Short communication

The influence of three antibiotics on the growth, intestinal enzyme activities, and immune response of the juvenile sea cucumber *Apostichopus japonicus* selenka

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ABSTRACT

The global abuse and misuse of antibiotics in the treatment and prevention of bacterial infections has resulted in the ubiquitous existence of these drugs in aquatic environments, which causes frequent antimicrobial resistance and pollution in ecosystems. However, the chronic effects of antimicrobial agents on aquatic animal growth and health have not been fully evaluated. In the present study, three typical antibiotics (tetracycline, erythromycin, and norfloxacin) were administered orally to juvenile sea cucumbers *Apostichopus japonicus* for 45 days, to mimic the long-term use of antibiotics. As a result, tetracycline and erythromycin promoted the growth and digestive activity of lipase, pepsin, and trypsin, but norfloxacin did not show significant prompting effect on digestive activity and even retarded the weight gain of the sea cucumbers. The mortality was higher in antibiotic treated groups between the 2nd and 4th days after challenge with *Vibrio splendidus*. At the same time, lower immune-related parameters were found in antibiotic feeding juveniles, suggesting that the use of antibiotics might weaken the immune defense system of sea cucumbers. This study revealed that antibiotic administration could facilitate the growth of sea cucumbers to varying degrees yet coupled with high risks of impaired immune function and compromised disease resistance.

1. Introduction

As the most influential innovation of the twentieth century, antibiotics are widely used in the treatment and prevention of bacterial infections, making significant contribution to the improvement of human and animal health over the last few decades. However, with the overuse and abuse of antibiotics in aquaculture and livestock husbandry all over the world, these compounds are emerging as new persistent environmental contaminants due to their limited biodegradability in organisms and inefficient removal in sewage treatment plants [1,2]. Numerous studies have reported that antibiotics were frequently detected in the ground water, rivers, lakes and seawater, especially in China [3–7]. Although the detected concentrations range from ng L^{-1} to mg L^{-1} in the aquatic environment, the antibiotics might induce the development of resistant bacteria and genes, so they could seriously present a great threat to human health and the safety of the entire ecosystem [8].

In addition to stimulating the spread of antimicrobial resistance, the ubiquitous presence of antibiotic residues in the aquatic environment might also raise serious concerns of biological toxicity, such as growth retardation, nephrotoxicity, and immunosuppression. There is a great deal of research indicating that these drugs lead to immune responses and injured function in fish. For example, the presence of sulfonamides could significantly damage the antioxidant defense system and neuronal function in zebrafish (*Danio rerio*) [9]; another study has reported that the administration of oxytetracycline led to liver damage in Atlantic salmon (*Salmo salar* L.) [10], while Lunden and Bylund found that oxolinic acid, oxytetracycline and florfenicol inhibited the mitogenic response of head kidney cells in rainbow trout (*Oncorhynchus mykiss*) [11].

The sea cucumber *Apostichopus japonicus* is an important marine economic species in Asian countries due to its profound nutritional and medicinal value [12]. With the rapid development of intensified artificial aquaculture of sea cucumbers, the use of antibiotics is still an

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inexpensive and dispensable way to treat pathogenic infections, especially during the nursery phase [13]. In the production practice, the oral administration of antibiotics by mixing the medicines with the aquatic feeds is the most common application method [14]. However, the influence of the long-term use of antibiotics on the sea cucumber's health remains unknown.

In this study, three typical antibiotics were administered orally to juveniles of *A. japonicus* for 45 days; growth performance was measured during the experiment, and then, pathogenic bacteria *Vibrio splendidus* were introduced to the sea cucumbers at the end of the trial to test the anti-infection ability. Furthermore, the digestive enzyme activity and immune response were also determined to evaluate the risk and benefit of the long-term use of antibiotics on sea cucumber growth and health.

