Environment International 115 (2018) 205–219

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Chronic exposure to low environmental concentrations and legal aquaculture doses of antibiotics cause systemic adverse effects in Nile tilapia and provoke differential human health risk

Samwel M. Limbu[a,b], Li Zhou[a], Sheng-Xiang Sun[a], Mei-Ling Zhang[a,*], Zhen-Yu Du[a,*]

[a] Laboratory of Aquaculture Nutrition and Environmental Health (LANEH), School of Life Sciences, East China Normal University, Shanghai, PR China
[b] Department of Aquatic Sciences and Fisheries Technology, University of Dar es Salaam, Dar es Salaam, Tanzania

ARTICLE INFO

Keywords:
Low environmental antibiotics
Legal aquaculture antibiotics
Toxicological effects
Human health risk

ABSTRACT

Background: Antibiotics used globally to treat human and animal diseases exist ubiquitously in the environment at low doses because of misuse, overdose and poor absorption after ingestion, coupled with their high-water solubility and degradation resistance. However, the systemic chronic effects of exposure to low environmental concentrations of antibiotics (LECAs) and legal aquaculture doses of antibiotics (LADAs) in fish and their human health risk are currently unknown.

Objective: To investigate the in vivo chronic effects of exposure to LECAs and LADAs using oxytetracycline (OTC) and sulfamethoxazole (SMZ) in Nile tilapia (*Oreochromis niloticus*) and their human health risk.

Methods: Twenty *O. niloticus* weighing 27.73 ± 0.81 g were exposed to water containing LECAs (OTC at 420 ng/L and SMZ at 260 ng/L) and diets supplemented with LADAs (OTC 80 mg/kg/day and SMZ 100 mg/kg/day) for twelve weeks. General physiological functions, metabolic activities, intestinal and hepatic health were systemically evaluated. The possible human health risks of the consumption of the experimental Nile tilapia fillets in adults and children were assessed by using risk quotient.

Results: After exposure, we observed retarded growth performance accompanied by reduced nutrients digestibility, feed efficiency, organ indices, and lipid body composition in treated fish. Antibiotics distorted intestinal morphological features subsequently induced microbiota dysbiosis and suppressed intestinal tight junction proteins. Exposure of fish to LECAs and LADAs induced oxidative stress, suppressed innate immunity, stimulated inflammatory and detoxification responses, concomitantly inhibited antioxidant capacity and caused lipid peroxidation in intestine and liver organs. Both LECAs and LADAs enhanced gluconeogenesis, inhibited lipogenesis and fatty acid beta oxidation in intestine and liver organs. The exposure of fish to LECAs and LADAs induced anaerobic glycolytic pathway and affected intestinal fat catabolism in intestine while halted aerobic glycolysis, increased hepatic fat catabolism, and induced DNA damage in liver. The hazard risk quotient in children for fish treated with OTCD was > 1 indicating human health risk.

Conclusion: Overall, both LECAs and LADAs impair general physiological functions, nutritional metabolism, and compromise fish immune system. Consumption of fish fed with legal OTC provokes health risk in children. Global stringent prohibition policy for use of antibiotics in aquaculture production and strategies to limit their release into the environment are urgently required to protect human health.

1. Introduction

Antibiotics misuse, overdose and poor absorption after medication, coupled with their high-water solubility, bioactivity, and persistent behavior represent a global environment pollution pandemic that has caused severe ecological sustainability and health problems (Brandt et al., 2015; Carvalho and Santos, 2016), particularly in fish, which plays a central role in human food safety, mainly through consumption. Globally, antibiotics consumption increased from 50 to 70 billion standard units in 2000 to 2010 (Van Boeckel et al., 2014), with 63 tons consumed by livestock in 2010 (Van Boeckel et al., 2015), and projection indicates up to 105 tons are expected to be consumed by 2030

Abbreviations: LECAs, low environmental concentrations of antibiotics; LADAs, legal aquaculture doses of antibiotics; OTCD, oxytetracycline diet; SMZD, sulfamethoxazole diet; OTCB, oxytetracycline bath; SMZB, sulfamethoxazole bath; LEC-OTC/SMZ, low environmental concentrations of OTC and SMZ; LAD-OTC/SMZ, legal aquaculture doses of OTC and SMZ

* Corresponding authors.
E-mail addresses: mlzhang@bio.ecnu.edu.cn (M.-L. Zhang), zydu@bio.ecnu.edu.cn (Z.-Y. Du).

https://doi.org/10.1016/j.envint.2018.03.034
Received 22 December 2017; Received in revised form 6 March 2018; Accepted 23 March 2018
0160-4120/ © 2018 Elsevier Ltd. All rights reserved.

(CDDEP, 2015). Antibiotics used in various human activities are released into the aquatic environment where they exist at low concentrations (Sotto et al., 2017). High concentrations of antibiotics existing in the environment provoke fish population disturbances and endanger species extinction (Sanchez et al., 2011), while those used for treatment of bacterial diseases in various fish species in aquaculture production result into controversial outcomes on growth performance (Reda et al., 2013; Gaikowski et al., 2015; Zhang et al., 2015), cause body malformation (Zhang et al., 2016), microbiota dysfunction (Navarrete et al., 2008; He et al., 2012), and suppress immunity (Guardiola et al., 2012; Han et al., 2014). Moreover, antibiotics used in aquaculture induce oxidative stress (Nunes et al., 2015; Wang et al., 2016), affect antioxidant capacity (Liu et al., 2015), and trigger DNA damage (Botelho et al., 2015). However, fish cannot avoid exposure to antibiotics because of 1) legal dietary medication to prevent and cure diseases (Dobšíková et al., 2013; Phu et al., 2015) and 2) existence of low environmental residues in aquatic environments (Sotto et al., 2017). Despite this, the potential chronic effects of low environmental concentrations of antibiotics (LECAs) and their legal aquaculture doses (LADAs) in fish and their human health have not been thoroughly evaluated.

Oxytetracycline (OTC) and sulfamethoxazole (SMZ) are bacteriostatic antibiotics, which limit bacteria growth by interfering with protein synthesis, for which OTC binds to 30S ribosomal subunits and prevents ammonia acyl tRNA from combining with ribosome (Zhang et al., 2015), while SMZ blocks dihydrofolate intermediate production, thereby restricts the normal bacterial folic acid synthesis (Johansson et al., 2014). Both OTC and SMZ are used for treatments of bacterial diseases in cultured fish species due to their broad-spectrum bacteria activities, cost effectiveness and legal availability (Navarrete et al., 2008; Dobšíková et al., 2013; Phu et al., 2015). The OTC is administered in feeds at 50 to 250 mg/kg fish body weight/day for 3 to 21 days (Gaikowski et al., 2003; Navarrete et al., 2008; Yonar et al., 2011), while SMZ is commonly administered at 100 to 200 mg/kg fish body weight/day for 5 days (Liu et al., 2017), depending on fish species, infection and country-specific legal requirements. Despite these legal directives, the two antibiotics are used for long periods in aquaculture production (Phu et al., 2015), sometimes on daily basis and in absence of diseases (Pham et al., 2015).

Previous studies have reported that, OTC and SMZ are poorly absorbed in the guts of animals after medication, subsequently are excreted in urine and feces, either unchanged or modified into metabolites, which are transported into surface waters through runoff and subsurface drainage systems (Zhang et al., 2016). Thus, they exist omnipresent in the aquatic environments at concentrations ranging from 174.9 ± 266.9 ng/L to 741.85 ng/L in freshwater (Bai et al., 2014; Dong et al., 2016) and 15,163 ng/L in sea water for OTC (Chen et al., 2015), whereas SMZ have been detected in freshwater at concentrations ranging from 259.60 ng/L to 385.00 ng/L (Chen and Zhou, 2014; Y. Li et al., 2016). These concentrations pose risks on aquatic species, raising global public concern on human health upon fish consumption (Johansson et al., 2014; Botelho et al., 2015; Yan et al., 2016; Chen et al., 2017). However, no study has extensively explored the chronic effects of LECAs and LADAs, particularly using OTC and SMZ in fish and their human health effects.

In this study, we subjected fish to low concentrations of antibiotics existing in the aquatic environment and their legal doses used in aquaculture. For this purpose, we exposed Nile tilapia (*Oreochromis niloticus*), a global economic, cultured and consumed species, to dietary and bath treatments containing OTC and SMZ for twelve weeks. The aim was to explore the chronic systemic effects of LEC-OTC/SMZ and LAD-OTC/SMZ on general body functions and intestinal and hepatic health, which are important organs in fish and assess their human health risks after fish consumption. Although we only exposed fish using two methods and explored two antibiotics, we anticipate that the strategy used could be applied extensively in the assessments of chronic effects of other antibiotics and veterinary drugs in fish and human health risks.

2. Materials and methods

2.1. Antibiotics and fish exposure

Sulfamethoxazole (SMZ, CAS: 723-46-6) and oxytetracycline (OTC, CAS: 615 3-64-6) were purchased from Yuanmu Biotechnology Co., Ltd., Shanghai, China. About 200 all-male *O. niloticus* fingerlings were purchased from Shanghai Ocean University (Shanghai, China). All male *O. niloticus* were used for the experiment because they grow faster and at a uniform size than the females, thus, many farmers world-wide are more likely to culture them in the practical aquaculture. The fish were acclimatized in four aseptic 200-litre tanks (50 fish each), supplied with dechlorinated water for two weeks. Fish in each tank were supplied with compressed air via air-stones from air pumps at a 10 h/14 h light/dark cycle and water temperature, dissolved oxygen, pH and total ammonia nitrogen were maintained at ranges from 26 to 28 °C, 4.8 to 6.4 mg/L, 7.5 to 7.9 and < 0.02 mg/L, respectively. During this period, the fish were hand-fed using a commercial diet (Chengdu, China) containing ≥33% protein and ≥5% lipid. After acclimatization, all *O. niloticus* were individually embedded with a tracking chip, which aided individual fish recognition through radio-frequency identification (RFID) technology (Voulodimos et al., 2010) and fish were randomly distributed into five sterile 220-litre tanks (30 fish per tank) filled with dechlorinated water to a volume of 130 L/tank and allowed to settle for one week.

Twenty-four hours before the experiment, total initial mean weights of fish were determined as 27.73 ± 0.81 g for all treatments. In one pair of the experiment, *O. niloticus* were exposed to LADAs in feeds supplemented with 2.00 g/kg diet of OTC (80 mg/kg body weight/day) and 2.50 g/kg diet of SMZ (100 mg/kg body weight/day), hereafter, OTCD and SMZD, respectively; Table S1. Another pair of fish were exposed to LECAs (bath treatments, referred to as OTCB and SMZB, respectively) containing similar types of antibiotics at concentrations of 420 ng/L for OTCB and 260 ng/L for SMZB to reflect low environmental relevant exposure situations (Bai et al., 2014; Chen and Zhou, 2014). A control tank was included in which fish were reared and treated similarly but deprived of antibiotics. The experimental conditions were similar to those used during the acclimatization period. All fish were hand-fed twice daily between 9:00 and 10:00 h and 17:00 and 18:00 h at 4% of their average body weight per day for 84 days and the feeds contained 0.10 g/kg/diet yttrium oxide (Yt_2O_3) marker for estimation of apparent nutrients digestibility (Table S1). Fish in the diet treatments were fed by using little amounts of feeds gradually until all the feeds per feeding ration were consumed completely without much dissolution of antibiotics from the diets. Fish in the control and bath treatments were hand-fed on the basal diet (Table S1). Half of the water in the dietary and control tanks were replaced with flesh-dechlorinated water every 24 h, while those in bath treatments – antibiotics were replaced with flesh-dechlorinated water containing the initial concentrations of antibiotics in order to maintain relatively stable concentrations as designed. The weight of individual fish was recorded fortnightly and the feed rations were adjusted accordingly.

2.2. Growth performance, survival rate, feed efficiency and organ indices

At the end of the experiment, all survived fish were fasted for 24 h, they were collected by using a scoop net and their individual weights determined for weight gain and final weight estimations. Any death of fish during the experiment was recorded for survival rate determination. The amount of feed and the weight of fish were used to calculate feed efficiency as the ratio of total wet weight gained by fish to total amount of feed fed in each treatment.

