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High fat diet worsens the adverse effects of antibiotic on intestinal health in juvenile Nile tilapia (*Oreochromis niloticus*)

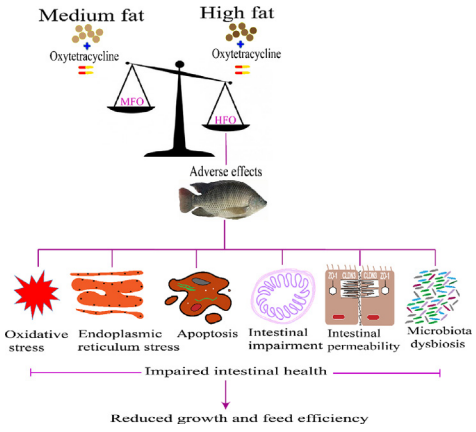
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HIGHLIGHTS

- The adverse effects of antibiotics on fish are dietary fat content-dependent.
- High fat diet worsens adverse effects of oxytetracycline on growth performance, feed efficiency and microbiota.
- High fat diet exacerbates adverse effects of oxytetracycline by inducing oxidative stress, endoplasmic reticulum stress and apoptosis.
- Antibiotics should be more strictly limited when high fat diets are used in aquaculture.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics and high fat diets are commonly used independently in global aquaculture production for fish health management and to spare the use of costly protein as energy sources, respectively, causing relatively similar metabolic effects and stresses. However, it is unknown whether dietary high fat worsens or attenuates the adverse effects caused by antibiotics in fish. We determined the ability of high fat diet to influence the adverse effects of oxytetracycline on Nile tilapia, *Oreochromis niloticus*. Thirty Nile tilapia weighing 8.45 ± 0.15 g were fed on medium fat (MF; 70 g/kg) and high fat (HF; 120 g/kg) diets and the same fat levels supplemented with 2.00 g/kg diet of OTC (80 mg/kg body weight/day) hereafter, MFO and HFO for 65 days. The general growth performance, feed efficiency and intestinal health of fish were evaluated. The Nile tilapia fed on HFO diet had significantly lower growth rate, body protein content and feed efficiency compared to those fed on MFO diet. Dietary HFO affected the intestine histomorphology, which decreased dramatically the tight junction proteins of Nile tilapia and induced microbiota dysbiosis compared to MFO diet. The Nile tilapia fed on HFO diet had increased oxidative stress, which stimulated drug detoxification response, caused endoplasmic reticulum stress and apoptosis compared to those fed on MFO diet. The new findings from our study demonstrate that, the adverse effects of antibiotics in fish are different at medium and high fat contents. Feeding fish with high fat diets with antibiotics worsen the adverse effects. This enlightens our understanding on the risks of antibiotics misuse and also suggests that antibiotics should be more strictly limited in aquaculture, in which high fat diets are currently widely used in fish production worldwide.

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1. Introduction

The use of antibiotics in global aquaculture for fish health management has become an integral part of the industry. Antibiotics are currently deemed as an emerging environmental pollutant posing human health risks due to their existence in skin and muscle of cultured fish from aquaculture (Li et al., 2017). Antibiotics used in aquaculture globally are administered abusively due to lack of knowledge about the purpose and proper application guidance by producers (Pham et al., 2015). Normally, antibiotics are applied in fish on a daily basis and in the absence of disease problems (Pham et al., 2015; Phu et al., 2015). More commonly, antibiotics are administered in fish through medicated feeds (Romero et al., 2012). Although their initial aim is to improve health of cultured fish, antibiotics cause multiple adverse effects especially on intestinal than their primary purpose.

Antibiotics administered in cultured fish are absorbed directly into the gastrointestinal tract where they accumulate and distribute causing various adverse effects in different fish species. Dietary oxytetracycline for instance, reduced growth performance in hybrid Striped bass (*Morone saxatilis* × *M. chrysops*) (Gaikowski et al., 2003) and feed intake in rainbow trout (*Oncorhynchus mykiss*) (Hustvedt et al., 1991) and Walleye (*Sander vitreus*) (Gaikowski et al., 2003). Furthermore, dietary oxytetracycline distorted intestinal histomorphology and tight junction proteins in Nile tilapia (*Oreochromis niloticus*) (Limbu et al., 2018) and induced gut microbiota dysbiosis in Atlantic salmon (*Salmo salar*) (Navarrete et al., 2008), Nile tilapia (Limbu et al., 2018) and zebrafish (*Danio rerio*) (Zhou et al., 2018a). Moreover, oxytetracycline caused oxidative stress and stimulated detoxification response in Nile tilapia (Limbu et al., 2018) and increased lipid peroxidation in zebrafish (Zhou et al., 2018a). Likewise, other antibiotics such as dietary flavomycin and florfenicol (He et al., 2010), flavomycin (Zhou et al., 2009) and florfenicol (He et al., 2012; He et al., 2011) also caused similar adverse effects on fish health intestine during chronic exposure. Therefore, the ability of antibiotics to impair fish health, particularly the intestinal is beyond doubt. This is undesirable due to critical functions performed by the fish intestine. The fish intestine executes vital functions on nutrition, immunology, detoxification and physiology to the host (Nayak, 2010). Its mucosal serves as a mechanical, chemical and biological barrier, protecting internal host body organs and tissues against xenobiotics (Kong et al., 2017). The fish intestine performs these key functions mainly due to the microbiota contained therein.