2. Materials and methods

2.1. Experimental animals and antibiotic exposure

Four hundred twenty healthy juvenile sea cucumbers with average weight of 2.57 ± 0.3 g were purchased from Oriental Ocean Technology Co., Ltd, Yantai, China. Before the experiment, sea cucumbers were allowed to acclimate for 3 days in 300 L aquariums. During acclimatization, the water temperature was maintained at 16 ± 2 °C and pH maintained between 7.9 and 8.1, with continuous aeration and daily seawater renewal. After acclimatization, the sea cucumbers were randomly allocated to four groups in 40 L tanks under the same condition as that during the acclimation. All juveniles were fed with a formula feed of 1.5% of their body weight every day, and the three experimental groups had three kinds of typical antibiotics (tetracycline, erythromycin, and norfloxacin) added as a 2% weight percentage of formula feed, while the control group had nothing but formula feed added. During the experiment, the sea cucumbers were fed once at 17:00 p.m., and the water from each tank was totally exchanged every day after the residual feeds and feces were removed. Tetracycline (CAS: 60-54-8), erythromycin (CAS: 114-07-8), and norfloxacin (70458-96-7) were purchased from MeiLun Biotechnology Co., Ltd, Dalian, China. Each treatment had three replicates. The experiment lasted 45 days, the weight of the juveniles in each tank was recorded during the experiment, and the quantity of feed was adjusted correspondingly.

2.2. Sample collection and bacterial challenge

Twelve individuals from each group were randomly sampled at 15 days, 30 days, and 45 days from each tank. The intestinal tract of sea cucumber samples was immediately stored in liquid nitrogen for further analysis. All juveniles were weighed, and the specific growth rate (SGR) was given as follows [15]:

$$SGR(\%) = \frac{\ln W_t - \ln W_0}{t} \times 100$$

Note: W_t and W_0 are the initial and final wet weight of sea cucumbers (g), and t is the duration of the sample time.

At the termination of the experiment, *Vibrio splendidus* was added to challenge the sea cucumbers in the control and three antibiotic treatment groups. *V. splendidus* was grown in liquid 2216E broth at 28 °C with shaking at 220 rpm for 14 h. After the overnight culture, *V. splendidus* was centrifuged at 1000 g for 5 min and resuspended in filtered seawater. Then, twenty juveniles from each aquarium after the feeding experiment were immersed in water containing the bacteria at a final concentration of 10^7 CFU/ml for 7 days. During the challenged period, the juvenile sea cucumbers were not fed with diets. The water and bacteria were completely changed every day; the mortality of sea cucumbers was observed, and dead ones were removed every day.

2.3. Enzyme analysis

The intestinal tracts of eight juvenile sea cucumbers in different antibiotic treatment groups were weighed and homogenized with 0.8% physiological saline and then centrifuged at 2500 × g at 4 °C for 15 min and the supernatant was collected for further enzyme analysis.

2.3.1. Digestive enzyme activities

A series of digestive enzyme activities including those of lipase, pepsin, trypsin, and cellulase was determined using assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer's instructions. The lipase was determined by the simplified turbidimetric assay [16] at a reaction temperature of 37 °C, and activities were expressed as U mg protein^{-1}. One unit of pepsin activity was defined as the amount of enzyme required for 1 μg of tyrosine to be liberated at 37 °C (U/mg protein/min); one unit of trypsin activity was defined as the amount of enzyme required for an OD values change of 0.003 at 37 °C and pH 8.0 (U/mg protein/min); one unit of cellulase activity was defined as the amount of enzyme required to liberate 1 μg of reducing sugar at 37 °C (U/mg protein/min).

2.3.2. Immune-related enzyme activities

A series of immune-related enzyme activities including superoxide dismutase (SOD) activity, catalase (CAT) activity, phosphatase (ACP) activity, and alkaline phosphatase (AKP) activity was determined using commercial assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer's instructions. One unit of SOD activity was defined as the amount of enzyme required when inhibition rate reached 50% in a 1 ml reaction system at 37 °C (U/mg protein/min); one unit of CAT activity was defined as the amount of enzyme required for 1 μmol of H_2O_2 to be liberated at 37 °C (U/mg protein/S); and one unit of ACP and AKP activity was defined as the amount of enzyme required for 1 g of phenol to be liberated at 37 °C (U/g protein/30 min). The results were recorded on a microplate reader (Epoch, BioTek, USA) according to the instructions of the manufacturer.