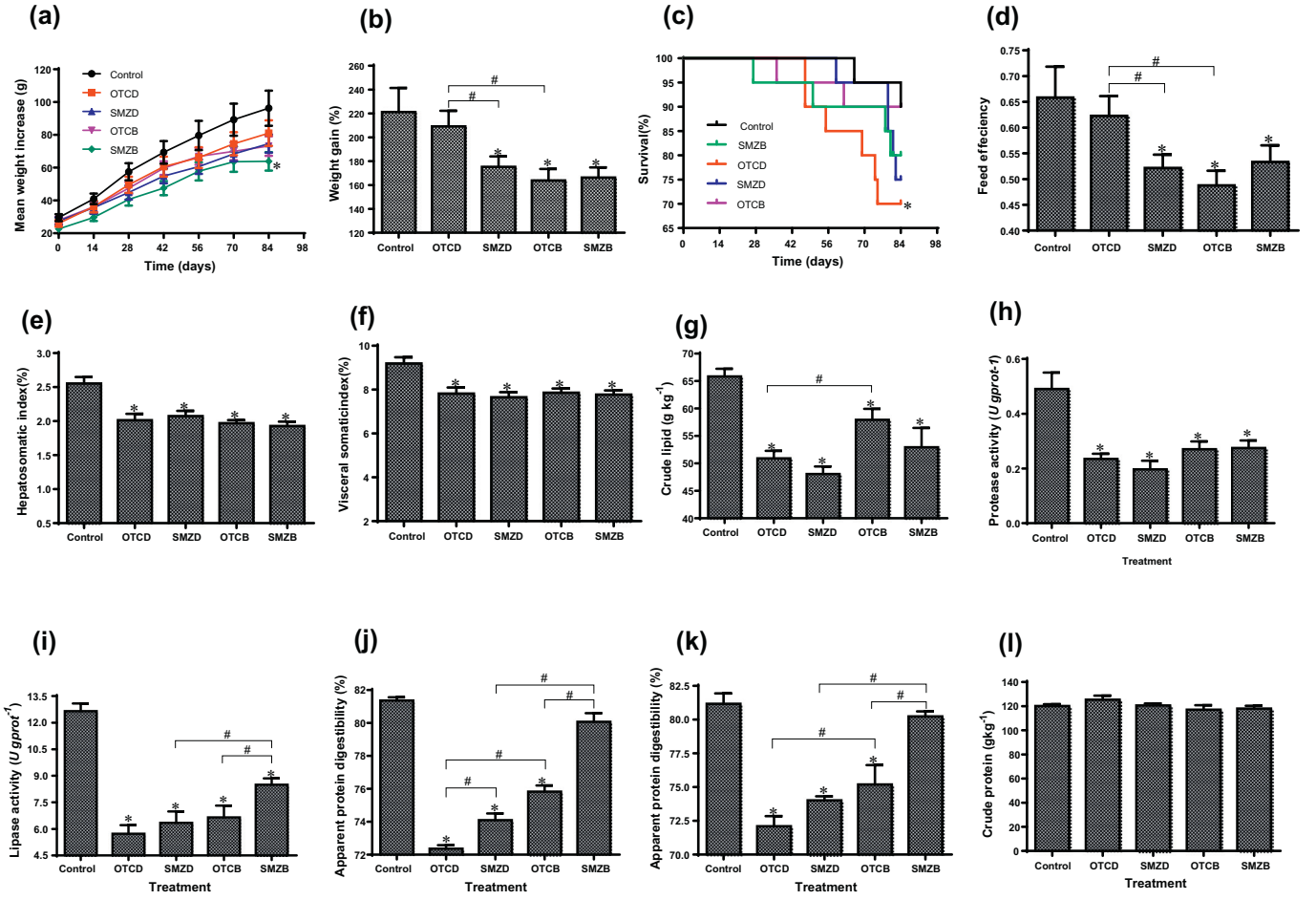


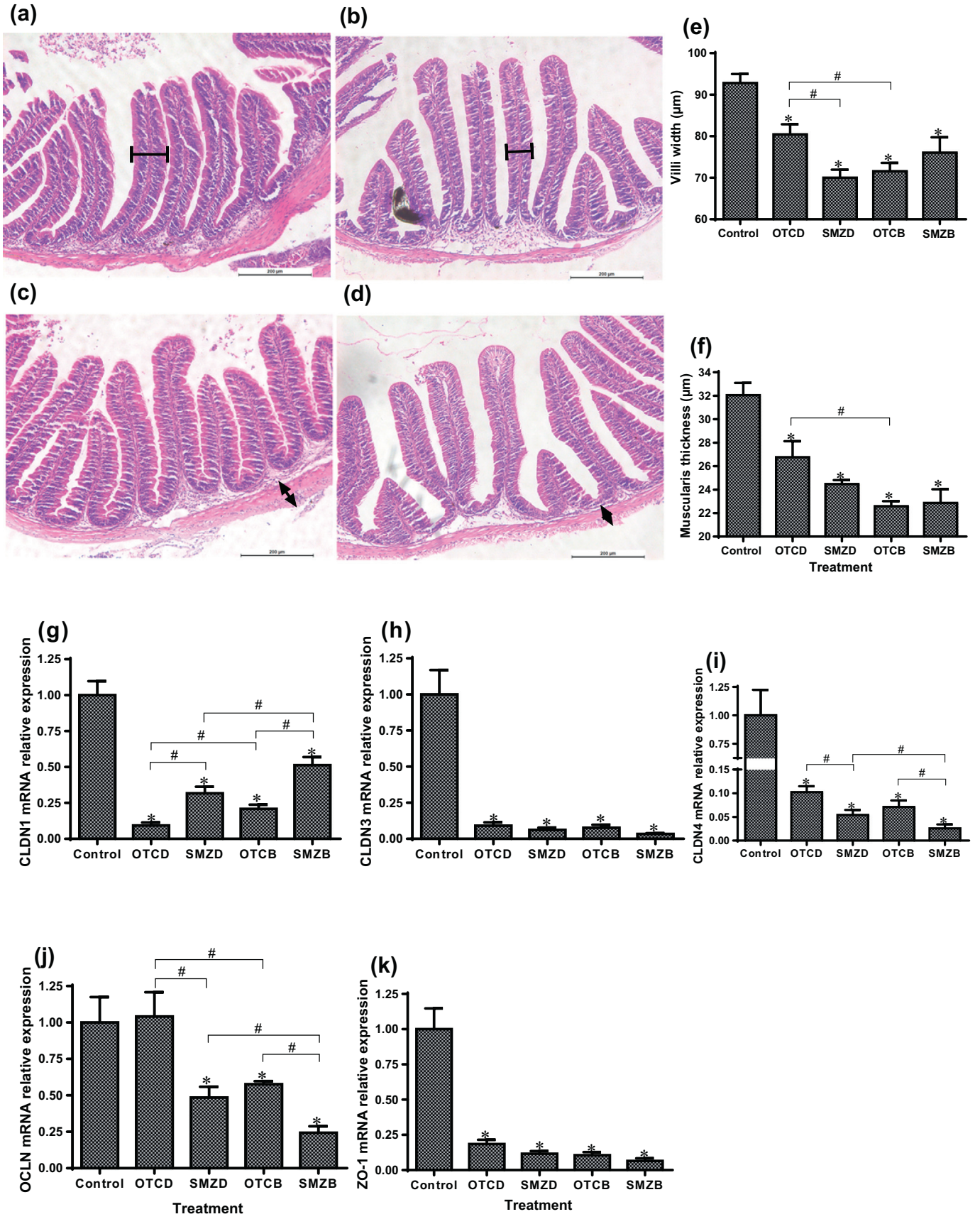
Fig. 1. Growth performance, feed efficiency, organ indices, body composition, nutrient digestibility, and digestive enzymes activities of Nile tilapia (*Oreochromis niloticus*) fed and exposed to oxytetracycline and sulfamethoxazole treatments; (a) mean weight increase, (b) weight gain, (c) survival rate, (d) feed efficiency, (e) hepatosomatic index, (f) visceral somatic index, (g) crude lipid dressed carcass fish body composition, (h) intestinal protease activity, (i) intestinal lipase activity, (j) apparent protein digestibility, (k) apparent lipid digestibility, and (l) crude protein dressed carcass fish body composition. Weight gain = [(final − initial weights) / initial weight] × 100, feed efficiency = total fish wet weight gain / total amount of feed, hepatosomatic index = (liver weight / fish body weight) × 100, visceral somatic index = (visceral weight / fish body weight) × 100, and nutrient digestibility (proteins, lipid, carbohydrate, %) = 100 − [100 × (Y_2O_3 in diet / Y_2O_3 in feces) − (nutrient in feces / nutrient in diet)]. Values are means ± SEM [n = 20 for (c), n = 14 for (a), (b) and (d), n = 8 for (h) and (i) and n = 6 for (g) and (j)–(l)]. The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB), sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs SMZD, OCTD vs OTCB, SMZD vs SMZB, and OTCB vs SMZB ($p < 0.05$).

2.3. Biochemical assays, body composition and nutrients digestibility

After weight measurements and counting, eight fish from each tank were sampled, individually anesthetized with MS-222 (20 mg/L) (tricaine methanesulfonate, Western Chemicals, Inc., Ferndale, Washington) and sacrificed for tissue samples collection for biochemical assays, nutrients body composition and digestibility measurements. Intestines, livers and skins from *O. niloticus* were excised and subsequently homogenized in a glass beds with 9 volumes (v/w) of 0.8% physiological saline. The homogenate was centrifuged (2500 rpm, at 4 °C for 10 min), the resulting supernatant was collected and subsequently frozen at −80 °C for biochemical assays according to manufacturers' instructions. One whole intestine, liver and a piece of skin from each fish were used in all analyses. On the days of analyses, malondialdehyde (MDA) concentration was determined by using enzyme-linked immune sorbent assay (ELISA) kit (Hengyuan, Shanghai). Protease, lipase, amylase, lactate dehydrogenase (LDH), glutathione *S*-transferase (GST), superoxide dismutase (SOD), total antioxidant capacity (TAC), acid phosphatase (ACP), and alkaline phosphatase (AKP) were measured by using specific commercial assay kits (Nanjing Jiancheng Institute, China), with their results recorded on a microplate reader (Epoch, BioTek, USA). The remaining fish (with viscera removed) were used for body composition and apparent nutrients digestibility measurements according to standard methods (AOAC, 1995).

2.4. Gut histomorphology

A 1-cm segment of intestine was taken from three individual fish per treatment and fixed with 4% paraformaldehyde for 24 h, followed by dehydration in a series of increasing ethanol concentrations at 50% (12 h), 70% (5 h), 80% (2 h), 90% (2 h), 95% (1 h), and 100% (30 min over two consecutive times), and then transferred to xylene for transparency, processed and embedded in paraffin wax. The intestines were sliced transversely into 5 μm sections and stained by using hematoxylin-eosin (H & E), and subsequently were observed under a microscope (Nikon, Eclipse, TS100). Villus width and muscularis thickness were measured from at least 30 segments in each treatment by using the Image Pro-Plus 6 software (Media Cybernetics, Inc., Rockville, MD USA).



(caption on next page)

Fig. 2. Nile tilapia (*Oreochromis niloticus*) representative intestinal histopathological structure and tight junction proteins mRNA expressions after feeding and exposure to oxytetracycline and sulfamethoxazole treatments; (a) control villi width, (b) OTCB villi width, (c) control muscularis thickness, (d) SMZD muscularis thickness, (e) results of villi width analysis, (f) results of muscularis thickness analysis, (g) claudin-1 (CLDN1), (h) claudin-3 (CLDN3), (i) claudin-4 (CLDN4), (j) occludin (OCLDN), and (k) zonula occludens-1 (ZO-1). Values are means ± SEM [n = 3 for (e) and (f) and n = 6 for (g)–(k)]. The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB), sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs SMZD, OCTD vs OTCB, SMZD vs SMZB, and OTCB vs SMZB ($p < 0.05$).

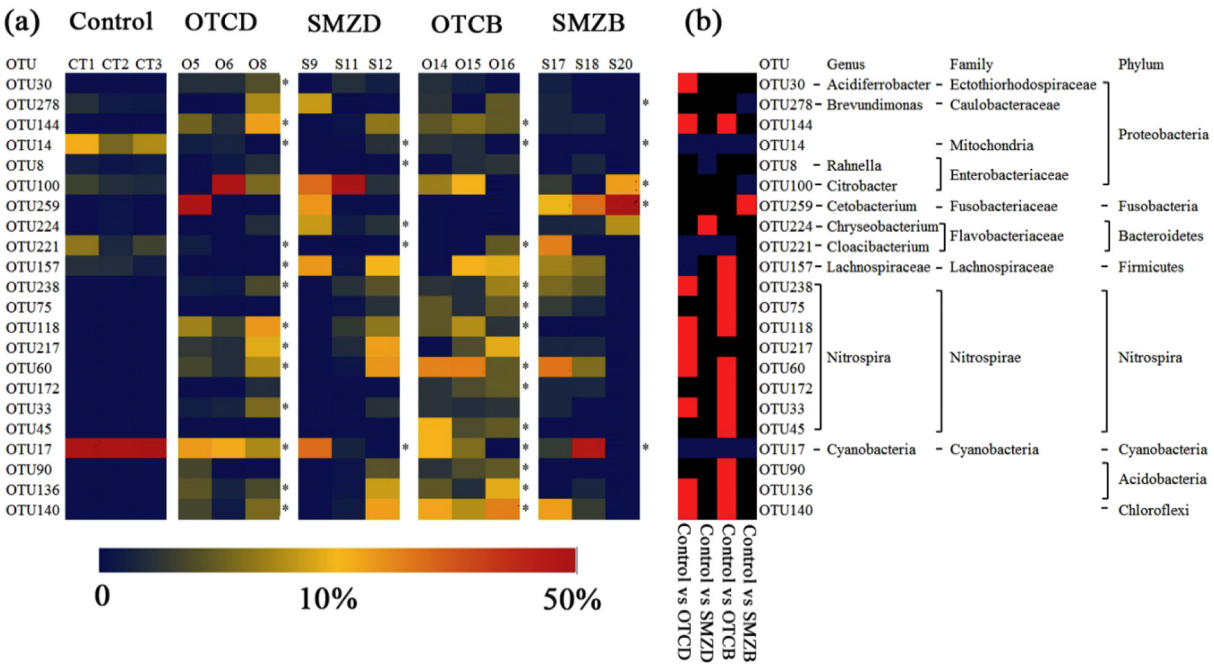


Fig. 3. Heatmap of the bacterial abundance in the intestine of Nile tilapia (*Oreochromis niloticus*) fed and exposed to oxytetracycline and sulfamethoxazole treatments; (a) heatmap figure of the 22 operational taxonomic units (OTUs) and (b) the changing direction of the 22 OTUs after dietary and exposure to antibiotics. Red and blue colors indicate the abundance of OTUs that were more and less abundant, respectively, while black color signifies no change in abundance in oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB) and sulfamethoxazole bath (SMZB) treatments relative to control. The taxonomy of the OTUs (genus, family and phylum) is depicted on the right. Asterisk (*) represents the OTU whose abundance was significantly changed by antibiotics administration relative to control (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Bacterial genomic DNA extraction

Three individual fish from each treatment were aseptically dissected and the intestinal contents were used for bacterial composition analysis. The total intestinal bacterial DNA was isolated using a E.Z.N.A.® Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. DNA quantity and quality were measured using a NanoDrop 2000 Spectrophotometer (Thermo, USA).