The adverse effects of antibiotics in fish intestine might be affected by several factors including dietary content. Fats are among the macronutrients that are well utilized by most fish species. They provide dense energy and essential fatty acids required by fish for normal growth, development and health maintenance (Jin et al., 2013; Lin and Shiau, 2003). Moreover, high fat diets are commonly used in modern aquaculture to spare the use of costly and limited availability protein as energy sources (He et al., 2015). However, high fat diets cause several metabolic effects and stresses in cultured fish, which affect their health (Chatzifotis et al., 2010; Du et al., 2006). Evidently, dietary high fat reduced growth performance, feed intake and feed efficiency in grass carp (*Ctenopharyngodon idella*) (Du et al., 2005) and induced lipid peroxidation and stress protein markers in turbot (*Scophthalmus maximus*) (Jia et al., 2017). Moreover, high dietary fat content caused oxidative stress in various fish species such as blunt snout bream (*Megalobrama amblycephala*) (Li et al., 2012) and yellow catfish (*Pelteobagrus fulvidraco*) (Wang et al., 2014). Altogether, it is apparent that, most of the metabolic effects and stresses induced by high fat diets are also caused by antibiotics in fish. Unfortunately, it is unknown whether dietary high fat worsens or attenuates the adverse effects caused by antibiotics in fish. In the present study we hypothesized that, high fat diet might be an important factor on worsening the adverse effects of antibiotic in fish general growth performance, feed efficiency and intestine health.

In our previous studies using medium fat content, we first exposed zebrafish to water treatments containing low environmental levels of sulfamethoxazole and oxytetracycline at concentrations of 260 ng/L and 420 ng/L, respectively and found that both antibiotics impaired intestinal structure and function (Zhou et al., 2018b). We then showed that, both antibiotics increased lipid peroxidation in intestine when zebrafish were fed on diets supplemented with sulfamethoxazole and oxytetracycline at 100 and 80 mg/kg body weight per day, respectively (Zhou et al., 2018a). To confirm further the intestinal effects, we fed and exposed an economic species, Nile tilapia to the two antibiotics at the same concentrations and found similar adverse effects in the intestine of treated fish (Limbu et al., 2018). Therefore, in the present study, we still used Nile tilapia a global economic, cultured and consumed species, which was fed to medium and high fat diets with or without oxytetracycline, a broad-spectrum antibiotic, widely authorized for use in aquaculture production globally, for nine weeks. The aim was to explore the ability of high fat diet to influence the adverse effects of oxytetracycline on Nile tilapia growth performance, feed efficiency and body composition. We further analyzed the intestinal health by using oxidative stress, microbiota composition, diversity and species richness, detoxification response, endoplasmic reticulum (ER) stress and apoptosis markers. To the best of our knowledge, this is the first study to investigate the influence of dietary fat levels on adverse effects of antibiotics in fish.

2. Materials and methods

2.1. Oxytetracycline acquisition and fish experimental set up

Oxytetracycline (OTC, CAS: 615 3–64 -6) was purchased from Yuanmu Biotechnology Co., Ltd., Shanghai, China. About 200 all-male Nile tilapia juveniles were purchased from Shanghai Ocean University (Shanghai, China). We used all male Nile tilapia because they grow faster and at a uniform size than females, practically, many farmers world-wide are more likely to culture them. All the fish were acclimatized in a 500-L aseptic tank, supplied with dechlorinated water for two weeks. The fish in the 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 Nile tilapia were individually embedded with a tracking chip, which aided individual fish recognition through radio-frequency identification (RFID) technology (Voulodimos et al., 2010). All the marked fish were randomly distributed into four treatments (30 fish per treatment) filled with dechlorinated water to allow them to settle for one week.

The total initial mean weight of fish in all treatments was determined as 8.45 ± 0.15 g twenty-four h before the start of the experiment. The Nile tilapia in two treatments were fed on medium fat (MF; 70 g/kg) and high fat (HF; 120 g/kg) diets. The fish in the other two treatments were fed on the same fat levels supplemented with 2.00 g/kg diet of OTC (80 mg/kg body weight/day) hereafter, MFO and HFO (Table A.1). We used 70 g/kg lipid because previous studies have shown that, 50 to 740 g/kg lipid is the optimum amount in diets for juvenile Nile tilapia (Chou and Shiau, 1996; He et al., 2015; Wang et al., 2005). The 120 g/kg lipid was chosen as a high lipid diet based on our earlier experiments on Nile tilapia (He et al., 2015; Xu et al., 2019). The lipid levels used in the present study have been utilized in previous studies in aquaculture (Du et al., 2005; He et al., 2015; Xu et al., 2019). The OTC concentration was selected depending on the legal permitted amount, which ranges from 50 to 250 mg/kg fish body weight/day (Gaikowski et al., 2003; Navarrete et al., 2008; Yonar et al., 2011), based on fish species, infection and country-specific legal requirements. We used 80 mg/kg body weight/day as a therapeutic dose administered in aquaculture (Trushenski et al., 2018). The same concentration has been used in previous studies (Limbu et al., 2018; Trushenski et al., 2018; Zhou et al., 2018a). 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 65 days. Half of the water in each tank was replaced with flesh-dechlorinated water every 24 h. The weight of individual fish was recorded fortnightly and the feed rations were adjusted accordingly.

2.2. Estimation of growth performance and feed efficiency

At the end of the experiment, all fish were fasted for 24 h, they were collected by using a scoop net and their individual weights were determined for weight gain estimation as the percentage increase in weight relative to the initial weight. The amount of feed fed on daily basis in each treatment was used to calculate feed intake according to Saravanan et al. (2012), while the feed conversion ratio (FCR) was estimated as the ratio of total feed fed to the total fish wet weight gained.