2.4. Quantitative real-time PCR

Total RNA was extracted from intestinal tract tissue of four juvenile sea cucumbers in each treat group using RNAiso Plus reagent following the manufacturer's instructions (TaKaRa, China) and was treated with RNase-free DNase (TaKaRa, China). The RNA integrity was confirmed by electrophoresis on 1% agarose gels, and the RNA concentration was measured by a NanoDrop 1000 (Thermo, USA). The first-strand cDNA was synthesized with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRA, Dalian, China) with 500 ng total RNA in each reaction. The gene expression patterns of several immune-related genes including *lysozyme* (*LZM*), *C type lectin* (*CLEC*), *NF-kB1* (*p105*) and *glutathione S-transferase* (*GST*) were determined by quantitative real-time PCR (qPCR) on an Applied Biosystems StepOne Real-time PCR System (Applied Biosystems, USA). The primers of the above genes were designed using Primer 3 online (http://frodo.wi.mit.edu/) (Table 1), and *β-tubulin* (TUBB) gene was used as reference control gene according to a previous study [17], and specificity of all primers used in our study were verified very well by PCR reactions before the experiment. The qPCR was carried out in a total volume of 20 μL using an SYBR Green® real-time PCR assay (SYBR PrimeScriptTM RT-PCR Kit II, TaKaRA), including 10 μL of SYBR Green Master Mix, 0.8 μL of each forward and reverse primer (10 μM), 2 μL of diluted cDNA, 0.4 μL ROX Reference Dye (50×) and 6 μL RNase-free water as follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. A melting curve analysis of the amplification products was performed to confirm that the unique PCR product was amplified and detected. Cycle threshold (Ct) values were recorded for further analysis.

Table 1
Primers used in this study.

Gene symbol	Gene name	Accession Number	Left Primer sequences (5′→3′)	Right Primer sequences (5′→3′)
TUBB	β-tubulin	MRZV01000406.1	GAAAGCCTTACGACGGAACA	CACCACGTGGACTCAAAATG
LZM	Lysozyme	KF773759	AGGGAGGTAGTCTGGATGGA	GCGCAAAATCCTCACAGGTA
CLEC	C-type lectin	JN133520	CCTGGTTGGGGTATGTCTGT	AGTGTCCGGTAGCTAAGTCG
p105	NF-kB1	JF828766	GCAACACACCCCTCCATCTT	TCTTCTTCGCTAACGTCACACC
GST	Glutathione S-transferase	KX503261	CAACCCACGGAAAAAGTTACCTG	TTGCTGTCTGTTTAGTGTCTGGG

2.5. Statistical analysis

Statistical analyses were performed using SPSS 18.0 software (SPSS Inc, Chicago, USA). All results are presented as mean ± standard error (SE). The differences of the corresponding values at the same sampling time were tested by a one-way analysis of variance (ANOVA) followed by a LSD test in the enzyme analysis and gene expression analysis. The Student's t-test was used to independently compare the SGR and mortality of each group treated with antibiotics with the control group. $p < 0.05$ was accepted as statistically significant.

3. Results and discussion

3.1. Effects of antibiotics on growth performance and mortality rate after bacterial challenge of sea cucumber

In our study, three antibiotics have different effects on the growth performance of juvenile sea cucumbers. As shown in Fig_1, juveniles fed diet supplement with erythromycin had the highest SGR in all groups all throughout the feeding experiment, with 15-day and 45-day SGRs that were significantly higher than those of control group ($p < 0.01$ and $p < 0.05$, respectively). However, sea cucumbers fed the norfloxacin diet had the lowest SGR ($p < 0.01$), even presented as negative growth. For the tetracycline diet group, this medicine had a significant growth-promoting effect on A. japonicas at the early stage of the experiment ($p < 0.05$), but this effect gradually waned over time. In culture industries, antibiotics have been widely used in the animal and aquatic feeds as growth promoters and prophylactics in the past, but the effects of antibiotics on the weight gain of animals remain inconsistent according to research reports. First, different kinds of antibiotics have various influences on the growth performance of aquatic animals. For example, tetracyclines generally play a beneficial role in growth promotion [18–20], while quinolones and sulfonamides do not show obvious growth-promoting effects, and sulfamerazine could even retard the growth of brook trout and zebrafish [21,22]. Second, one antibiotic can have different effects on different species; for example, oxytetracycline helped accelerate the weight gain of Ictalurus punctatus, Oreochromis niloticus and Xiphophorus helleri, but this same effect was not observed in zebrafish and Carassius auratu [18–20,23]. In the current study, dietary tetracycline and erythromycin promoted the growth of sea cucumbers to a certain degree, but dietary norfloxacin induced lower weight gains in juveniles, and the mechanisms by which antibiotics affect growth are currently not clearly understood.