2.6. Illumina high-throughput sequencing of barcoded 16S rRNA genes

The V3–V4 regions of the bacteria 16S ribosomal RNA genes were amplified by polymerase chain reaction (PCR) using primers 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGCTACHVGGGTWTCTAAT-3′ based on their ability to amplify most bacterial sequences as previously reported (Sun et al., 2013). Unique eight-base barcodes were added to each primer to distinguish PCR products. PCR reactions were performed in 20 μL mixture containing 4 μL of 5 × Fast Pfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of Fast Pfu Polymerase (Trans Gen, China), and 10 ng of template DNA. The PCR conditions were as follows: 95 °C for 3 min for DNA denaturation, with the amplification lasting 29 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s), and final extension for 10 min at 72 °C. Purified PCR products were subjected to high-throughput sequencing using Illumina HiSeq 2500 (Illumina, San Diego, USA).

2.7. Bioinformatics analysis

The raw pair-end readings obtained were subjected to quality-control procedures by using the quantitative insights into microbial ecology (QIIME, version 1.17). The qualified reads were clustered to generate operational taxonomic units (OTUs) at the 97% similarity level by using UPARSE (version 7.1). Chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project (RDP) Classifier against the silva 16S rRNA database using confidence threshold of 70%. Taxonomic richness and diversity estimators were determined using a Mothur software. The sequences obtained for microbiota in this study are available in the https://www.ncbi.nlm.nih.gov/sra/SRP126609.

2.8. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR)

Total RNA from intestine, liver and skin tissues for mRNA expressions to evaluate tight junction proteins, oxidative stress, detoxification response, inflammation, innate immunity, DNA damage, and nutrients metabolism were isolated by using a Tri Pure Reagent (Aidlab, China) according to the manufacturer's protocol. The quality and quantity of total RNA were tested using a NanoDrop 2000 Spectrophotometer (Thermo, USA) and the OD260/280 values of all RNA samples ranged from 1.86 to 2.02, showing good quality. First-strand cDNAs were synthesized using a PrimerScript™ RT reagent Kit with a gDNA Eraser

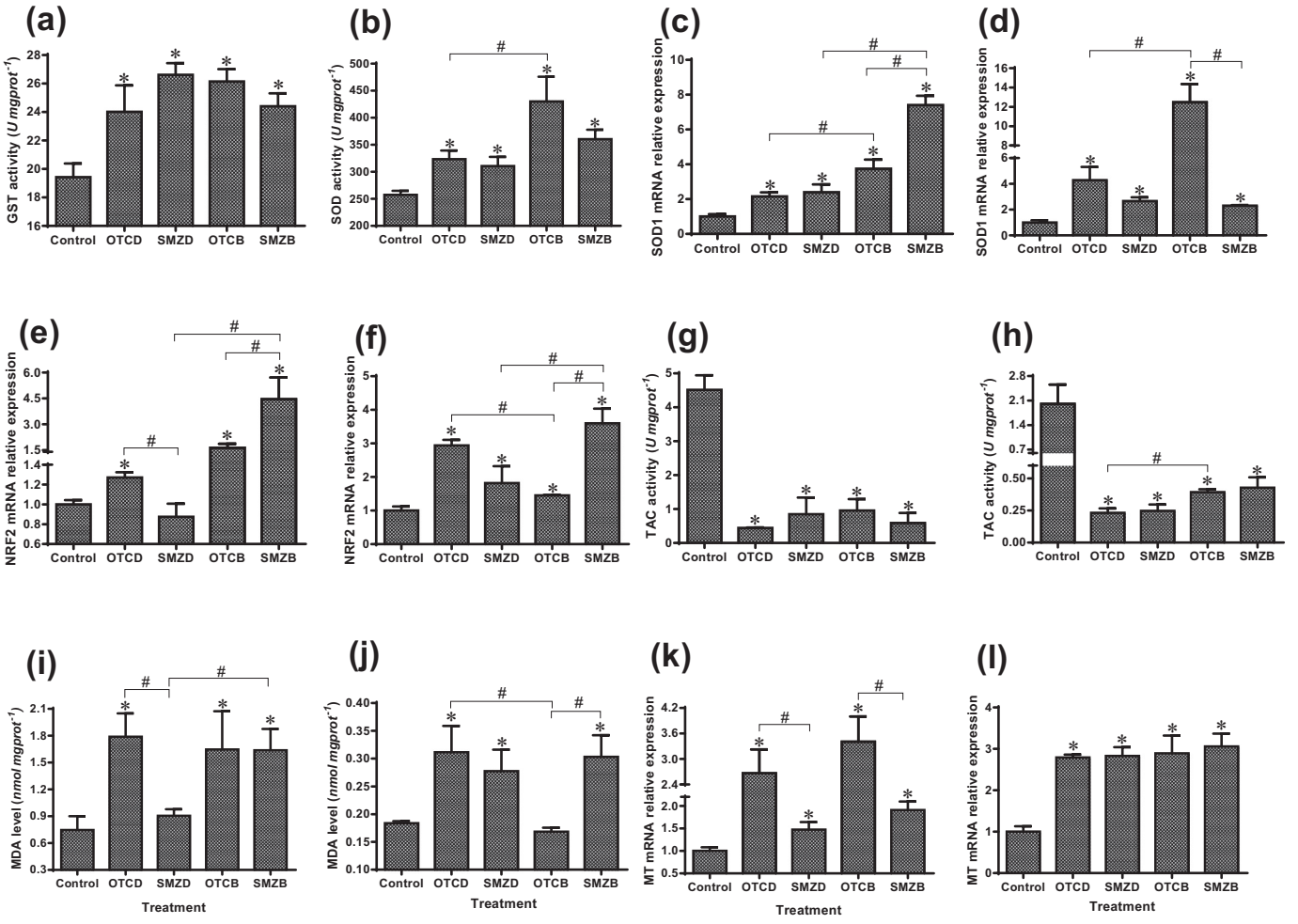


Fig. 4. Intestinal and hepatic oxidative stress responses of Nile tilapia (*Oreochromis niloticus*) fed and exposed to oxytetracycline and sulfamethoxazole treatments; (a) glutathione *S*-transferase (GST) activity in the intestine, (b) superoxide dismutase (SOD) activity in the intestine, (c) superoxide dismutase 1 soluble (SOD1) mRNA expression in the intestine, (d) SOD1 soluble mRNA expression in the liver, (e) nuclear factor (erythroid-derived 2)-like 2 (NRF2) mRNA expression in the intestine, (f) NRF2 mRNA expression in the intestine, (g) total antioxidant capacity (TAC) activity in the intestine, (h) TAC activity in the liver, (i) malondialdehyde (MDA) concentration in the intestine, (j) MDA concentration in the liver, (k) metallothionein (MT) mRNA expression in the intestine, and (l) MT mRNA expression in the liver. Values are means ± SEM (n = 8). The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB), sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs SMZD, OTCD vs OTCB, SMZD vs SMZB, and OTCB vs SMZB ($p < 0.05$).

(Perfect Real Time) (Takara, Japan) by using S1000TM Thermal Cycler (Bio-Rad, USA) by following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis for genes were performed by mixing 1 µl synthesized cDNA, 1.6 µl forward and reverse primers specific for genes, 10 µl 2 × Ultra SYBR Mixture (CWbio, China), and 7.4 µl nuclease-free water, and run in the CFX Connect Real-Time System (Bio-Rad) according to the manufacturer's protocol. Elongation factor 1 alpha (EF1α) and β-actin were used as reference genes. To ensure specificity on intended genes, all the primers for qRT-PCR were designed to span an intron. All the primers sequences used for the qRT-PCR analyses of genes were synthesized by Sangon Biotech (Shanghai, China) and are listed in Table S2. During the study, the qRT-PCR efficiency was between 99% and 102% and the correlation coefficient was above 0.98 for each gene. Each qRT-PCR run was performed in triplicate and negative controls (no cDNA) were conducted. The relative expressions of genes were calculated by using the $2^{-\Delta Ct}$ method, thereof, $\Delta Ct = Ct_{target} - (Ct_{EF1\alpha} + Ct_{\beta\text{-actin}}) / 2$.

2.9. Detection of antibiotics in fish muscle, water samples and human health risk evaluation

Muscles tissues from four fish and four water samples at three different culture periods were collected from each treatment for analysis of antibiotics concentrations by using specific ELISA kits for the two antibiotics according to manufacturer's instructions (REAGEN LLC, Moorestown, New Jersey, USA). The three-time points (28, 56 and 84 days) for measuring water samples were designed to include days before and after water exchange in order to realize the actual concentrations of antibiotics during the 24 h period in the whole feeding trial (Table S1). The potential human health risk of consuming fish treated with antibiotics was assessed based on target hazard quotient (THQ) according to Wang et al. (2005) using the following equation:

$$THQ = \left(\frac{E_F \times E_D \times F_{IR} \times C}{R_{FD} \times W_{AB} \times T_A}\right) \times 10^{-3}$$

where E_F is exposure frequency (365 days/year); E_D is the exposure duration (70 years), equivalent to the average lifetime; F_{IR} is the fish consumption rate (g/person/day), a value of 62.3 g for adults and 60% of adult (37.38 g) for children was adopted (Du et al., 2012); C is the

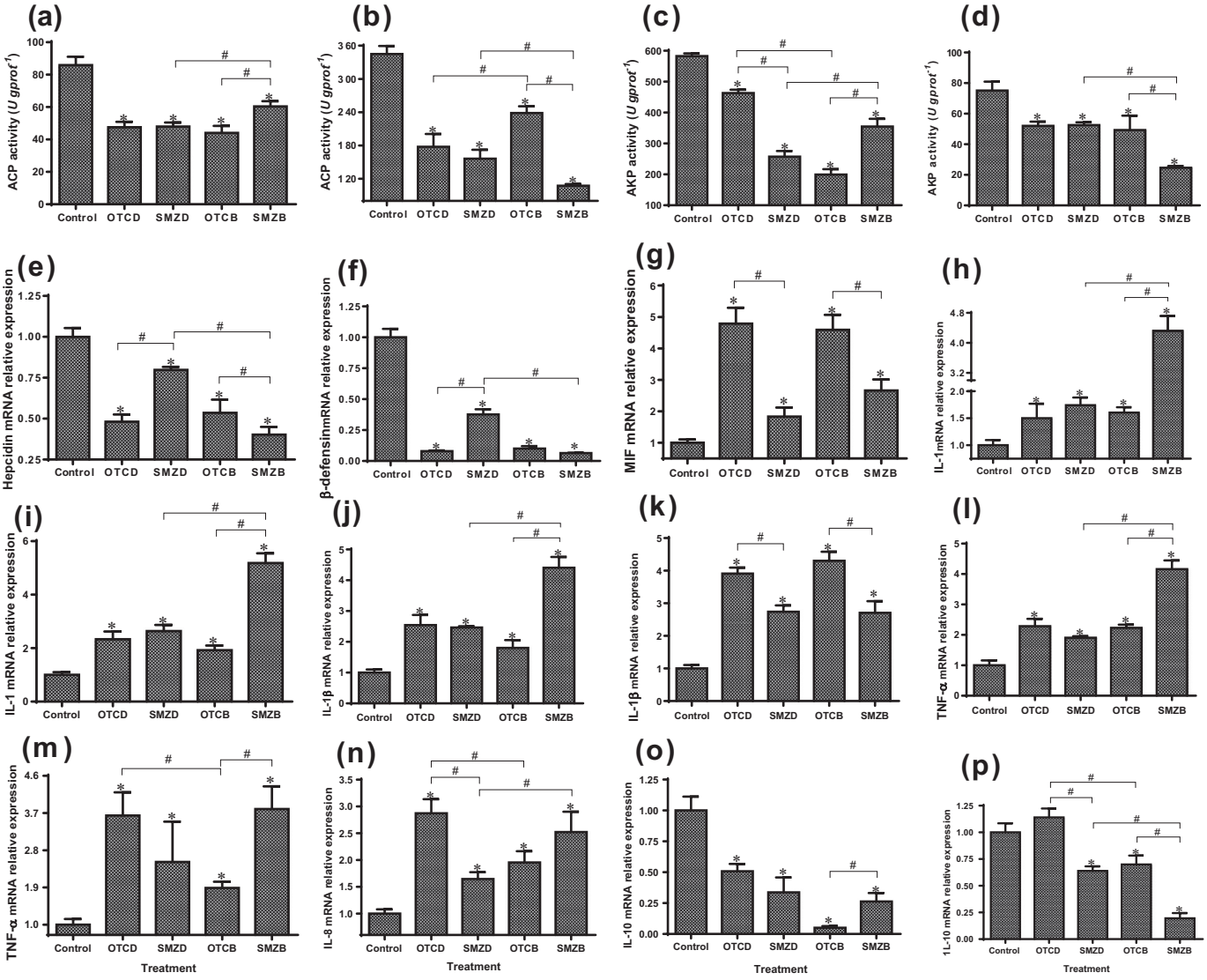


Fig. 5. Intestinal innate immunity and inflammatory responses of Nile tilapia (*Oreochromis niloticus*) fed and exposed to oxytetracycline and sulfamethoxazole treatments; (a) acid phosphatase (ACP) activity in the intestine, (b) ACP activity in the liver, (c) alkaline phosphatase (AKP) activity in the intestine, (d) AKP activity in the liver, (e) hepcidin mRNA expression in the intestine, (f) β-defensin mRNA expression in the intestine, (g) macrophage migration inhibitory factor (MIF) mRNA expression in the liver, (h) interleukin 1 (IL-1) mRNA expression in the intestine, (i) IL-1 mRNA expression in the liver, (j) interleukin 1 beta (IL-1 β) mRNA expression in the intestine, (k) IL-1β mRNA expression in the liver (l) tumor necrosis factor alpha (TNF-α) mRNA expression in the intestine, (m) TNF-α mRNA expression in the liver, (n) interleukin 8 (IL-8) mRNA expression in the liver, (o) interleukin 10 (IL-10) mRNA expression in the intestine, and (p) IL-10 mRNA expression in the liver. Values are means ± SEM (n = 8). The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB) and sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs SMZD, OTCD vs OTCB, SMZD vs SMZB, and OTCB vs SMZB ($p < 0.05$).

