the Experiment 1 results, 150 mg kg^{-1} olaquindox could induce fish growth fast and more mortality after pathogen challenge during the 14 days continual feeding. Therefore, the experiment 2 is set below: Zebrafish at 1 month were allocated to two groups (CON, 0 mg kg^{-1} olaquindox and T, 150 mg kg^{-1} olaquindox) with 18 fish per10-L tank. All groups were fed as described in Experiment 1 for 14 days. After 6 h feeding on the final day (Figure S3 in Supporting Information), the zebrafish were euthanized. The gut samples were immediately transferred into tubes containing 1 mL of RNA later (Qiagen, USA), and stored at −80°C until gene expression analysis.

Aeromonas hydrophila challenge

A. hydrophila strain ZJB-1 (Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences), was isolated in epidemic outbreak aquaculture pond, and screened as the most invasive strain from dozens of different *A. hydrophila* strains. ZJB-1 was grown overnight at 28°C in trypticase soy broth medium with agitation. The overnight culture was suspended in water to reach 10^7 CFU mL^{-1}. After the feeding trial, a group of 30 fish were immersed for 96 h in 500 mL of the ZJB-1 containing water after challenge and fish mortality was monitored every 12 h for four days. The kidney from six zebrafish of each group were sampled at 6, 12, and 24 h post infection, and the *A. hydrophila* strain ZJB-1

counts were determined on ampicilin plates at 28°C.

Respiratory burst activity of kidney

Fish were euthanized with 4 mg mL^{-1} tricaine (Sigma, USA) and the kidneys were surgically removed. Phorbol myristate acetate (PMA)-inducible ROS were detected by oxidation of a nonfluorescent dye 2V,7V-dihydrodichlorofluorescein diacetate (H2DCFDA) to dichlorofluorescein (DCF) (Hermann et al., 2004). A whole kidney sampled from one fish was transferred to a well in a 96 well microplate containing 100 μL of Dulbecco's modified Eagle's medium/F-12 (50% DMEM, 50% F-12, without phenol red; Gibco BRL, Life Technologies, USA). After incubation for 30 min at 28°C, 100 μL of 1 μg mL^{-1} H2DCFDA (Molecular Probes, USA) in 0.4% DMSO and 400 ng mL^{-1} PMA (Molecular Probes) were added to each well to a final concentration of 500 ng mL^{-1} H2DCFDA, 0.2% DMSO and 200 ng mL^{-1} PMA. Fluorescence was measured with excitation and emission filters set at 485 and 530 nm. 10% DMSO was used as background values.

Gut digesta microbiota

To avoid individual variation, 24 fish were randomly chosen from each group. About 200 mg gut digesta sample was taken from 12 fish and pooled together at 6 h post the final feeding. Thus each group includes two replicates. The sample was homogenized using bead beating procedure at 30 Hz. Bacterial genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's recommendations. The quality and integrity of the DNA samples was determined by electrophoresis in 1% agarose gel with Tris-acetate-EDTA (TAE) buffer. DNA concentration was quantified using NanoDrop ND-2000 spectrophotometer (Termo Scientific, USA). The V3-V4 region of the 16S rRNA gene was amplified for each DNA sample by PCR according to the previously described methods (Zhu et al., 2015). The sequencing was performed in AllWeGene, Beijing, China. The sequence data were processed using QIIME Pipeline-Version 1.7.0 (http://qiime.org/). The sequences with an average phred score lower than 20, ambiguous bases, homopolymer runs exceeding 8 bp, primer mismatches, or sequence lengths shorter than 150 bp were removed. Only the sequences with an overlap longer than 10 bp and without any mismatch were assembled according to their overlap sequence. The reads that could not be assembled were discarded. All the sequence reads were trimmed and assigned to each sample, based on their barcodes. The barcode and sequencing primers were trimmed from the assembled sequence, and aligned with the Bacterial SILVA database (SILVA SSU 123). Alpha diversity and beta diversity metrics were calculated on rarefied OTU tables with OIIME to assess sampling depth coverage using observed species, phylogenetic diversity, Cho1, and Shannon's diversity index. The heatmap was constructed using the heatmap 2 function of the R gplots package.

RNA extraction and quantitative real-time PCR

The gut samples obtained in Experiment 2 were subjected to RNA extraction. Total RNA was extracted using the TRIzol Reagent RNA kit (Promega, Germany) following the manufacturer's instructions. cDNA was subsequently synthesized using the ReverTra Ace-α-RT-PCR kit (TOYOBO, Shanghai). Real-time PCR was performed using SYBR Green Premix EX Taq TM11 (TaKaRa, Japan) and the ABI 7500 real-time PCR detection system (Applied Biosystems, USA). The real time PCR reactions utilized the following conditions: 95 °C for 3 min and then 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Dissociation curves were analyzed to assess the melting temperature for each PCR product. Ribosomal protein S11 (rps11) gene was selected as the reference gene. The experiment was repeated for at least three times and data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Zhang et al., 2015a).