As the results show in Table 2, antibiotic diet groups were observed to have obviously higher mortality than that of the control group after 2 days of infection, and the significant difference began to appear between the antibiotic groups (tetracycline diet group and norfloxacin diet group) and the control group on the third day after the bacterial challenge ($p < 0.01$). As the strain of V. splendidus is a major pathogenic bacterium for skin ulceration syndrome [24], it was used to test the disease resistance of sea cucumbers after antibiotic treatment. The current data show that juveniles in the antibiotic groups had higher mortality than the control group, indicating that the antibiotic treatment could leave sea cucumbers more vulnerable to infections. Similar results were also reported in rainbow trout and zebrafish [23,25].

3.2. Effects of antibiotics on digestive enzyme activity of sea cucumber

Considering the disparate growth performance in antibiotic-diet groups, we examined the main digestive enzyme activity levels of sea cucumber gut, and the results indicated that the three antibiotics also had different effects on digestive activities (shown in Fig_2). It was found that dietary addition of tetracycline significantly increased the pepsin activity, lipase activity, and trypsin activity at first 15 days ($p < 0.01$), and though the growth of enzyme activity fell afterwards, it was still significantly higher than that in controls in 30 days ($p < 0.05$). Similarly, the diet with added erythromycin also had varying degrees of promotional effects on the above three digestive enzyme activities of juveniles, and lipase activity at 30 days, the pepsin activity at 30 days and trypsin activity at 30 days and 45 days showed significantly higher enzyme activity than that in the controls ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively). However, the above three digestive enzyme activities were not significantly altered in the sea cucumbers fed a diet with norfloxacin. For cellulase, sea cucumbers showed lower enzyme activity under antibiotic exposure compared to that of the control group, and the difference reached significant levels in 15 days for the norfloxacin-diet group ($p < 0.01$) and 45 days for the erythromycin-diet group ($p < 0.05$).

The abilities of marine species to absorb and utilize nutrients depend on the activities of digestive enzymes, and the enzyme activity is proven to be closely related with feeding levels and growth performance [26,27]. As previous studies reported, digestive enzyme activity was affected by various factors, such as water salinity, water temperature, environmental and intestinal microflora, diet formula, feeding rate, and the degree of gut development [28–30]. In our study, dietary addition of tetracycline and erythromycin enhanced the activity of pepsin, lipase, and trypsin, with similar results observed in antibiotic treatments of zebrafish and pigs [23,31]. In contrast, it is worth noting
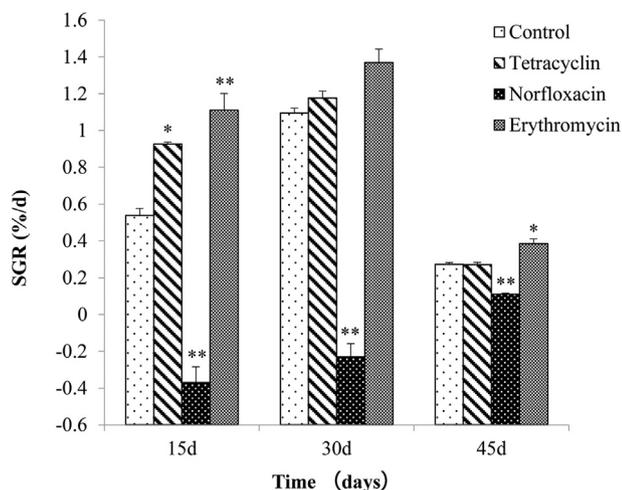
Fig. 1. Influence of feeding tetracycline, erythromycin, and norfloxacin on the growth performance sea cucumbers during the 45-day experimental period. Data was presented as mean ± SE. Student's t-test was used to detect the significant differences between the control and antibiotic exposed groups, * represents $p < 0.05$, ** represents $p < 0.01$.

Table 2
The mortality of sea cucumber challenged with *V. splendidus* after different antibiotic treatment.