antibiotic concentration in fish muscle (μg/g); R_{FD} is the oral reference dose (mg/kg/day) which is 1 and 0.01 mg/kg/day for adults and children, respectively for OTC based on U.S. Environmental Protection Agency (U.S. EPA, 2017). We used an oral reference dose of 1.2 mg/kg/day for adults and children for SMZ as advised by the Minnesota Department of Health (MDH, 2015) which is for a related sulfonamide (sulfamethazine) with similar chemical structures, metabolites, metabolic pathways, and have comparable toxicological profiles (MDH, 2013) due to unavailability of specific R_{FD} for SMZ during the study period. The term W_{AB} is the average body weight (55.9 kg for adults and 32.7 kg for children) and T_A is the averaging exposure time for non-antibiotics (365 days/year × number of exposure years, assuming 70 years in this study). It was assumed that cooking of fish has no effect on the human health risk of antibiotics. A THQ ≤ 1 means the exposure population is unlikely to experience obvious adverse health risk.

2.10. Statistical analyses

Results are presented as means ± standard error of means (SEM). The mean values for each measured parameter were compared by using independent *t*-test for Windows (SPSS version 20, IBM, Armonk, NY, USA). Each treatment was first compared to control followed by comparisons between and within dietary and bath treatments. Two-way analysis of variance (ANOVA) was used to compare the effects of antibiotics and exposure methods on measured parameters. Survival rate was analyzed by using Log-rank (Mantel-Cox) test (GraphPad Prism 6, GraphPad Software, Inc. La Jolla, CA, USA). Abundance of microbiota was analyzed by using ACE and Chao1 indices while microbiota species diversity was assessed by using Shannon and Simpson indices. All microbiota indices were estimated based on OTU abundance matrices. Significant differences were judged at a probability level of $p \leq 0.05$ for all analyzes.

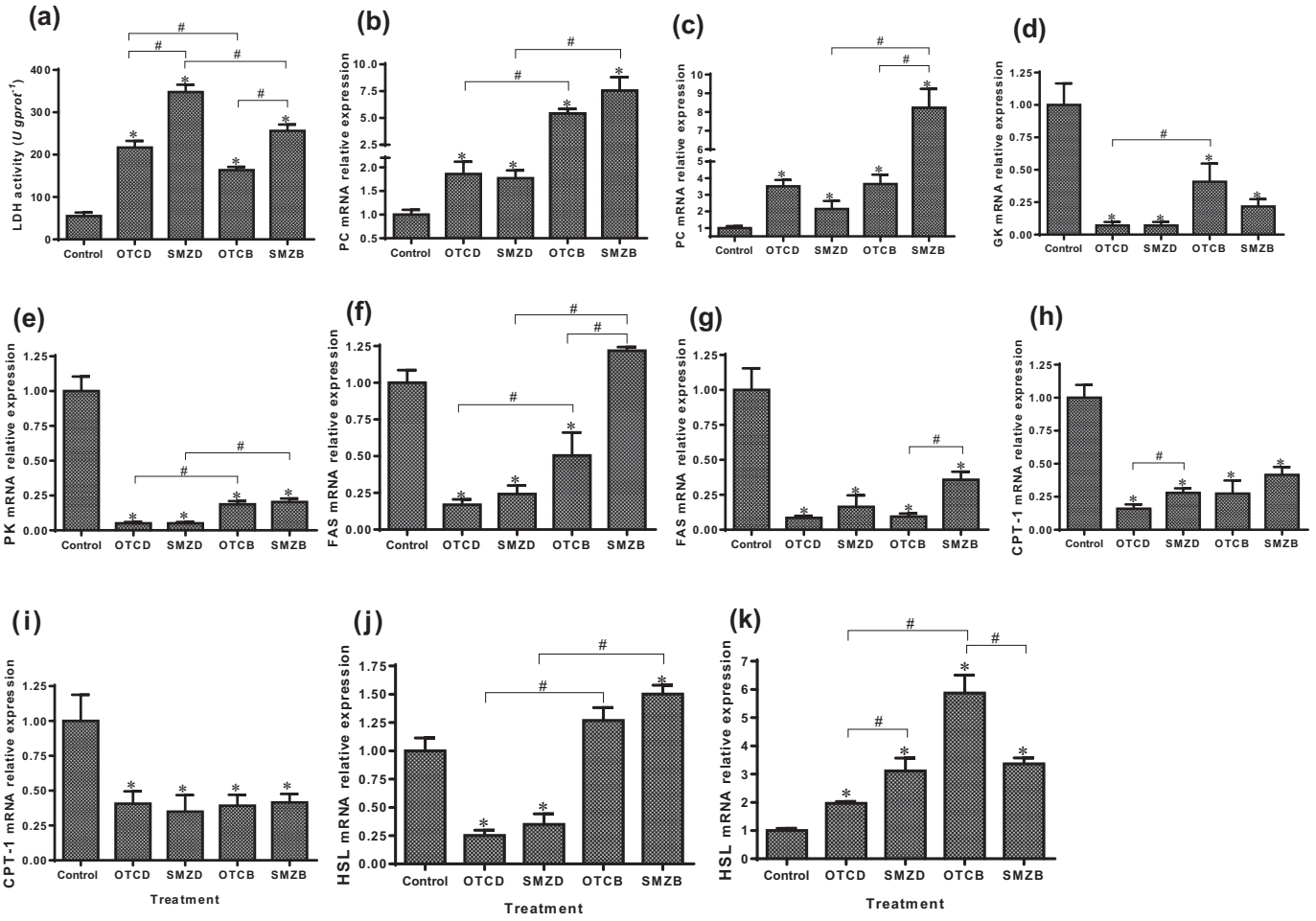


Fig. 6. Intestinal glucose and lipid metabolism of Nile tilapia (*Oreochromis niloticus*) fed and exposed to oxytetracycline and sulfamethoxazole treatments; (a) lactate dehydrogenase (LDH) activity in the intestine, (b) pyruvate carboxylase (PC) mRNA expression in the intestine, (c) PC mRNA expression in the liver, (d) glucokinase (GK) mRNA expression in the liver, (e) pyruvate kinase (PK) mRNA expression in the liver, (f) fatty acid synthetase (FAS) mRNA expression in the intestine, (g) FAS mRNA expression in the intestine, (h) carnitine palmitoyltransferase 1 (CPT-1) mRNA expression in the intestine, (i) CPT-1 mRNA expression in the liver, (j) hormone sensitive lipase (HSL) mRNA expression in the intestine, and (k) lipase (HSL) mRNA expression in the liver. Values are means ± SEM (n = 8). The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB) and sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs SMZD, OTCD vs OTCB, SMZD vs SMZB, and OTCB vs SMZB ($p < 0.05$).

3. Results

3.1. Low environmental concentrations and legal aquaculture doses of antibiotics impaired growth, body composition and nutrients digestibility

Growth performance is one of the most important aspects to fish farmers and environmentalists because it affects aquaculture production and perpetuation of species in the environment, respectively. With exception of OTCD, exposure of *O. niloticus* to LEC-SMZ and LAD-OTC/SMZ retarded growth performance and reduced feed efficiency ($p < 0.05$; Fig. 1b, d). The antibiotics and exposure methods interacted to reduce weight gain and feed efficiency (Table S3). The *O. niloticus* exposed to LEC-OTC/SMZ and LAD-OTC/SMZ had atrophied liver and lower viscera than control fish ($p < 0.05$; Fig. 1e, f). Exposure of *O. niloticus* to LEC-OTC/SMZ and LAD-OTC/SMZ diminished dressed carcass crude lipid composition ($p < 0.05$; Fig. 1g).

Concurrently, LEC-OTC/SMZ and LAD-OTC/SMZ reduced significantly protease and lipase enzymes activities ($p < 0.05$; Fig. 1h, i). With exception of SMZB, fish exposed to the remaining LEC-OTC and LAD-OTC/SMZ had reduced apparent protein and lipid digestibility ($p < 0.05$; Fig. 1j, k). Indeed, antibiotics and exposure methods interacted to impair apparent protein digestibility ($p < 0.05$; Table S3). However, LEC-OTC/SMZ and LAD-OTC/SMZ did not affect crude protein ($p > 0.05$; Fig. 1l), crude carbohydrate and amylase activity ($p > 0.05$; Fig. 1Sa, b). These results indicate that, chronic exposure to LECAs and LADAs have adverse effects on growth performance, lipid body composition and nutrient digestibility. In addition, liver is one of the main target organs for antibiotics effects in *O. niloticus*.

3.2. Low environmental concentrations and legal aquaculture doses of antibiotics damaged intestinal integrity and induced microbiota dysbiosis

Intestinal histomorphological features provide a quick diagnosis to detect abnormalities in gut structure after exposure to xenobiotics; thereof, the intestine structure was assessed in the present study. Chronic exposure to LEC-OTC/SMZ and LAD-OTC/SMZ distorted the gut histomorphology by reducing villi width and muscularis thickness in the treated *O. niloticus* relative to control ($p < 0.05$; Fig. 2a–f).

Tight junction proteins preserve the structural integrity of the intestinal mucosal and act as epithelial cell barriers regulating the passage of molecules, ions and xenobiotics between plasma membranes of adjacent cells. Thus, tight junction proteins allow precise control over which substances can pass through a particular tissue, subsequently playing a vital role in intestinal health of fish. Accordingly, we assessed

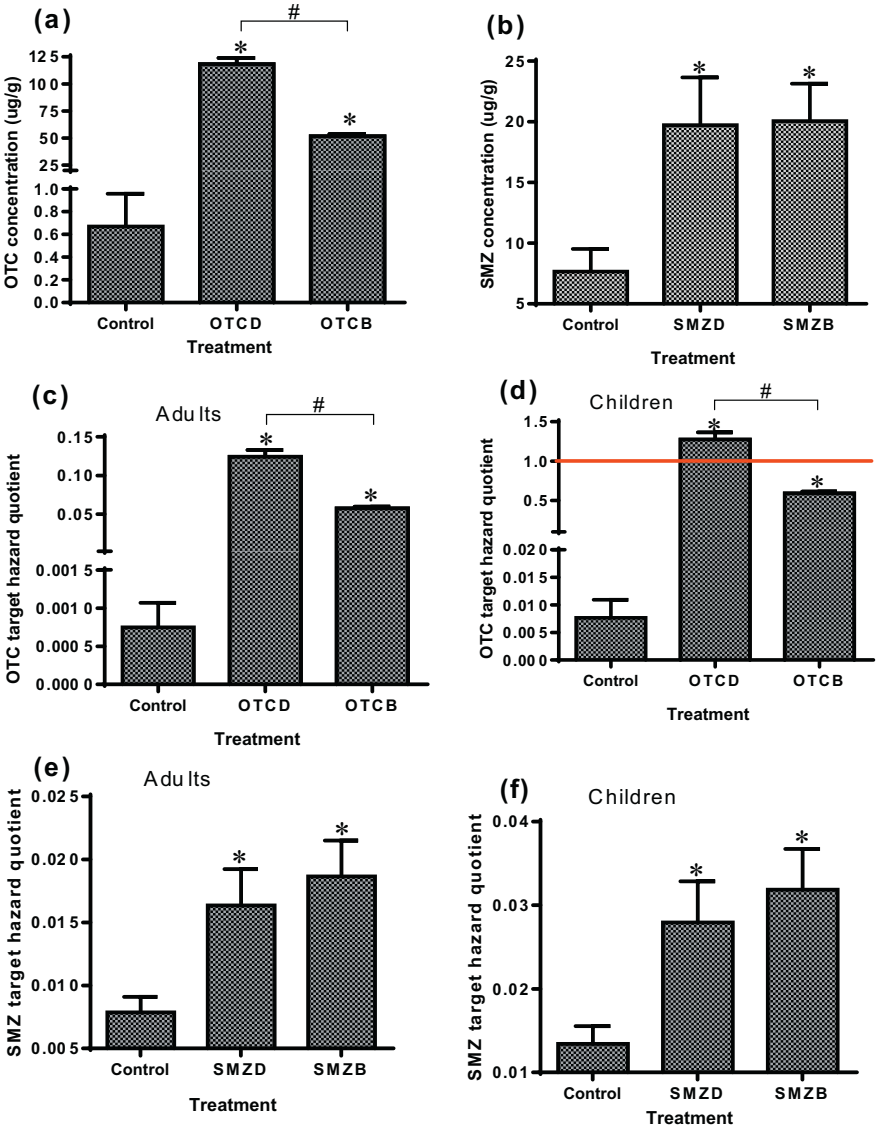


Fig. 7. The concentration of antibiotics in fish muscle and their human health risks arising from consumption of fish contaminated with low environmental concentrations and treated with legal aquaculture doses of antibiotics; (a) oxytetracycline concentration in fish muscles, (b) sulfamethoxazole concentration in fish muscles, (c) oxytetracycline hazard quotients in adults, (d) oxytetracycline hazard quotients in children, (e) sulfamethoxazole hazard quotients in adults, and (f) sulfamethoxazole hazard quotients in children. The asterisk (*) indicates significant differences between control and oxytetracycline diet (OTCD), sulfamethoxazole diet (SMZD), oxytetracycline bath (OTCB) and sulfamethoxazole bath (SMZB), whereas the symbol (#) above a line indicates differences between fish treated with OTCD vs OTCB ($p < 0.05$).