2.3. Mesenteric fat index, biochemical assays and body composition analyses

After weight measurements, eight fish from each treatment were sampled, anesthetized individually by using MS-222 (20 mg/L) (tricaine methanesulfonate, Western Chemicals, Inc., Ferndale, Washington) and sacrificed for mesenteric fat index (MFI), intestinal collection for biochemical assays and body composition measurements. The MFI was determined as the percentage of mesenteric fat weight to the individual fish weight. The small intestines from Nile tilapia were excised and subsequently homogenized in glass beds with 9 volumes (*v*/*w*) of 0.8% physiological saline for biochemical assays. 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 manufacturer's instructions. One whole small intestine from each fish was used in all analyses. On the days of analyses, malondialdehyde (MDA) concentration, the activities of glutathione S-transferase (GST) and superoxide dismutase (SOD) 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 measurements according to standard methods (AOAC, 1995).

2.4. Gut histomorphology assessment

A 1-cm segment of small intestine was taken from three individual fish per treatment and processed for hematoxylin-eosin (H & E) analyis as we reported previously (Limbu et al., 2018), and subsequently were observed and digitized using a microscope Nikon Microscope ECLIPSE N*i* (Nikon corporation, Tokyo, Japan). Muscularis thickness (40×), villus height (20×) and villus width (20×) were measured from at least 30 segments in each treatment by using the Nikon Microscope ECLIPSE N*i*. Goblet cells were quantified by examining five different areas in each slide by using the Nikon Microscope ECLIPSE N*i* at 40× magnification as reported previously (Bosi et al., 2017). The number of goblet cells was estimated as numbers counted on each of the field viewed divided by the area. The diameter of the cycle (and thus the radius, r = d/2) for calculation of the area was obtained by using the following formula; d = FN/Mo, where FN = field number (22 for the Nikon microscope used), d = diameter and Mo = objective magnification (40×).

2.5. Bacterial genomic DNA extraction

Five individual fish from each treatment were aseptically dissected and the small intestine contents were used for bacterial composition analysis as we reported previously (Limbu et al., 2018). The total intestinal bacterial DNA was isolated using a TIANamp Marine Animals DNA Kit (TIANGEN) according to the manufacturer's instructions. The DNA quantity and quality were measured by using a NanoDrop 2000 Spectrophotometer (Thermo, USA). The OD260/280 ratio ranged from 1.92 to 2.15 showing good quality. The quality of the extracted DNA was further confirmed by using agarose gel electrophoresis.

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 reported previously (Sun et al., 2013). Unique eight-base barcodes were added to each primer to distinguish PCR products. The 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 by 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 by using the Mothur software. The sequences obtained for microbiota in this study are available in the https://www.ncbi.nlm.nih.gov/sra/PRJNA493566.

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

Total RNA from small intestine tissues for mRNA expressions to evaluate tight junction proteins, detoxification response, ER stress and apoptosis 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 ratio values of all RNA samples ranged from 1.86 to 2.03, showing good quality. First-strand cDNAs were synthesized using a PrimerScript™ RT reagent Kit with a gDNA Eraser (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. The β-actin and elongation factor 1 alpha (EF-1α) were used as reference genes due to their stability, specificity and sensitivity results obtained during the preliminary experiment on reference gene validation process. 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 and Shanghai Rui Mian Biological Technology Co., Ltd. (Shanghai, China). The primers used in the present are listed in Table A.2. 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. Prediction of microbiota functional pathways

We predicted the functions of the altered pathways after feeding Nile tilapia with medium and high fat diets with or without oxytetracycline by using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). The expression levels of unigenes were calculated and normalized using the fragments per kilobase of exon per million fragments mapped (FPKM) method. Significant differentially expressed genes (DEGs) among libraries were determined using edgeR package (http://www.rproject.org/) with a false discovery rate (FDR) threshold <0.05 and absolute fold change ≥2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were conducted based on the DEGs.

2.10. Statistical analyses

Results are presented as means ± standard error of means (SEM) and data were tested for normality and homogeneity of variances by using Shapiro–Wilk and Levene's tests, respectively. The mean values for each measured parameter were compared by using independent *t*-test for Windows (SPSS version 20, IBM, Armonk, NY, USA). Statistical comparisons were made for the mean values between dietary MF and MFO; HF and HFO; MF and HF; and MFO and HFO. The microbiota species richness was analyzed by using the abundance coverage-based estimator (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. The correlations between measured parameters and microbiota species for MF vs MFO and HF vs HFO were conducted by using Pearson correlation. Significant differences were judged at a probability level of $p \leq 0.05$ for all analyzes.

3. Results

3.1. High fat diet worsens the adverse effects of oxytetracycline on growth performance, feed efficiency and body composition of fish

We determined the growth performance and related parameters of Nile tilapia fed on medium and high fat diets with or without oxytetracycline. Feeding Nile tilapia on MFO and HFO diets decreased weight gain (Fig. 1a), feed intake (Fig. 1b), MFI (Fig. 1d) and carcass body lipid content (Fig. 1f) compared to those fed on MF and HF diets, respectively. The Nile tilapia fed on HFO diet had significantly lower weight gain, feed intake and carcass body protein content (Fig. 1e) while they had higher FCR (Fig. 1c) compared to those fed on MFO diet. These results indicate that, high fat diet exacerbate the adverse effects of oxytetracycline in fish growth by reducing feed efficiency and body nutrients content.