Time	Mortality(%)							
	0d	1d	2d	3d	4d	5d	6d	7d
Control	0 ± 0	3.33 ± 0.96	13.33 ± 0.96	18.33 ± 0.96	41.67 ± 2.55	71.67 ± 2.55	86.67 ± 0.96	93.33 ± 1.92
Tetracycline	0 ± 0	6.67 ± 0.96	20 ± 1.67	40 ± 1.67**	55 ± 1.67	75 ± 1.67	86.67 ± 0.96	96.67 ± 1.92
Norfloxacin	0 ± 0	6.67 ± 0.96	21.67 ± 0.96	36.67 ± 0.96**	70 ± 1.67	81.67 ± 0.96	91.67 ± 0.96	100 ± 0
Erythromycin	0 ± 0	5 ± 0	15 ± 1.67	26.67 ± 0.96	48.33 ± 2.55	76.67 ± 0.96	86.67 ± 0.96	95 ± 1.67

*Note: data was presented as mean ± SE. Student's t-test was used to detect the significant differences between the control and antibiotic exposed groups, * represents $p < 0.05$, ** represents $p < 0.01$.

that the three antibiotics all showed cellulose inhibitory activity in the present study. We speculate that this effect is likely due to pepsin, lipase, and trypsin being secreted from the gut enterocyte while cellulose is produced by gut microbiota [32]. Since antibiotic treatment can greatly modulate the gut microbial ecology, which may in turn influence this digestive enzyme. Our unpublished data have further reinforced that the above antibiotic treatments all induced the lower abundances of *Psychromonas* in gut microbiota of sea cucumber, which can produce cold-tolerant cellulose.

3.3. Effects of antibiotics on immune response of sea cucumber

The faster killing rate by *V. splendidus* is probably related to damaged immune function, so several immune-related parameters were detected in our study. ACP and AKP are important components of the nonspecific immune system in sea cucumbers [33,34], and the results in our present study indicated that ACP activities in the three antibiotic-diet groups all declined significantly during the first 15 days of the feeding experiment (Fig_3C, $p < 0.01$), though they increased briefly after 30 days in the norfloxacin and erythromycin groups and again decreased at 45 days. A similar trend was observed in AKP activities, but the recovery rate appeared to be better in the tetracycline and erythromycin diet groups at 45 days (Fig_3D). The mRNA expression of several important immune-related genes in the gut was also tested to

evaluate the influence of antibiotic diet addition on immune response at a molecular level. Compared to that of the control group, the relative expression of *p105* and *LZM* showed a significant decline in all antibiotic-diet groups under early 15-day exposure ($p < 0.01$, Fig_4A and Fig_4B). After a short-term upregulation, the expression levels of *p105* decreased again in the antibiotic diet groups, while the expression levels of *LZM* in the tetracycline and erythromycin diet groups exhibited a rapid recovery in the late stage of the experiment. For the gene *CLEC*, the expression continuously decreased with prolonged feeding time in all the antibiotic-diet groups, and the difference reached a significant level at 45 days compared to that of the control ($p < 0.01$, Fig_4C). In contrast, an early diet supplemented with antibiotics seemed to induce the rapid upregulation of *GST* compared to that of controls, but the expression level decreased sharply and even significantly reversed the latter expression levels in the norfloxacin-diet group and tetracycline-diet group (Fig_4D).