mRNA expressions of the tight junction proteins of fish after antibiotics treatments. The exposure of *O. niloticus* to chronic LEC-OTC/SMZ and LAD- OTC/SMZ suppressed the mRNA expressions of intestinal tight junction proteins such as claudin-1 (CLDN1), claudin-3 (CLDN3), claudin-4 (CLDN4), occludin (OCLDN) and zonula occludens-1 (ZO-1) ($p < 0.05$; Fig. 2g–k). Relatively similar results on suppression of mRNA expressions for tight junction proteins were obtained in skin of *O. niloticus* ($p < 0.05$; Fig. 2Sa, b). Indeed, antibiotics and exposure methods interacted to inhibit the mRNA expressions of claudin-1 ($p = 0.002$), claudin-4 ($p = 0.014$), occludin ($p = 0.006$) and ZO-1 ($p = 0.006$) in skin of treated *O. niloticus*.

Gut microbiota are augmenting recognized for their critical roles on host nutrition, physiological, immunity and metabolic processes. Therefore, we analyzed the abundance and diversity of microbiota in *O. niloticus* after exposure to LEC-OTC/SMZ and LAD-OTC/SMZ. Regardless of the treatments, the microbiota of *O. niloticus* in the intestine were composed of 16 phyla, dominated by Firmicutes (87.68%) and Proteobacteria (10.54%), with other phyla representing < 1% (Table S4). Exposure of *O. niloticus* to OTCB and SMZB increased significantly the abundance (Ace) of microbiota ($p < 0.05$; Table S5), while the former also increased Chao1 index ($p < 0.05$; Table S5). However, the diversity indices (Shannon and Simpson) of microbiota were comparable among treatments ($p > 0.05$; Table S5). Based on the results of independent *t*-test, 22 OTUs in treated fish depicted significant differences relative to control, and these were selected for drawing a heatmap (Fig. 3a). Exposure of fish to LEC-OTC/SMZ and LAD-OTC/SMZ eradicated OTU14 and OTU17 belonging to Proteobacteria and Cyanobacteria phyla, respectively (Fig. 3b). Exposure of *O. niloticus* to OTCD and OTCB disrupted 15 microbiota OTUs including eradication of OTU221 (Bacteroidetes), and OTU157 (Firmicutes) relative to control, compared to only six OTUs that were affected when *O. niloticus* were exposure to SMZD and SMZB (Fig. 3b), suggesting oxytetracycline had greater influence on gut microbiota than sulfamethoxazole treatments. Exposure of *O. niloticus* to SMZD decreased OTU8 (Proteobacteria), and OTU221 (Bacteroidetes) while SMZB decreased OTU278, and OTU100 (Proteobacteria) and increased OTU259 (Fusobacteria) (Fig. 3b). These results suggest that, chronic exposure to antibiotics impair intestinal morphology, permeability and induce microbiota dysbiosis.

3.3. Low environmental concentrations and legal aquaculture doses of antibiotics elicited intestinal and hepatic oxidative stress and caused DNA damage

Antibiotics treatments in fish commonly induces oxidative stress, thus some oxidative stress markers were measured in the present study

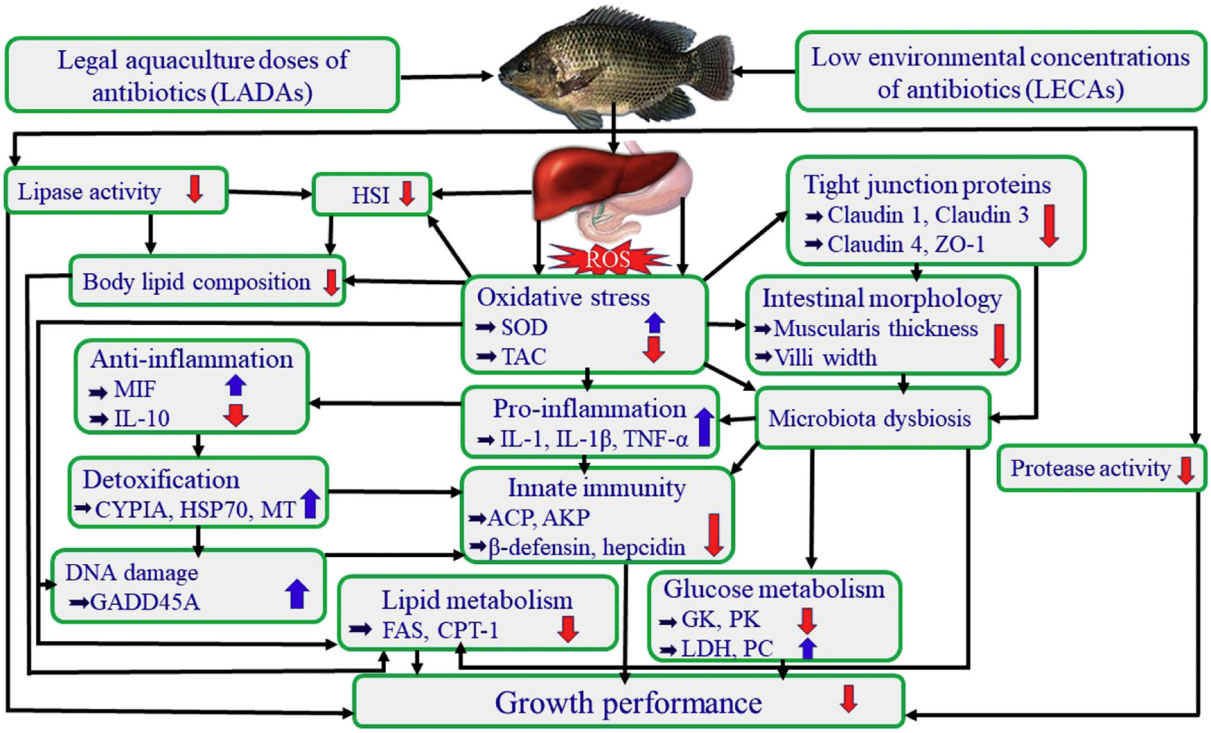


Fig. 8. Chronic systemic impairing pathway for Nile tilapia (*Oreochromis niloticus*) exposed to low environmental concentrations and legal aquaculture doses of antibiotics. Blue and red arrows indicate increased ad decreased responses, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

at biochemical and molecular levels in intestine and liver. Exposure of *O. niloticus* to LEC-OTC/SMZ and LAD-OTC/SMZ increased significantly the activities of GST and SOD in intestine ($p < 0.05$; Fig. 4a, b), and mRNA expressions levels of SOD1 in the intestine ($p < 0.05$; Fig. 4c) and liver ($p < 0.05$; Fig. 4d) and nuclear factor (erythroid-derived 2)-like 2 (NRF2) in intestine ($p < 0.05$; Fig. 4e) and liver ($p < 0.05$; Fig. 4f), and consequently reduced TAC in intestine ($p < 0.05$; Fig. 4g) and liver ($p < 0.05$; Fig. 4h). Most LEC-OTC/SMZ and LAD-OTC/SMZ increased MDA concentrations in the intestine ($p < 0.05$; Fig. 4i) and liver ($p < 0.05$; Fig. 4j). Indeed, antibiotics and exposure methods interacted to elevate MDA levels in intestine of treated fish (Table S3). Moreover, both LEC-OTC/SMZ and LAD-OTC/SMZ increased the expression of metallothionein (MT) gene in intestine ($p < 0.05$; Fig. 4k) and liver ($p < 0.05$; Fig. 4l), which functions primarily in storage, transport, and detoxification of xenobiotics, consequently, they stimulated heat shock proteins 70 (HSP70) ($p < 0.05$; Fig. 3Sa), an intracellular stress signaling marker, and cytochrome P450 (CYP1A) ($p < 0.05$; Fig. 3Sb), a xenobiotics detoxification marker, genes expressions in the liver. In response to the impaired detoxification mechanism, the expression of growth arrest and DNA-damage 45 alpha (GADD45A) gene, a DNA damage marker was upregulated in the liver of treated fish ($p < 0.05$; Fig. 3Sc). Overall, antibiotics and exposure methods interacted to affect SOD1 and CYP1A expressions in the liver (Table S6). These results show that, exposure of fish to chronic antibiotics induce oxidative stress which elicit detoxification response and cause DNA damage.

3.4. Low environmental concentrations and legal aquaculture doses of antibiotics suppressed intestinal and hepatic innate immunity and increased inflammation

The induced oxidative stress in fish after xenobiotics administration frequently causes impaired immune function and higher inflammation; therefore, some immunity and inflammatory indices were measured in intestine and liver. Results revealed that, both LEC-OTC/SMZ and LAD-OTC/SMZ suppressed ACP enzymes activities in the intestine ($p < 0.05$; Fig. 5a) and liver ($p < 0.05$; Fig. 5b), AKP in the intestine ($p < 0.05$; Fig. 5c) and liver ($p < 0.05$; Fig. 5d) as well as mRNA expression genes of hepcidin ($p < 0.05$; Fig. 5e) and β-defensin ($p < 0.05$; Fig. 5f) in the intestine of *O. niloticus*. Both LEC-OTC/SMZ and LAD-OTC/SMZ stimulated the macrophage migration inhibitory factor (MIF) gene in liver ($p < 0.05$; Fig. 5g), which induced upregulation of pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 1 beta (IL-1 β), and tumor necrosis factor-α (TNF-α) in the intestine ($p < 0.05$; Fig. 5h, j, l) and liver ($p < 0.05$; Fig. 5i, k, m) and interleukin 8 (IL-8) in the liver ($p < 0.05$; Fig. 5n), lastly down-regulated IL-10, an anti-inflammatory gene in most *O. niloticus* treated with antibiotics relative to control in the intestine ($p < 0.05$; Fig. 5o) and liver ($p < 0.05$; Fig. 5p). The antibiotics and exposure methods interacted to affect AKP activity in intestine (Table S3) and liver (Table S6), hepcidin, β-defensin, IL-1, IL-1 β, and TNF-α in intestine (Tables S3) and IL-1 and IL-8 in the liver (Table S6). These results indicate that, the immune functions were depressed and inflammation was increased in *O. niloticus* treated with chronic LECAs and LADAs.

3.5. Low environmental concentrations and legal aquaculture doses of antibiotics disturbed glucose and lipid metabolism in intestine and liver organs

This study revealed reduced lipid body composition, lipase activity and apparent lipid digestibility in the previous sections. To further delineate the systemic chronic effects of LECAs and LADAs, we detected the activity of an anaerobic glycolysis enzyme and mRNA expressions of genes involved in glucose and lipid metabolism in intestine and liver. Results indicated elevated LDH activity in intestine ($p < 0.05$; Fig. 6a), an indicator of anaerobic respiration and cellular damage and pyruvate carboxylase (PC) mRNA expression, an important enzyme in gluconeogenesis in intestine ($p < 0.05$; Fig. 6b) and liver ($p < 0.05$; Fig. 6c). In addition, antibiotics and exposure methods interacted to affect PC expression in intestine (Table 3S). The LEC-OTC/SMZ and

Table 1
The general variations in toxicological effects between LECAs and LADAs.