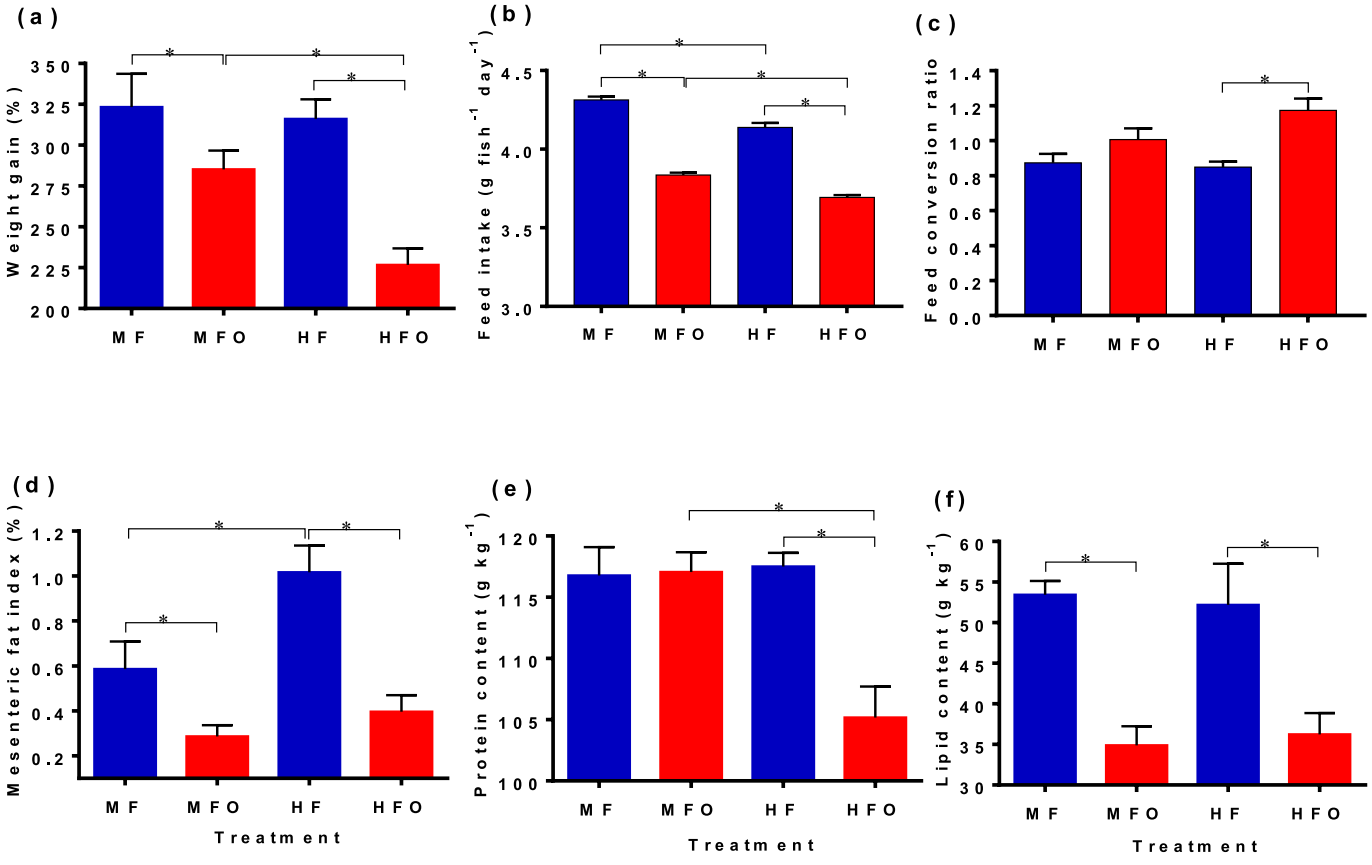


Fig. 1. Growth performance, feed efficiency, and body composition of Nile tilapia (*Oreochromis niloticus*) fed on medium and high fat diets with or without oxytetracycline; (a) weight gain, (b) feed intake, (c) feed conversion ratio (FCR), (d) mesenteric fat index (MFI), (e) carcass protein content, and (f) carcass lipid content. Weight gain = [(Final – initial weights)/initial weight] × 100, Feed intake = (Total dry feed fed – dry feed recovered)/ (geometric mean body weight × number of fish × time), Feed conversion ratio = Total amount of dry feed fed/Total fish wet weight gain, and Mesenteric fat index = (Mesenteric fat weight/fish body weight) × 100. Values are means ± SEM [n = 20–30 for (a) - (c) and n = 6–8 for (d) - (f)]. The asterisk (*) indicates significant differences between medium fat (MF) and medium fat with oxytetracycline (MFO), high fat (HF) and high fat with oxytetracycline (HFO), MF vs HF and MFO vs HFO diets ($p < 0.05$).

3.2. High fat diet with oxytetracycline impairs intestinal histomorphology and tight junction proteins

Feeding Nile tilapia on MF (2a) and HF (2c) diets increased muscularis thickness (Fig. 2i), villi height (Fig. 2j) and villi width (Fig. 2k) compared to those fed on MFO (2b) and HFO (2d) diets, respectively. Similarly, fish fed on MF (e) and HF (g) had many goblet cells (Fig. 2l) compared to those fed on MFO (f) and HFO (h), respectively. Dietary HFO decreased significantly muscularis thickness and increased villi height and goblet cells of Nile tilapia compared MFO diet.

To identify whether the changes in intestine morphology affected the intestine permeability, we measured the mRNA expression of tight junction proteins. Results affirmed that, feeding Nile tilapia with both MFO and HFO diets reduced claudin-3 (*CLDN3*) mRNA expression (Fig. 2m) and zonula occludens-1 (*ZO-1*) mRNA expression (Fig. 2n) compared to those fed on MF and HF diets, respectively. Dietary HFO decreased dramatically the mRNA expressions of *CLDN3* and *ZO-1* genes of Nile tilapia compared to MFO diet. These data suggest that, high fat diet with oxytetracycline impairs intestinal histomorphology, which increases intestinal permeability by affecting tight junction proteins.