Lacking adaptive immunity, echinoderms mainly reply on a non-specific immune system against pathogens and infection [35]. As the interface between external environment and immune system, the intestine is an important barrier in the innate immune defense of sea cucumbers [36]. The current study found that administration of antibiotics resulted in decreased levels of major immune enzyme activities and important immune-related genes in the gut of *A. japonicas*. Additionally, among these immune indexes, some appeared to recover and
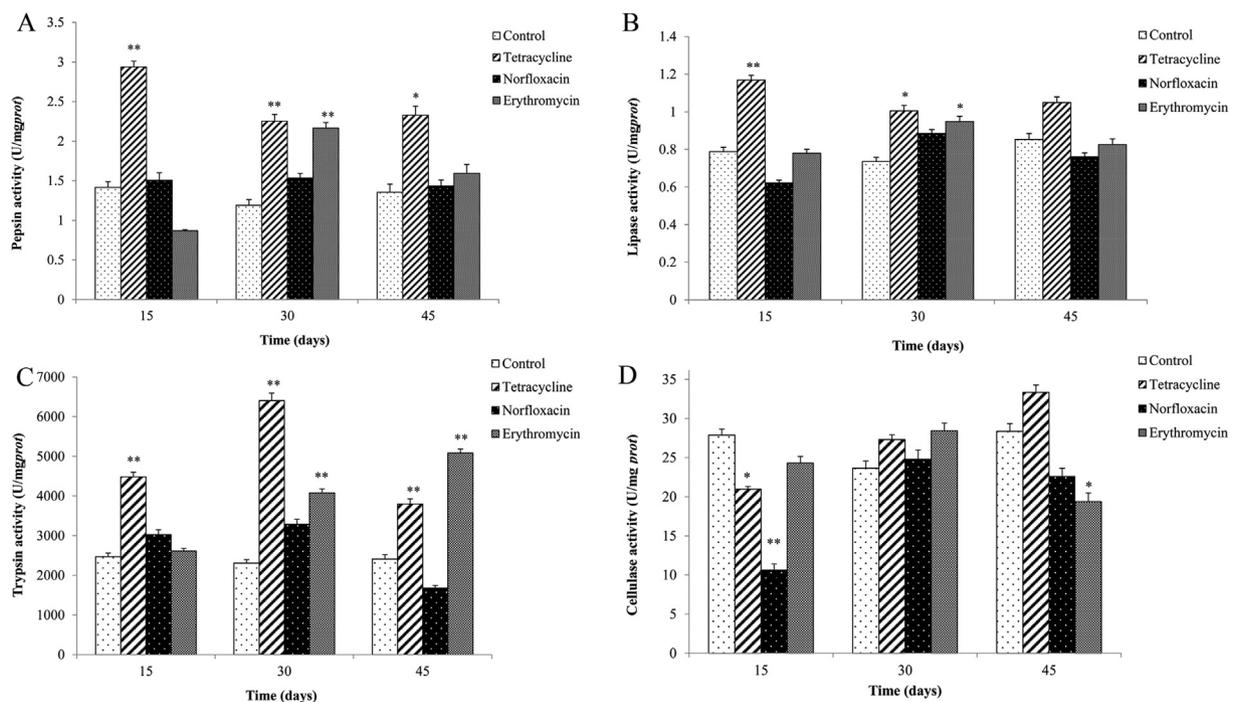