Type	Toxicological effects		Tissue	Reference figure
	LEC-OTC/SMZ	LAD-OTC/SMZ		
Body composition		Lipid composition ↓	General body	Fig. 1g
Tight junction proteins	Occludin ↓	Claudin 1 ↓	Intestine	Fig. 2j, g
Oxidative stress	SOD1 ↑		Intestine	Fig. 4c
Anti-inflammation	IL-10 ↓		Liver	Fig. 5p
Glucose metabolism	LDH ↑	PC ↑	Intestine and liver	Fig. 6a, c
Lipid metabolism	HSL ↑	FAS ↓	Intestine	Fig. 6j, f
Glucose metabolism		PK ↓	Liver	Fig. 6e
	OTCB	OTCD		
Growth and feed efficiency	Weight gain ↓ Feed efficiency ↓		General body	Fig. 1b, d
Intestine histomorphology	Villi width ↓ Muscularis thickness ↓		Intestine	Fig. 2e, f
Oxidative stress	SOD, SOD1 ↑		Intestine and liver	Fig. 4c, d
		NRF2 ↑ TAC ↓	Liver	Fig. 4f, h
Lipid peroxidation		MDA ↑	Liver	Fig. 4j
Intracellular stress	HSP70 ↑		Liver	Fig. 3Sb
Innate immunity	AKP ↓		Intestine	Fig. 5c
		ACP ↓	Liver	Fig. 5b
Pro-inflammation		TNF-α, IL-8 ↑	Liver	Fig. 5m, n
Glucose metabolism		GK ↓	Liver	Fig. 6d
Lipid metabolism	HSL ↑		Liver	Fig. 6k
	SMZB	SMZD		
Digestive enzyme		Lipase ↓	Intestine	Fig. 1i
Tight junction proteins	Claudin-4 ↓		Intestine	Fig. 2i
Lipid peroxidation	MDA ↑		Intestine	Fig. 4i
Oxidative stress	NRF2 ↑		Intestine and liver	Fig. 4e, f
	TNF-α, IL-8 ↑		Liver	Fig. 5m, n
Pro-inflammation	IL-1 ↑		Intestine and liver	Fig. 5h, i
Innate immunity	Hepcidin ↓ B-defensin ↓		Intestine	Fig. 5e, f
	ACP, AKP ↓		Liver	Fig. 5b, d
		ACP, AKP ↓	Intestine	Fig. 5a, c
Glucose metabolism	GK ↑		Liver	Fig. 6d

LAD-OTC/SMZ reduced glucokinase (GK) and pyruvate kinase (PK) genes in treated *O. niloticus* relative to control in the liver ($p < 0.05$; Fig. 6d, e). They also down-regulated the fatty acid synthetase (FAS) in the intestine ($p < 0.05$; Fig. 6f) and liver ($p < 0.05$; Fig. 6g) and carnitine palmitoyltransferase 1 (CPT-1) mRNA expressions in the intestine ($p < 0.05$; Fig. 6h) and liver ($p < 0.05$; Fig. 6i), suggesting inhibited lipogenesis and fatty acid β-oxidation, respectively. Correspondingly, hormone sensitive lipase (HSL) gene expression was increased in fish exposed to LEC-SMZB in intestine ($p < 0.05$; Fig. 6j) and both LEC-OTC/SMZ and LAD-OTC/SMZ in the liver ($p < 0.05$; Fig. 6k). In fact, antibiotics and exposure methods interacted to affect expression of FAS in intestine (Table 3S) and HSL gene in the liver of treated fish (Table S6). These results suggest that, exposure to chronic antibiotics disrupt glucose and lipid metabolism in fish.

3.6. Low environmental concentrations and legal aquaculture doses of antibiotics pose differential human health risk after fish consumption

In order to evaluate the potential human health risk to fish consumers, the accumulation of the antibiotics in fillets in the experimental *O. niloticus* was assayed. The levels of antibiotics in fillets of fish treated with LEC-OTC/SMZ and LAD-OTC/SMZ were higher than those in the control ($p < 0.05$), both for OTC ($p < 0.05$; Fig. 7a) and SMZ ($p < 0.05$; Fig. 7b). For OTC, the fish fed the diet supplemented with the legal aquaculture dose contained significantly higher amount than those exposed to low environment concentration ($p < 0.05$; Fig. 7a), while similar concentrations existed for those fed and exposed to SMZ ($p > 0.05$; Fig. 7b).

We then assessed the potential human health risk for adults and children after consuming fish fillets. The target hazard quotients for fish fed on LEC-OTC/SMZ and those exposed to LAD-OTC/SMZ for the two antibiotics were higher than the control, both for adults and children ($p < 0.05$; Fig. 7c–f). For OTC, the hazard quotients for OTCD were higher than that of OTCB ($p < 0.05$; Fig. 7c, d). Indeed, the target hazard quotient for OTCD in children was > 1 indicating human health risk to this group (Fig. 7d). For SMZ, the hazard quotients for fish fed on SMZD and those exposed to SMZB for adults and children were statistically comparable ($p > 0.05$). All hazard quotients for LEC and LAD for SMZ were lower than a unit, indicating human health safety (Fig. 7e, f). These results indicate that, consumption of fish treated with chronic legal aquaculture antibiotics, particularly OTCD in children pose human health risk.

4. Discussion

4.1. Metabolic toxicological characteristics of the fish exposed to low environmental concentrations and legal aquaculture doses of antibiotics

We systematically identified three main metabolic toxicological characteristics after chronic exposure to LECAs and LADAs in *O. niloticus* (Fig. 8). The first main metabolic toxicological feature is nutritional impairment, which is a multifaced phenomenon attributed to; 1) liver atrophy, which led to hepatic steatosis and necrosis, consequently impaired glucose and lipid metabolism; 2) distorted gut histomorphology through reduced villi width and muscularis thickness as an attempt to facilitate absorption of more nutrients and enhance nutrient digestibility under stressful conditions; and 3) intestinal microbiota dysbiosis such as the eradication of Lachnospiraceae family (Firmicutes) and *Cloacibacterium* species (Bacteroidetes), which participate in carbohydrate metabolism (Allen et al., 2006; Scott et al., 2014). In general, the observed nutritional dysfunctions indicate stressful conditions after chronic exposure to LEC-OTC/SMZ and LAD-OTC/SMZ. Antibiotics exposure in treated fish inhibited aerobic glycolysis, enhanced gluconeogenesis and suppressed lipogenesis and fatty acid β-oxidation in liver and intestine due to their ability to increase generation of endogenous reactive oxygen species (ROS) such as oxygen ions, free radicals and peroxides, which induced oxidative stress (Nazeri et al., 2017). Clear evidences exist in the literature indicating that antibiotics particularly OTC and SMZ are metabolized in the body of various fish species resulting into generation of ROS in *Oncorhynchus mykiss* (Yonar et al., 2011; Rodrigues et al., 2017a; Rodrigues et al., 2017b), *Sparus aurata* (Guardiola et al., 2012), *Carassius auratus* (Li et al., 2012), *Danio rerio* (Oliveira et al., 2013; Yan et al., 2016), *Gambusia holbrooki* (Nunes et al., 2015). The ROS generated by antibiotics cause alterations and damages at biochemical, cellular and tissues levels (Nunes et al., 2015), including inhibition of mitochondrial β-oxidation and disruption of the respiratory chain (Rodrigues et al., 2017b).

Evidently, the inhibited aerobic glycolysis due to ROS generation led to enhanced gluconeogenesis as a compensatory mechanism to generate more energy. In the same vein, the liver of fish exposed to LEC-OTC/SMZ and LAD-OTC/SMZ reacted by enhancing HSL gene expression, which hydrolyzes stored triglycerides to free fatty acids, when the body needs to mobilize energy stores under stressful conditions as a response to reduced lipogenesis and fatty acid β-oxidation coupled with reduced lipid body composition and lipase activity. Since lipogenesis and fatty acid β-oxidation are critical for lipid storage in the liver, the

Table 2
The differences in the toxicological mechanisms between low-dose and high-dose exposures.

Antibiotic	Species/size	Dose	Duration (days)	Effect	Reference
Oxytetracycline	*Oryzias latipes*/larvae 30 dph	0.005 to 50 mg/L	90	No effect on growth and survival rate.	Ji et al. (2012)
	Labeo rohita/9 g	80 mg/L	25	Time-specific increase in LDH activity.	Ambili et al. (2013)
	Danio rerio/larvae 48 hpf	750 ppm	4	High inflammation	Barros-Becker et al. (2012)
	Danio rerio/2 ± 1 cm	1 to 100 mg/L	4	Dose and tissue-specific increase in GST and LDH activities.	Oliveira et al. (2013)
	Oncorhynchus mykiss/9.24 ± 0.23 g	0.005 to 50 mg/L	4	Dose-dependent increase in TBARS levels, and DNA damage.	Rodrigues et al. (2017a), Rodrigues et al. (2017b)
	Oncorhynchus mykiss/7.75 ± 0.53 g	0.3125 to 5 µg/L	28	Increased liver pathological index and DNA damage.	Rodrigues et al. (2017a), Rodrigues et al. (2017b)
	Oreochromis niloticus	8000 ng/L	4	DNA damage	Botelho et al. (2015)
Sulfamethoxazole	*Danio rerio*/adult fish	533 µg/L	21	Lower nuclear hepatocytes	Madureira et al. (2012)
	Danio rerio/14 dpf	2 to 200 µg/L	150	No differences in body weight, length, and condition factor. Increased EROD and SOD activities. Dose-dependent lower survival rate.	Yan et al. (2016)
	Carassius auratus/39.50 ± 2.55 g	0.016 to 50 mg/L	7	Time-dependent increase in EROD. Dose-dependent increase in GST activities. All doses increased SOD activity.	Li et al. (2012)

Note: dph = days post-hatching, EROD = 7-ethoxyresorufin *O*-dealkylase, GST = glutathione *s*-transferase, hpf = hours post-fertilization, LDH = lactate dehydrogenase, SOD = superoxide dismutase, TBARS = thiobarbituric acid reactive substances.

down-regulation of FAS and CPT-1 genes might explain the liver atrophy observed in treated fish. Taken together, exposure to LEC-OTC/SMZ and LAD-OTC/SMZ exert nutritional malabsorption which may lead to malnutrition, affecting the quality of fish produced.

The second metabolic toxicological feature of chronic exposure to LEC-OTC/SMZ and LAD-OTC/SMZ in *O. niloticus* is their effect on antioxidant capacity, detoxification mechanism and DNA damage. In the present study, LEC-OTC/SMZ and LAD-OTC/SMZ generated ROS, which increased antioxidant enzymes both at biochemical (GST and SOD) level in intestine and molecular level (SOD1) in intestine and liver through stimulation of NRF2, a regulator of antioxidant enzymes in liver, which exhausted the total antioxidant capacity (TAC) in both liver and intestine, consequently, elicited intracellular stress (HSP70) and induced detoxification responses (MT and CYPIA) in the liver and intestine. Evidently, both antioxidant and detoxification mechanisms were insufficient to clear the oxidative stress caused by antibiotics because GADD45A gene expression was elevated in treated fish. These results suggest severe intestine and liver tissue damages which might lead to increased toxicity of antibiotics, damaging muscle, nervous system, cellular membrane, metabolism, DNA, and eventually cell death.

The last general metabolic toxicological attribute of chronic exposure to LEC-OTC/SMZ and LAD-OTC/SMZ in *O. niloticus* is innate immunity suppression, ascribed to distorted tight junction proteins, both in intestine (Fig. 2g–k) and skin (Fig. 2Sa, b) tissues by increased oxidative stress, which broke down microtubule and F-actin cytoskeletons, as previous indicated in fish (Syakuri et al., 2013) and mice (Nevado et al., 2015). The impaired intestinal tight junctions caused a "leaky gut" that facilitated attack of lysosomal membranes, lymphatic channel and hepatic sinus (F. Li et al., 2016) by antibiotics-induced oxidative stress which limited the secretions of ACP and AKP in intestine and liver as well as interfered with mRNA expression levels of hepcidin and β-defensin genes in the intestine, which are first line innate immunity defense enzymes and markers of xenobiotics exposure. Responsively, *O. niloticus* treated with LEC-OTC/SMZ and LAD-OTC/SMZ stimulated neutrophils and macrophages to release MIF in the liver which induced upregulation of pro-inflammatory cytokines in the intestine and liver as a protective response and completed the inflammation as indicated by down-regulation of an anti-inflammatory gene (Fig. 5o) in the intestine. These results imply that, chronic exposure to LEC-OTC/SMZ and LAD-OTC/SMZ in fish suppresses innate immunity through impaired intestinal barrier function and first line defense enzymes resulting into increased permeability, which may further affect fish health.