***Ex vivo* intestine assay**

To measure *ex vivo* bacterial (pathogens) binding and growth in the fish gut treated with antibiotics, fish intestines were sampled under sterile conditions and immediately bath in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS) and 0.5% d-mannose. The intestinal specimens were dissected and the digesta were removed to expose the intestinal inner surface.

For the binding assay, as described as (Liu et al., 2016), 100 μL of *A. hydrophila* ZJB-1 (10^8 CFU mL^{-1}) was applied to the inner surface of the intestine, and the samples were incubated at 28 °C for 30 min in DMEM. After incubation, the intestinal tissue was rinsed three times in 1 × PBS buffer. Each rinse was followed by a 10 s vortex step to ensure that mucosal bacteria were separated from mucus bacteria. Next, the tissues were homogenized and cell counts were determined on LB plates (for *A. hydrophila* ZJB-1).

To assess *A. hydrophila* ZJB-1 growth in fish intestinal mucosa (Liu et al., 2016), intestines were collected and dissected to remove the digesta. The samples were subsequently immersed into sterilized 1 × PBS buffer (1:1, weight/volume), and were vigorously mixed by vortexing for 3 min (Qilinbeier Voatex, Haimen Qilinbeier, Haimen) to facilitate mucosa extraction. An overnight *A. hydrophila* ZJB-1 culture (2 μL) was inoculated to 200 μL of the extracted mucosa, and the mixture was incubated at 37 °C for 12 h. The number of bacteria in the mixture was determined by serial dilution, plating on LB agar, and incubation.

Preparation of gnotobiotic zebrafish

GF zebrafish were prepared following established protocols

(Oyarbide et al., 2015) with some modifications. Briefly, embryos 6 h post-fertilization were soaked in 0.1% polyvinyl pyrrolidoneiodine (Sigma) for 2 min and washed three times in gnotobiotic zebrafish media (GZM). Thereafter, the embryos were further soaked in 0.003% bleach for 10 min and washed by GZM. Lastly, GF embryos were transferred to a 25-cm^2 cell culture flask (Nest Biotechnology Co., Wuxi) containing 30 mL of sterile GZM.

Transfer of gut bacteria from adult zebrafish to GF recipients

The gut digesta of fish fed control or olaquindox supplemented diets (150 mg kg^{-1}) for 2 weeks were collected. Briefly, distal contents were pooled from 6 fish and suspended in 1 mL of PBS. Then the bacterial suspension was added to GZM containing 3-d post fertilization (dpf) GF zebrafish at a final concentration of 10^6 CFU mL^{-1} of GZM. The concentration of bacteria in GZM was confirmed by culture on lysogeny broth (aerobic) and brain-heart infusion-blood (anaerobic) agar for 24 h at 28°C. For this experiment, three treatments were included: GF (GF zebrafish with no microbial inoculation), CK (GF zebrafish colonized with gut bacteria of control), T (GF zebrafish colonized with gut bacteria of olaquindox-treated fish). Each treatment contained 12 replicates with 10 zebrafish/replicate. At 6 dpf, eight replicates were sampled from each of the three treatments and stored in TRIzol at −80°C. Total RNA was exacted following published protocol. Thereafter, quantitative RT-PCR analysis was conducted to evaluate expression of selected gene markers related to immunity (Table S2 in Supporting Information). Lastly, the zebrafish were challenged with *A. hydrophila* ZJB-1 as described above.

Direct effect of oliquindox on GF zebrafish

Olaquindox was added to GZM to make 0, 1, 10 and 100 μg L^{-1} solution to the 3-dpf GF zebrafish. Each treatment contained 6 replicates with 30 fish. The protection effect of the oliquindox was evaluated by *A. hydrophila* ZJB-1 challenge as described above.

Respiratory burst assay using whole zebrafish larvae

The production of ROS was measured in GF zebrafish larvae at 6 dpf. Conditions for detection of ROS by fluorescence measurements were detected as described as above (Hermann et al., 2004), except that all solutions were prepared in egg water. Data from six individual larvae were averaged, and standard errors of the mean were calculated for each experiment.

Statistical analysis

Results are expressed as means with pooled SEMs. Differences between treatment means were determined by the Duncan's multiple range test. All statistical analyses were performed by using the SPSS 18.0 software. $P<0.05$ were taken to indicate significance.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 The total number of bacteria in gut digesta of zebrafish after satiation feeding.

Figure S2 Quantitative PCR analysis of the expression of cytokine genes in GF larvae colonized by different microbiotas at 10^6 CFU mL^{-1}.

Figure S3 Microbial composition changes in the gut digesta of zebrafish fed OLA-supplemented diet for fourteen days.

Table S1 Feed formulation and chemical composition

Table S2 Primers for real-time PCR

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