Fig. 2. Influence of diet antibiotics on the activities of intestinal digestive enzymes. (A) Pepsin activity, (B) Lipase activity, (C) Trypsin activity, (D) Cellulase activity. Data was presented as mean ± SE. The difference analysis was carried out by a one-way analysis of variance (ANOVA) and the differences among treatments were tested by LSD test. * represents $p < 0.05$, ** represents $p < 0.01$.
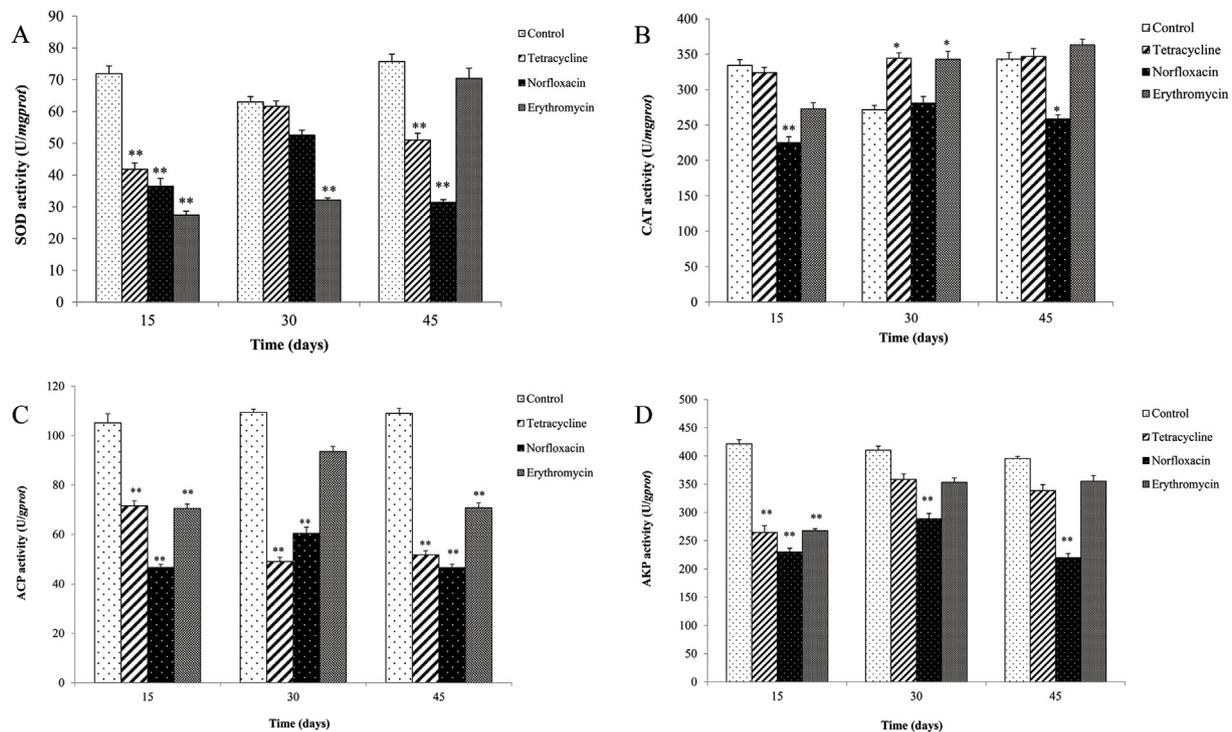
Fig. 3. Influence of diet antibiotics on the immune-related and antioxidant enzymes. (A) SOD activity, (B) CAT activity, (C) ACP activity, (D) AKP activity. Data was presented as mean ± SE. The difference analysis was carried out by a one-way analysis of variance (ANOVA) and the differences among treatments were tested by LSD test. * represents $p < 0.05$, ** represents $p < 0.01$.
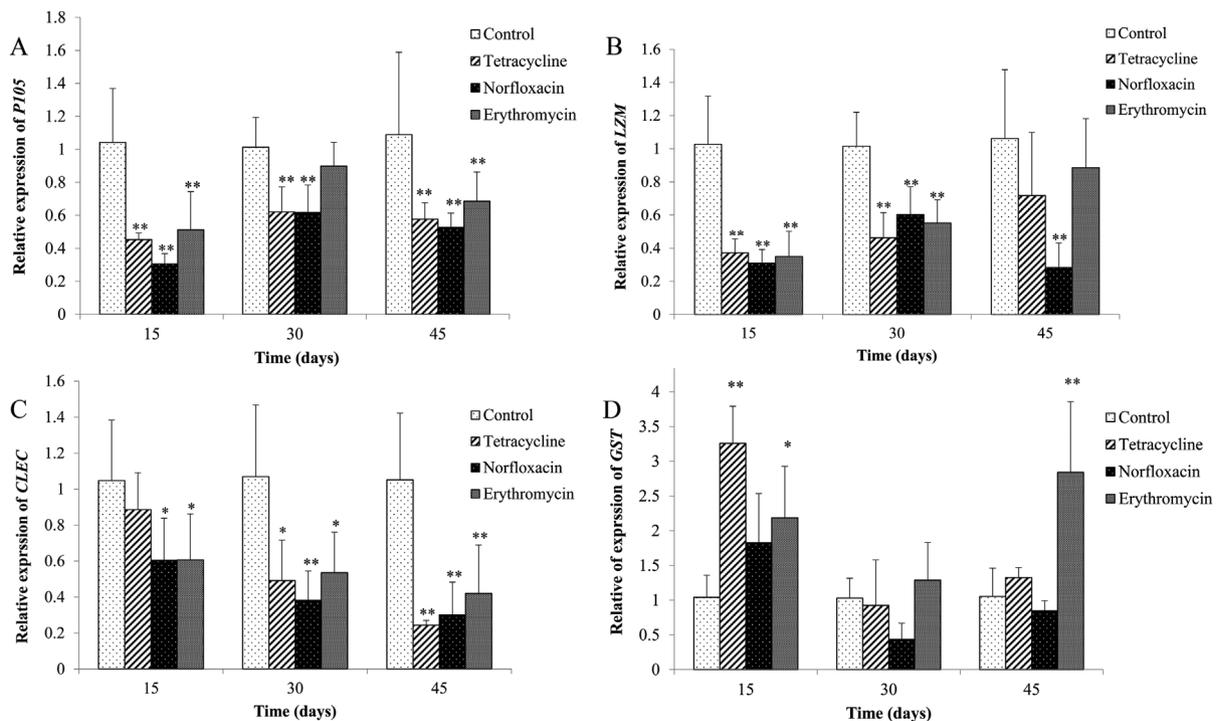