3.3. High fat diet promotes the adverse effects of oxytetracycline by inducing oxidative stress, endoplasmic reticulum stress and apoptosis

Antioxidant enzymes such as SOD and GST counteract the toxicity generated by antibiotics. Thus, we determined the activities of SOD and GST in fish fed on medium and high fat with or without oxytetracycline. Feeding Nile tilapia on MFO and HFO diets increased the activities of SOD (Fig. 3a) and GST (Fig. 3b) compared to those fed on MF and HF diets, respectively, indicating oxidative stress. The Nile tilapia fed on HFO diet had lower SOD activity compared to those fed on MFO diet. Furthermore, the fish fed on HFO diet had higher MDA content (Fig. 3c) than those fed on HF diet, suggesting lipid peroxidation. We assessed the mRNA expression of cytochrome P450 1A (*CYP1A*) gene, which is involved in drug metabolism. We found that, both MFO and HFO diets elevated the mRNA expression of *CYPIA* gene (Fig. 3d) in Nile tilapia compared to MF and HF diets, respectively. The Nile tilapia fed on HFO diet had higher mRNA expression of *CYPIA* gene compared to those fed on MFO diet.

We further analyzed ER stress markers by using mRNA expression. Results showed that, feeding Nile tilapia on MFO and HFO diets elevated

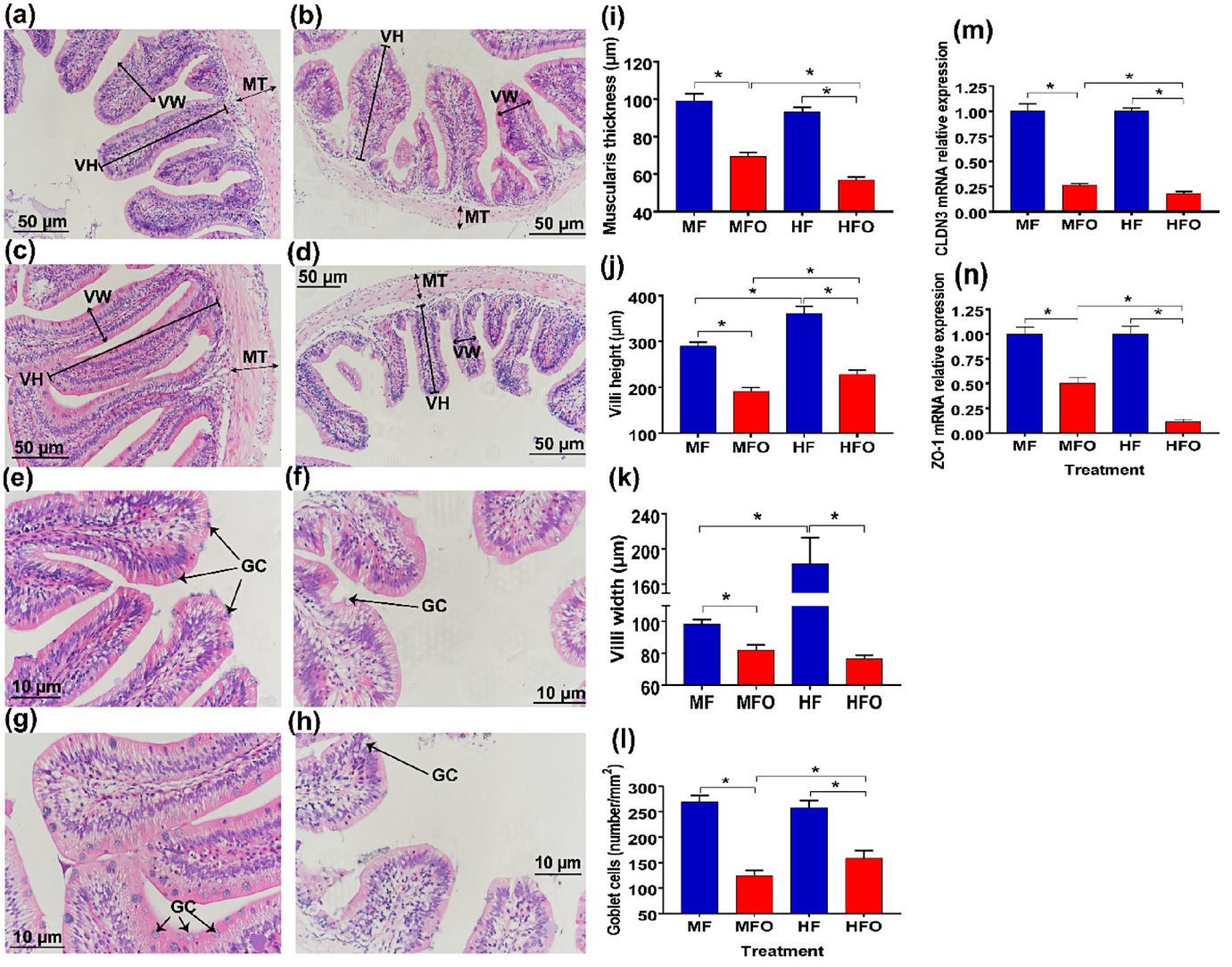


Fig. 2. Representative intestinal histomorphological features of Nile tilapia (*Oreochromis niloticus*) fed on medium and high fat diets with or without oxytetracycline; (a) MF villi width (VW), villi height (VH) and muscularis thickness (MT), (b) MFO villi width (VW), villi height (VH) and muscularis thickness (MT), (c) HF villi width (VW), villi height (VH) and muscularis thickness (MT), (d) HFO villi width (VW), villi height (VH) and muscularis thickness (MT), (e) MF goblet cells (GC), (f) MFO goblet cells (GC), (g) HF goblet cells (GC), (h) HFO goblet cells (GC), (i) results of muscularis thickness analysis, (j) results of villi height analysis, (k) results of villi width analysis, (l) results of goblet cells analysis, (m) claudin-3 (*CLDN3*) mRNA expression, and (e) zonula occludens-1 (*ZO-1*) mRNA expression. Values are means ± SEM ($n = 8$). The asterisk (*) indicates significant differences between medium fat (MF) and medium fat with oxytetracycline diets (MFO), high fat (HF) and high fat with oxytetracycline (HFO), MF vs HF, and MFO vs HFO diets ($p < 0.05$).