4.2. The systemic differences in the toxicological mechanisms between low environmental concentrations and legal aquaculture doses of antibiotics

Despite the long-term exposure of fish to LECAs and LADAs, no study in the literature has attempted to elaborate critically their variations in toxicological effects. A systemic synthesis of all results in our study indicates that, LEC-OTC/SMZ and LAD-OTC/SMZ produce relatively similar number of toxicological effects when the two antibiotics are considered together (Table 1). Nonetheless, individual LEC-OTC/SMZ and LAD-OTC/SMZ of each antibiotic vary in toxicological effects; thereof, LAD-OTC/SMZ produce limited tissue-specific effects while LEC-OTC/SMZ induce substantial toxicological effects for the two tissues sampled. The OTCD had toxicological effects that were limited to the liver, consistent with its accumulation in this tissue in fish (Yuan et al., 2014). Meanwhile, SMZD toxicological effects were only detected in the intestine, an observation which requires further study to elucidate. Remarkably, individual LEC-OTC/SMZ for each antibiotic elicited profound toxicological effects in the two organs sampled than LAD-OTC/SMZ (Table 1). We attribute the ability of LEC-OTC/SMZ to cause extensive toxicological effects in treated fish to four mechanisms; 1) LECAs are easy to digest due to their existence in liquid state compared to LADAs that are in solid states in feeds which require digestion process to set them available for biochemical reactions; 2) LECAs have higher solubility because they are already suspended in solution, which is an initial step for absorption of substances by cells; and 3) LECAs have higher assimilation rates because they are readily absorbed than LADAs, thus penetrate and become widely distributed in various tissues after exposure, resulting into multiple toxicological effects. Moreover, since LADAs are scientifically and experimentally evaluated before they are legally licensed for use, inherently they pose less toxicological effects compared to LECAs, which are environmentally site-specific pollutants. These results imply that, globally both LECAs and LADAs lead to relatively similar toxicological effects, however LECAs of individual antibiotics cause diverse toxicological effects, which impair multiple organs than LADAs, suggesting severe effects of low environmental residues of antibiotics on fish in the aquatic environments.

4.3. The distinctive toxicological effects of low environmental concentrations of antibiotics

In the aquatic environment, information regarding the effects of LECAs in fish is unavailable, presumably because of the notion that they do not cause severe toxicological effects than high levels of antibiotics. We related the results of previous studies obtained in various fish species exposed to high concentrations of OTC and SMZ to the present study (Table 2). Noteworthy, some of the previous studies, which used higher environmental concentrations of the two antibiotics for extended periods did not find differences in growth performance (Ji et al., 2012; Yan et al., 2016), contrary to the reduced weight gain obtained in the present study (Fig. 1b). This observation unveils our understanding that, exposure of fish to even very low environmental concentrations of antibiotics as those used in the present study impair growth performance.

Interestingly, exposure of fish to high environmental concentrations resulted into several similar toxicological effects to low concentrations obtained in the present study. For instance, the two antibiotics caused liver toxicological effects (Madureira et al., 2012; Rodrigues et al., 2017a; Rodrigues et al., 2017b), similar to atrophied liver (Fig. 1e). Moreover, the reported oxidative stress (Li et al., 2012; Oliveira et al., 2013; Yan et al., 2016) are consistent with the results in the intestine and liver (Fig. 4a–d). Similarly, lipid peroxidation in the liver (Rodrigues et al., 2017a) is coherent with higher MDA concentration for fish exposed to LEC-OTC/SMZ in intestine (Fig. 4i). Furthermore, the widespread inflammation reported by Barros-Becker et al. (2012), is similar to results obtained in intestine and liver (Fig. 5h–p), while the induced detoxification response (Li et al., 2012; Yan et al., 2016) is similar to elevated CYPIA gene expression results obtained in the liver (Fig. 3Sb). Likewise, anaerobic respiration and cellular damage in gill, muscle and liver at various periods (Ambili et al., 2013; Oliveira et al., 2013) are similar to present results in Fig. 6a in the intestine. Lastly, the induced DNA damage obtained in LEC-OTC/SMZ in the liver (Fig. 3Sc), has been reported when high concentrations of OTC were used (Botelho et al., 2015; Rodrigues et al., 2017a; Rodrigues et al., 2017b).

Clearly, these results should unfold scientists thinking and realize that, very low environmental concentrations (ng/L) of antibiotics may not have distinctive toxicological effects of their own, but are biologically active and act as potential micropollutants in the aquatic environments causing similar toxicological effects as high concentrations. Responsibly, intentional strict global policies limiting the release and prohibiting the use of antibiotics in animal production are required to reduce environmental effects on fish and protect human health.

4.4. Potential human health risk and environmental relevance

The consumption of fish fillets from aquaculture has been criticized globally to contain unsafe levels of antibiotics due to environmental pollution and antibiotics usage. Despite the global accusation of antibiotics, direct evidences are hardly available, because previous studies mostly focused on ecological effects or environmental toxicity of antibiotics, with little or no priority directed towards human health risks (Ambili et al., 2013; Brandt et al., 2015; Chen et al., 2015; Dong et al., 2016). We showed clearly that the consumption of fish fed on legal aquaculture dose of dietary OTC poses a health risk in children who are particularly vulnerable to the toxic burdens from xenobiotics. Moreover, the risk quotient for fish fillets exposed to the low environmental concentration of OTC in children was > 0.5, highlighting the existence of low risk. However, this risk may be higher in practical settings because OTC concentrations up to 741.85 ng/L (Bai et al., 2014) and 15,163 ng/L (Chen et al., 2015) have been detected in some aquatic environments, which are far higher than 420 ng/L used in the current human health risk assessment. Although the health risks of consuming antibiotics-treated fish on children are currently limited, studies using other exposure methods on animal models indicate potential human health risks such as psychological abnormalities (Wang et al., 2015), stroke (Winek et al., 2016), decrease in neurogenesis and cognitive function (Möhle et al., 2016) and brain damage (Siswanto et al., 2016). These damages are also possibly happening in children due to the long-term consumption of antibiotics-treated fish because antibiotics have been found in urine (Li et al., 2017) and are associated with increased risk of asthma in children (Metsälä et al., 2015). Most importantly, consumption of fish treated with antibiotics could cause antibiotics resistance bacteria and genes to human, particularly in children, because their exposure life is longer than adults (Huddleston, 2014).

Practically, although human health risk was not found after consumption of fish treated with LEC-OTC/SMZ and those treated with OTC for adults, yet the absence of evidence does not mean that there is no risk because of: 1) higher doses of antibiotics are likely to be used in aquaculture and on daily basis (Pham et al., 2015; Phu et al., 2015) due to inadequate knowledge, high stocking densities and diverse number of pathogens; 2) the possible existence of “synergistic effects” or “cocktail effects” resulting from mixture of antibiotics and other pollutants; 3) the presence of specific human populations, who consume higher amount of fish such as fish farmers than that used in the current analysis (Du et al., 2012); and 4) the higher concentrations of OTC in the muscles of treated fish (Fig. 7a) than the established tolerable levels (2 μg/g) (U.S. FDA, 2017). Responsibly, condemning the daily use of antibiotics by imposing strict regulations and production of antibiotics-free fish by prohibiting their use in aquaculture are welcome precautionary approaches to safeguard human health.

The aquatic environments act as the final sink for the majority of antibiotics produced from human activities either directly or indirectly, consequently leading into long-term adverse effects on the aquatic living resources. For the first time, our study generated four important pieces of information for environmentalists and policy makers. First, the general chronic systemic toxicological effects of fish exposure to low environmental doses of antibiotics and their legal aquaculture concentrations intend to apprise the minds of environmentalists and policy makers to protect aquatic living resources by formulating policies, which limit the uses of antibiotics on animal multinational food production industry. Secondly, environmentalists should be alarmed by the multiple effects caused by low environmental concentrations of antibiotics due to their immanent higher bioavailability, with potential long-term effects on aquatic ecosystem. Thirdly, environmentalists should not only be concerned about high concentrations of antibiotics in the aquatic environments but rather any existence of antibiotics should be deemed as an alarming situation because chronic low and high environmental concentrations of antibiotics induce comparatively similar toxicological effects. Finally, and more importantly, consumption of fish treated with OTC at legal dose provokes direct health risk in children thus, pose public health concerns.

Despite the important contributions to knowledge generated in the present study, our findings should be considered in the context of the following limitations. First, we used juvenile *O. niloticus* which is not commercially traded and consumed world-wide. Apparently, adult fish may have variations in physiological and metabolic mechanisms to detoxify antibiotics that may differ from juvenile fish, which limits the scope of interpretations of our results to particular fish size. Secondly, we noticed that the dietary ingredients and city water used were already contaminated with antibiotics as indicated by their levels in control diet, water (Table S1) and muscles in the fish fed with the control diet (Fig. 7a, b), which possibly caused additive effects on antibiotics in the treated fish groups, or covered possible differences in fish physiological or toxicological indices between control and antibiotics treatments. Thirdly, the present study is a laboratory-based in which conditions such as water quality are easily monitored and controlled compared to practical large scale commercial production and wild environments where there is little or no control of variables and multiple pollution factors affect fish and human health. Certainly, in practice effects may be worse (for synergistic effects of different

antibiotics and other pollutants) or least when different factors work antagonistically to affect fish and human health risk. Fourth, the present study did not consider withdraw period because we aimed to establish the potential chronic effects of the antibiotics on fish and human health due to violation of the legal guidance by fish farmers (Pham et al., 2015; Phu et al., 2015). Therefore, the results and interpretations given in our study are limited to fish farmers who apply antibiotics for extended periods without following withdraw periods. Thus, to consolidate the results, further studies that consider different fish sizes, which are raised in large scale production and considering withdraw periods are needed to elucidate the different chronic toxicological effects of antibiotics in fish and human health risk.

5. Conclusion

For the first time we show that, regardless of the exposure route, chronic low environmental concentrations and legal aquaculture doses antibiotics severely impair general body physiological functions, nutritional metabolism, fish health and compromise their immune system. Although both legal aquaculture doses and low environmental concentrations of antibiotics cause toxicological effects, the latter pose severe risks than the former, due to their higher bioavailability, indicating grievous environmental risks than legal aquaculture antibiotics. Noticeable, chronic low environmental concentrations of antibiotics are biologically toxic, cause comparatively similar and in some cases, serious toxicological effects than high concentrations. Our results provide substantial evidence that low environmental concentrations and their legal aquaculture doses of antibiotics cause severely systemic fish effects and the latter, particularly, dietary OTC pose direct human health risk in children, requiring global public stringent prohibition policies on their use in animal food production and strategies to limit release of antibiotics in the aquatic environments.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

We are grateful to the Chinese government for funding this Ph.D. work through China Scholarship Council (CSC) without influence on the content. This work was also funded by the Fundamental Research Funds for the Central Universities, to whom we are thankful.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.03.034.

References

Allen, T.D., Lawson, P.A., Collins, M.D., Falsen, E., Tanner, R.S., 2006. *Cloacibacterium normanense* gen. nov., sp. nov., a novel bacterium in the family Flavobacteriaceae isolated from municipal wastewater. Int. J. Syst. Evol. Microbiol. 56, 1311–1316.

Ambili, T.R., Saravanan, M., Ramesh, M., Abhijith, D.B., Poopal, R.K., 2013. Toxicological effects of the antibiotic oxytetracycline to an Indian major carp *Labeo rohita*. Arch. Environ. Contam. Toxicol. 64, 494–503.

AOAC (Ed.), 1995. Official Methods of Analysis, 16th ed. AOAC International, Arlington, Virginia, USA.

Bai, Y., Meng, W., Xu, J., Zhang, Y., Guo, C., 2014. Occurrence, distribution and bioaccumulation of antibiotics in the Liao River Basin in China. Environ. Sci.: Processes Impacts 16, 586–593.

Barros-Becker, F., Romero, J., Pulgar, A., Feijóo, C.G., 2012. Persistent oxytetracycline exposure induces an inflammatory process that improves regenerative capacity in zebrafish larvae. PLoS One 7, e36827.

Botelho, R.G., Christofoletti, C.A., Correia, J.E., Ansoar, Y., Olinda, R.A., Tornisielo, V.L., 2015. Genotoxic responses of juvenile tilapia (*Oreochromis niloticus*) exposed to florfenicol and oxytetracycline. Chemosphere 132, 206–212.

Brandt, K.K., Amézquita, A., Backhaus, T., Boxall, A., Coors, A., Heberer, T., Lawrence, J.R., Lazorchak, J., Schönfeld, J., Snape, J.R., Zhu, Y.-G., Topp, E., 2015. Ecotoxicological assessment of antibiotics: a call for improved consideration of microorganisms. Environ. Int. 85, 189–205.

Carvalho, I.T., Santos, L., 2016. Antibiotics in the aquatic environments: a review of the European scenario. Environ. Int. 94, 736–757.

CDDEP, 2015. State of the World's Antibiotics, 2015. pp. 79 (Washington, D.C.).

Chen, K., Zhou, J.L., 2014. Occurrence and behavior of antibiotics in water and sediments from the Huangpu River, Shanghai, China. Chemosphere 95, 604–612.

Chen, H., Liu, S., Xu, X.-R., Zhou, G.-J., Liu, S.-S., Yue, W.-Z., Sun, K.-F., Ying, G.-G., 2015. Antibiotics in the coastal environment of the Hailing Bay region, South China Sea: spatial distribution, source analysis and ecological risks. Mar. Pollut. Bull. 95, 365–373.

Chen, Y., Zhou, J.L., Cheng, L., Zheng, Y.Y., Xu, J., 2017. Sediment and salinity effects on the bioaccumulation of sulfamethoxazole in zebrafish (*Danio rerio*). Chemosphere 180, 467–475.

Dobšíková, R., Blahová, J., Mikulíková, I., Modrá, H., Prášková, E., Svobodová, Z., Škorič, M., Jarkovský, J., Siwicki, A.-K., 2013. The effect of oyster mushroom β-1.3/1.6-D-glucan and oxytetracycline antibiotic on biometrical, haematological, biochemical, and immunological indices, and histopathological changes in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 35, 1813–1823.

Dong, D., Zhang, L., Liu, S., Guo, Z., Hua, X., 2016. Antibiotics in water and sediments from Liao River in Jilin Province, China: occurrence, distribution, and risk assessment. Environ. Earth Sci. 75, 1202.