Fig. 4. Influence of diet antibiotics on the relative mRNA expression of immune-related genes. (A) *p105*, (B) *LZM,* (C) *CLEC*, (D) *GST*. Data was presented as mean ± SE. The difference analysis was carried out by a one-way analysis of variance (ANOVA), and the differences among treatments were tested by LSD test. * represents $p < 0.05$, ** represents $p < 0.01$.

increase over the treatment time, but others presented a sustained decline, which suggested that antibiotic addition could induce the disruption of immune function accompanied by some irreversible damage. Similarly, numerous reports have previously confirmed the immunosuppressive effects of antibiotics in fish. For example, Siwicki et al

found that oxytetracycline induced a decline in specific and nonspecific immune functions in salmonids [37]; Rijkers also demonstrated that oxytetracycline exposure suppressed both humoural and cellular immune responses in carp (*Cyprinus carpio*) [38,39]; Lunden reported that both oxytetracycline and oxolinic acid significantly suppressed

antibody production as well as the level of circulating white cells, while florfenicol caused a suppression in chemiluminescence response/phagocytic cells 5–6 weeks after treatment [25,40]. A recent study by Zhou showed that long-term dietary oxytetracycline treatment could cause intestinal inflammatory disorders and decreased nonspecific immune activities in zebrafish [23].

Meanwhile, a high level of reactive oxygen species (ROS) may be generated in organisms when confronted with environmental stress. Compared to those of the control group, the SOD activities of sea cucumbers decreased significantly under the first 15 days of three antibiotic exposure ($p < 0.01$, Fig_3A) and then dropped sharply again after a brief rally in the tetracycline and norfloxacin groups after 45 days of feeding; only the SOD activities of the sea cucumbers in the erythromycin diet group recovered to a similar level as that of the controls. Norfloxacin significantly inhibited CAT activity at 15 days of feeding ($p < 0.01$, Fig_3B), and this suppressive effect had not been relieved after 45 days of the experiment. The antioxidant defense system is a well-developed regulatory mechanism scavenging ROS, including enzymes SOD and CAT. SOD is a group of metalloenzymes that catalyze the dismutation of superoxide radicals and constitute the primary defense system against ROS, and CAT is an enzyme located in peroxisomes that converts hydrogen peroxide to water and oxygen [41,42]. In the present study, the decreased SOD activity and CAT activity reflected a weakened defense of sea cucumbers against free radical reactions after antibiotic exposure. This result was supported by previous studies; for example, the administration of oxytetracycline resulted in significantly decreased SOD and CAT activity in various tissues of treated rainbow trout [43], and Lin et al also revealed in a zebrafish study that their antioxidant defense was significantly damaged by the presences of sulfonamides [9]. Furthermore, it can be speculated that the reduction function of immune and antioxidant defense may be the reasons leading to the higher mortality of *A. japonicas* after antibiotic treatment, which suggests that oral antibiotics might facilitate growth promotion of sea cucumbers to some extent but that this benefit comes at the expense of impaired immune function and compromised disease resistance in the long term.

In summary, this work evaluated the risk and benefit of long-term dietary antibiotic treatment on sea cucumber growth and health. As a result, tetracycline and erythromycin promoted the growth and digestive activity of lipase, pepsin, and trypsin, but norfloxacin did not show significant promoting effects on digestive activity and even retarded the weight gain of the sea cucumbers. The mortality was higher in the antibiotic-treated groups on 2nd–4th day after being challenged with *V. splendidus*. At the same time, lower immune-related parameters were found in antibiotic-fed juveniles, suggesting that the use of antibiotics might weaken the immune defense system of sea cucumbers. The findings provide an avenue into understanding how the growth, digestive function and immune response of sea cucumbers are influenced by three antibiotics at low concentrations after long-term exposure.

Declarations of interest

None.

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