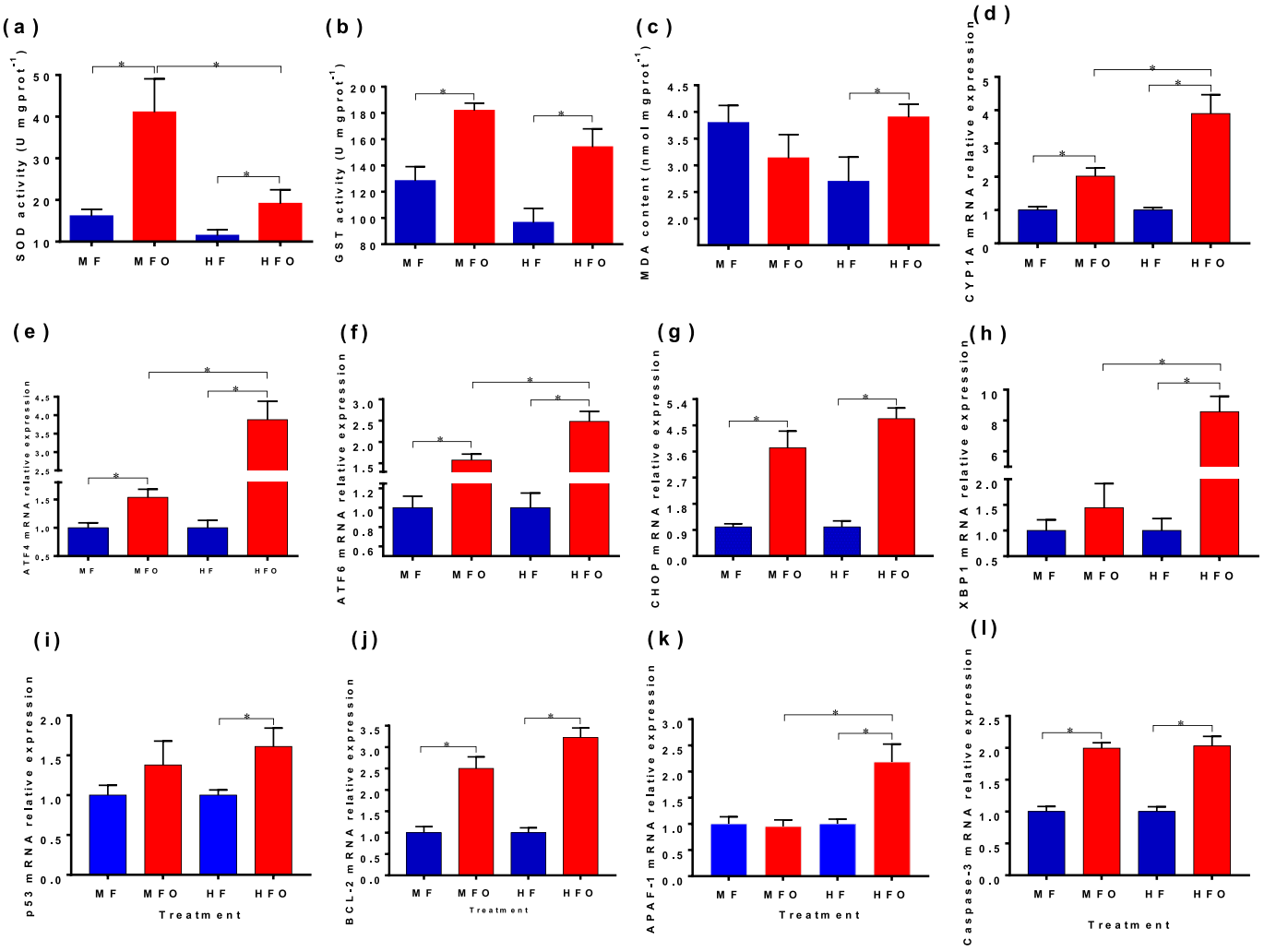


Fig. 3. Intestinal oxidative stress, endoplasmic reticulum stress and apoptotic responses of Nile tilapia (*Oreochromis niloticus*) fed on medium and high fat diets with or without oxytetracycline; (a) superoxide dismutase (SOD) activity, (b) glutathione S-transferase (GST) activity, (c) malondialdehyde (MDA) concentration, (d) cytochrome P450 1A (*CYP1A*) mRNA expression, (e) activating transcription factor 4 (*ATF4*) mRNA expression, (f) activating transcription factor 6 (*ATF6*) mRNA expression, (g) C/EBP homologous protein (*CHOP*) mRNA expression, (h) X-box binding protein 1 (*XBP1*) mRNA expression, (i) tumor suppressor protein p53 (*p53*) mRNA expression, (j) B-cell lymphoma 2 (*BCL-2*) mRNA expression, (k) apoptotic peptidase activating factor 1 (*APAF-1*) mRNA expression, and (l) cysteine-aspartic acid protease 3 (*caspase-3*) mRNA expression. Values are means ± SEM (n = 8). The asterisk (*) indicates significant differences between fish fed on medium fat (MF) and medium fat with oxytetracycline (MFO), high fat (HF) and high fat with oxytetracycline (HFO), MF vs HF, and MFO vs HFO diets ($p < 0.05$).