Du, Z.-Y., Zhang, J., Wang, C., Li, L., Man, Q., Lundebye, A.-K., Frøyland, L., 2012. Risk–benefit evaluation of fish from Chinese markets: nutrients and contaminants in 24 fish species from five big cities and related assessment for human health. Sci. Total Environ. 416, 187–199.

Gaikowski, M.P., Wolf, J.C., Schleis, S.M., Gingerich, W.H., 2003. Safety of oxytetracycline (Terramycin TM-100F) administered in feed to hybrid Striped bass, Walleyes, and Yellow perch. J. Aquat. Anim. Health 15, 274–286.

Gaikowski, M.P., Whitsel, M.K., Charles, S., Schleis, S.M., Crouch, L.S., Endris, R.G., 2015. Depletion of florfenicol amine in tilapia (*Oreochromis* sp.) maintained in a recirculating aquaculture system following Aquaflor®-medicated feed therapy. Aquac. Res. 46, 1842–1857.

Guardiola, F.A., Cerezuela, R., Meseguer, J., Esteban, M.A., 2012. Modulation of the immune parameters and expression of genes of gilthead seabream (*Sparus aurata* L.) by dietary administration of oxytetracycline. Aquaculture 334–337, 51–57.

Han, J., Zhang, L., Yang, S., Wang, J., Tan, D., 2014. Detrimental effects of metronidazole on selected innate immunological indicators in common carp (*Cyprinus carpio* L.). Bull. Environ. Contam. Toxicol. 92, 196–201.

He, S., Zhou, Z., Liu, Y., Cao, Y., Meng, K., Shi, P., Yao, B., Ringø, E., 2012. Do dietary betaine and the antibiotic florfenicol influence the intestinal autochthonous bacterial community in hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂)? World J. Microbiol. Biotechnol. 28, 785–791.

Huddleston, J.R., 2014. Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. Infect. Drug Resist. 7, 167–176.

Ji, K., Kim, S., Han, S., Seo, J., Lee, S., Park, Y., Choi, K., Kho, Y.-L., Kim, P.-G., Park, J., Choi, K., 2012. Risk assessment of chlortetracycline, oxytetracycline, sulfamethazine, sulfathiazole, and erythromycin in aquatic environment: are the current environmental concentrations safe? Ecotoxicology 21, 2031–2050.

Johansson, C.H., Janmar, L., Backhaus, T., 2014. Toxicity of ciprofloxacin and sulfamethoxazole to marine periphytic algae and bacteria. Aquat. Toxicol. 156, 248–258.

Li, Z., Lu, G., Yang, X., Wang, C., 2012. Single and combined effects of selected pharmaceuticals at sublethal concentrations on multiple biomarkers in *Carassius auratus*. Ecotoxicology 21, 353–361.

Li, F., Wang, H., Liu, J., Lin, J., Zeng, A., Ai, W., Wang, X., Dahlgren, R.A., Wang, H., 2016a. Immunotoxicity of β-Diketone antibiotic mixtures to zebrafish (*Danio rerio*) by transcriptome analysis. PLoS One 11, e0152530.

Li, Y., Liu, B., Zhang, X., Wang, J., Gao, S., 2016b. The distribution of veterinary antibiotics in the river system in a livestock-producing region and interactions between different phases. Environ. Sci. Pollut. Res. 23, 16542–16551.

Li, N., Ho, K.W.K., Ying, G.-G., Deng, W.-J., 2017. Veterinary antibiotics in food, drinking water, and the urine of preschool children in Hong Kong. Environ. Int. 108, 246–252.

Liu, B., Cui, Y., Brown, P.B., Ge, X., Xie, J., Xu, P., 2015. Cytotoxic effects and apoptosis induction of enrofloxacin in hepatic cell line of grass carp (*Ctenopharyngodon idellus*). Fish Shellfish Immunol. 47, 639–644.

Liu, X., Steele, J.C., Meng, X.-Z., 2017. Usage, residue, and human health risk of antibiotics in Chinese aquaculture: a review. Environ. Pollut. 223, 161–169.

Madureira, T.V., Rocha, M.J., Cruzeiro, C., Rodrigues, I., Monteiro, R.A.F., Rocha, E., 2012. The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): evaluation of impacts on fish liver, by histopathology, stereology, vitellogenin and CYP1A immunohistochemistry, after sub-acute exposures of the zebrafish model. Environ. Toxicol. Pharmacol. 34, 34–45.

MDH, 2013. Health Based Value for Groundwater. Unit, H.R.A. Environmental Health Division, Minnesota, USA, pp. 6. Available from: http://www.health.state.mn.us/divs/eh/risk/guidance/gw/sulfamethoxsum.pdf, Accessed date: 9 December 2017.

MDH, 2015. Health Based Guidance for Water. Unit, H.R.A. Environmental Health Division, Minnesota, USA, pp. 7. Available from: http://www.health.state.mn.us/divs/eh/risk/guidance/gw/sulfamethsum.pdf, Accessed date: 9 December 2017.

Metsälä, J., Lundqvist, A., Virta, L.J., Kaila, M., Gissler, M., Virtanen, S.M., 2015. Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. Clin. Exp. Allergy 45, 137–145.

Möhle, L., Mattei, D., Heimesaat, Markus M., Bereswill, S., Fischer, A., Alutis, M., French, T., Hambardzumyan, D., Matzinger, P., Dunay, Ildiko R., Wolf, Susanne A., 2016. Ly6Chi monocytes provide a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis. Cell Rep. 15, 1945–1956.

Navarrete, P., Mardones, P., Opazo, R., Espejo, R., Romero, J., 2008. Oxytetracycline

treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. J. Aquat. Anim. Health 20, 177–183.
Nazeri, S., Farhangi, M., Modarres, S., 2017. The effect of different dietary inclusion levels of rutin (a flavonoid) on some liver enzyme activities and oxidative stress indices in rainbow trout, *Oncorhynchus mykiss* (Walbaum) exposed to oxytetracycline. Aquac. Res. 48, 4356–4362.
Nevado, R., Forcén, R., Layunta, E., Murillo, M.D., Grasa, L., 2015. Neomycin and bacitracin reduce the intestinal permeability in mice and increase the expression of some tight-junction proteins. Rev. Esp. Enferm. Dig. 107, 672–676.
Nunes, B., Antunes, S.C., Gomes, R., Campos, J.C., Braga, M.R., Ramos, A.S., Correia, A.T., 2015. Acute effects of tetracycline exposure in the freshwater fish *Gambusia holbrooki*: antioxidant effects, neurotoxicity and histological alterations. Arch. Environ. Contam. Toxicol. 68, 371–381.
Oliveira, R., McDonough, S., Ladewig, J.C.L., Soares, A.M.V.M., Nogueira, A.J.A., Domingues, I., 2013. Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 36, 903–912.
Pham, D.K., Chu, J., Do, N.T., Brose, F., Degand, G., Delahaut, P., De Pauw, E., Douny, C., Van Nguyen, K., Vu, T.D., Scippo, M.-L., Wertheim, H.F.L., 2015. Monitoring antibiotic use and residue in freshwater aquaculture for domestic use in Vietnam. EcoHealth 12, 480–489.
Phu, T.M., Scippo, M.-L., Phuong, N.T., Tien, C.T.K., Son, C.H., Dalsgaard, A., 2015. Withdrawal time for sulfamethoxazole and trimethoprim following treatment of striped catfish (*Pangasianodon hypophthalmus*) and hybrid red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*). Aquaculture 437, 256–262.
Reda, R.M., Ibrahim, R.E., Ahmed, E.-N.G., El-Bouhy, Z.M., 2013. Effect of oxytetracycline and florfenicol as growth promoters on the health status of cultured *Oreochromis niloticus*. Egypt. J. Aquat. Res. 39, 241–248.
Rodrigues, S., Antunes, S.C., Correia, A.T., Nunes, B., 2017a. Rainbow trout (*Oncorhynchus mykiss*) pro-oxidant and genotoxic responses following acute and chronic exposure to the antibiotic oxytetracycline. Ecotoxicology 26, 104–117.
Rodrigues, S., Antunes, S.C., Nunes, B., Correia, A.T., 2017b. Histological alterations in gills and liver of rainbow trout (*Oncorhynchus mykiss*) after exposure to the antibiotic oxytetracycline. Environ. Toxicol. Pharmacol. 53, 164–176.
Sanchez, W., Sremski, W., Piccini, B., Palluel, O., Maillot-Maréchal, E., Betoulle, S., Jaffal, A., Aït-Aïssa, S., Brion, F., Thybaud, E., Hinfray, N., Porcher, J.-M., 2011. Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. Environ. Int. 37, 1342–1348.
Scott, K.P., Martin, J.C., Duncan, S.H., Flint, H.J., 2014. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. FEMS Microbiol. Ecol. 87, 30–40.
Siswanto, S., Arozal, W., Juniantito, V., Grace, A., Agustini, F.D., Nafrialdi, 2016. The effect of mangiferin against brain damage caused by oxidative stress and inflammation induced by doxorubicin. HAYATI J. Biosci. 23, 51–55.
Sotto, R.B.D., Medriano, C.D., Cho, Y., Kim, H., Chung, I.-Y., Seok, K.-S., Song, K.G., Hong, S.W., Park, Y., Kim, S., 2017. Sub-lethal pharmaceutical hazard tracking in adult zebrafish using untargeted LC–MS environmental metabolomics. J. Hazard. Mater. 339, 63–72.
Sun, D.-L., Jiang, X., Wu, Q.L., Zhou, N.-Y., 2013. Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. Appl. Environ. Microbiol. 79, 5962–5969.
Syakuri, H., Adamek, M., Brogden, G., Rakus, K.Ł., Matras, M., Irnazarow, I., Steinhagen, D., 2013. Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: molecular identification and regulation of the mRNA expression of claudin encoding genes. Fish Shellfish Immunol. 34, 305–314.
U.S. EPA, 2017. Federal Register/Vol. 82, No. 46/Friday, March 10, 2017/Rules and Regulations, Environmental Protection Agency (EPA). Federal Register, Washington DC, USA, pp. 13245–13251. Available from: https://www.gpo.gov/fdsys/pkg/FR-2017-03-10/pdf/2017-04795.pdf, Accessed date: 9 December 2017.
U.S. FDA, 2017. CFR - Code of Federal Regulations Title 21. Animal Drugs, Feeds, and Related Products. Tolerances for Residues of New Animal Drugs in Food. Specific Tolerances for Residues of New Animal Drugs, Department of Health and Human Services. U.S. Food and Drug Administration, New Hampshire Avenue, Washington, D.C., USA Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=556.500, Accessed date: 12 December 2017.
Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infect. Dis. 14, 742–750.
Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. U. S. A. 112, 5649–5654.
Voulodimos, A.S., Patrikakis, C.Z., Sideridis, A.B., Ntafis, V.A., Xylouri, E.M., 2010. A complete farm management system based on animal identification using RFID technology. Comput. Electron. Agric. 70, 380–388.
Wang, X., Sato, T., Xing, B., Tao, S., 2005. Health risks of heavy metals to the general public in Tianjin, China via consumption of vegetables and fish. Sci. Total Environ. 350, 28–37.
Wang, T., Hu, X., Liang, S., Li, W., Wu, X., Wang, L., Jin, F., 2015. *Lactobacillus fermentum* NS9 restores the antibiotic induced physiological and psychological abnormalities in rats. Benefic. Microbes 6, 707–717.
Wang, H., Yin, X., Li, F., Dahlgren, R.A., Zhang, Y., Zhang, H., Wang, X., 2016. Chronic toxicological effects of β-diketone antibiotics on zebrafish (*Danio rerio*) using transcriptome profiling of deep sequencing. Environ. Toxicol. 31, 1357–1371.
Winek, K., Engel, O., Koduah, P., Heimesaat, M.M., Fischer, A., Bereswill, S., Dames, C., Kershaw, O., Gruber, A.D., Curato, C., Oyama, N., Meisel, C., Meisel, A., Dirnagl, U., 2016. Depletion of cultivatable gut microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke. Stroke 47, 1354.
Yan, Z., Lu, G., Ye, Q., Liu, J., 2016. Long-term effects of antibiotics, norfloxacin, and sulfamethoxazole, in a partial life-cycle study with zebrafish (*Danio rerio*): effects on growth, development, and reproduction. Environ. Sci. Pollut. Res. 23, 18222–18228.
Yonar, E.M., Yonar, M.S., Sibel, S., 2011. Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (*Oncorhynchus mykiss*, W.). Fish Shellfish Immunol. 31, 318–325.
Yuan, J., Li, R.Q., Shi, Y., Peng, X.Y., Chen, X.X., Wu, Z.X., 2014. Pharmacokinetics of oxytetracycline in yellow catfish [*Pelteobagrus fulvidraco* (Richardson, 1846)] with a single and multiple-dose oral administration. J. Appl. Ichthyol. 30, 109–113.
Zhang, Q., Cheng, J., Xin, Q., 2015. Effects of tetracycline on developmental toxicity and molecular responses in zebrafish (*Danio rerio*) embryos. Ecotoxicology 24, 707–719.
Zhang, Y., Wang, X., Yin, X., Shi, M., Dahlgren, R.A., Wang, H., 2016. Toxicity assessment of combined fluoroquinolone and tetracycline exposure in zebrafish (*Danio rerio*). Environ. Toxicol. 31, 736–750.