the mRNA expression of activating transcription factor 4 (*ATF4*) (Fig. 3e), activating transcription factor 6 (*ATF6*) (Fig. 3f), and C/EBP homologous protein (*CHOP*) (Fig. 3g) genes compared to MF and HF diets, respectively, indicating ER stress. Moreover, fish fed on HFO diet upregulated X-box binding protein 1 (*XBP1*) gene compared to those fed on HF diet (Fig. 3h). The Nile tilapia fed on HFO diet had higher mRNA expression of *ATF4*, *ATF6* and *XBP1* compared to those fed on MFO diet. Apoptosis-related genes were also assayed in Nile tilapia fed on medium and high fat diets with or without oxytetracycline. Results showed that, both MFO and HFO diets upregulated the mRNA expression of B-cell lymphoma 2 (*BCL-2*) (Fig. 3j) and cysteine-aspartic acid protease 3 (*caspase-3*) (Fig. 3l) in Nile tilapia compared to MF and HF diets, respectively. In addition, feeding Nile tilapia on HFO diet elevated the mRNA expression of tumor suppressor protein p53 (*p53*) (Fig. 3i) and apoptotic peptidase activating factor 1 (*APAF-1*) (Fig. 3k) compared to those fed on HF diet. The Nile tilapia fed on the HFO diet increased the mRNA expression of *APAF-1* gene (Fig. 3 k) compared to MFO diet. In general, these results indicate that, high fat diet promotes the adverse effects of oxytetracycline by inducing oxidative stress, detoxification response, ER stress and apoptosis.

3.4. High fat diet with oxytetracycline causes microbiota dysbiosis

Intestinal health correlates tightly with microbiota composition, therefore, we analyzed the intestinal bacterial abundance, richness and diversity in Nile tilapia after feeding them with medium and high fat with or without oxytetracycline. A total of 1,010,720 sequences were obtained from Nile tilapia intestinal bacteria. The abundance of microbiota was assessed based on the dominant phyla representing >1% of the total bacterial count. Clear differences existed in microbiota composition between Nile tilapia fed on diets with and without oxytetracycline (Fig. 4a). The Nile tilapia fed on MF and HF diets increased Fusobacteria species while those fed on MFO and HFO diets had higher abundance of Bacteroidetes species. We further analyzed in details the variations in microbiota phyla among the different dietary treatments (Fig. 4b-d). Results showed that, Nile tilapia fed on MFO and HFO diets had higher abundance of Bacteroidetes phylum compared to those fed MF and HF diets, respectively (Fig. 4b). Detailed analysis showed that, Bacteroidetes phylum was mainly composed of Poryphyromonads species (87%) and Macellibacteroides species (13%) (Fig. A.1). Contrary to Bacteroidetes, Nile tilapia fed on MFO and HFO diets had lower abundance of

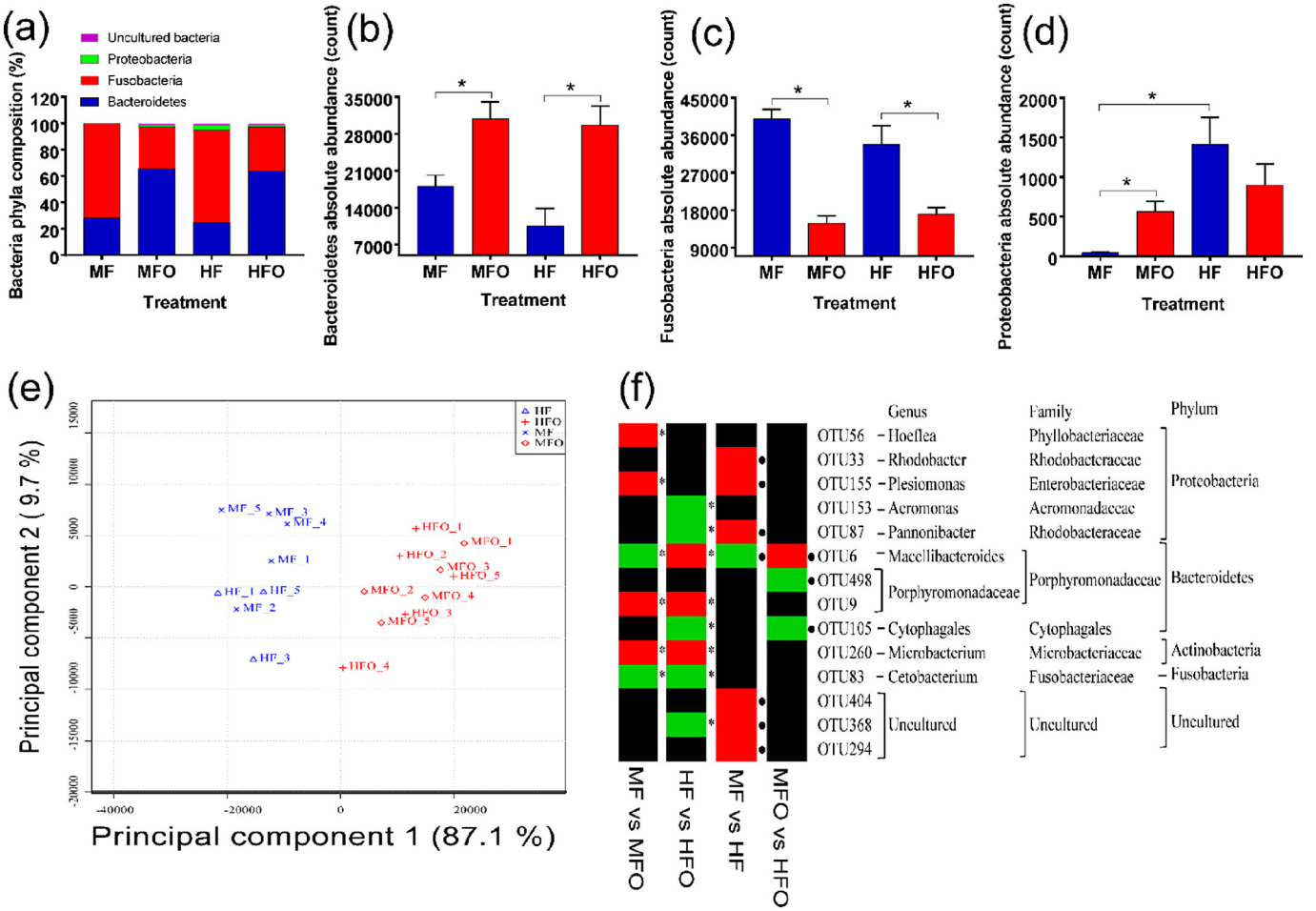


Fig. 4. Microbiota dysbiosis of Nile tilapia (*Oreochromis niloticus*) fed on medium and high fat diets with or without oxytetracycline; (a) bacteria phyla abundance composition, (b) Bacteroidetes absolute abundance, (c) Fusobacteria absolute abundance, (d) Proteobacteria absolute abundance (e) principal component analysis (PCA), and (g) the changing direction of the 14 OTUs after dietary treatment. Red color in (f) indicates the abundance of OTUs that increased in MFO, HFO, HF and HFO compared to MF, HF, MF and MFO, respectively whereas green color indicates the abundance of OTUs that increased in MF, HF, MF and MFO compared to MFO, HFO, HF and HFO, respectively, while black color signifies no change in abundance. Values are means ± SEM ($n = 5$). The asterisk (*) indicates significant differences between medium fat (MF) and medium fat with oxytetracycline (MFO) and high fat (HF) and high fat with oxytetracycline (HFO), while a black dot (●) indicates significant differences between MF vs HF and MFO vs HFO diets ($p < 0.05$).

Fusobacteria phylum compared to those fed on MF and HF diets, respectively (Fig. 4c). The abundance of Proteobacteria phylum was higher for Nile tilapia fed on MFO diet than MF diet, with fish fed on the latter also showed lower abundance compared to those fed on HF diet (Fig. 4d).

To determine the influence of fat content and oxytetracycline on bacterial phylotype distribution, a principal component analysis (PCA) was performed on OTUs. The bacteria communities from the dietary treatments were divided into two clear groups, with those in Nile tilapia fed on MF and HF diets clustering distinctly from those fed on MFO and HFO diets (Fig. 4e). The bacterial community were mainly attributed to fat content along the PCA1, which explained 87.10% whereas the combined effect of fat content and oxytetracycline along the PCA2 explained only 9.70% of total variations. We further analyzed the species richness and diversity based on OTUs. In accordance with ACE results, feeding Nile tilapia on MFO diet increased the OTU richness as compared to MF diet, while the HFO diet reduced the OTU richness compared to HF diet (Table A.3). The Simpson index results showed that, feeding Nile tilapia on both MFO and HFO diets reduced significantly the species diversity compared to those fed on MF and HF diets, respectively (Table A.3). The Nile tilapia fed on MFO diet had an increased diversity of bacteria based on Shannon diversity index than those fed on MF diet (Table A.3).

To further explore the microbiota variations in details, all microbiota with OTUs abundance >0.01% in all intestinal samples from Nile tilapia were compared among the different treatments. The results obtained were used to construct a heatmap (Fig. 4f), which was further classified to genus level. A comparison between Nile tilapia fed on HF vs HFO diets resulted into dysbiosis of eight microbiota genera compared to six that were changed when the fish were fed on MF vs MFO diets. In particular, feeding Nile tilapia with MFO and HFO diets increased the abundance of OTU9, a *Porphyromonas* species (Bacteroidetes) and OTU260, a *Microbacterium* species (Actinobacteria), while they increased OTU83, which belongs to *Cetobacterium* species (Fusobacteria) compared to MF and HF diets, respectively. The MFO diet decreased OTU6, which belongs to *Macellibacteroides* species compared to MF diet, while HFO diet eradicated completely OTU105, which belongs to *Cytophagales* species compared to MF and MFO diets, which are both Bacteroidetes. In addition, HFO diet increased *Macellibacteroides* species compared to HF and MFO diets, but decreased *Porphyromonas* species compared to MFO diet. These results suggest that, high fat diet with oxytetracycline causes microbiota dysbiosis, which may affect the host nutritional, immunological, detoxification mechanism and physiological functions.

3.5. High fat diet exacerbates adverse effects of oxytetracycline by disturbing functional pathways

The use of KEGG has facilitated the understanding of high-level functions of the biological system. We predicted the functions of the altered pathways after feeding Nile tilapia with medium and high fat diets with

or without oxytetracycline by using PICRUSt. A total of 405,032,109 pathways were changed in all treatments dominated by metabolism (MTB, >50%) followed by genetic information processing (GIP, 18%), unclassified (14%), environmental information processing (EIP, 11%) and cellular processes (CLP, about 2%), with the remaining pathways representing <1% (Fig. 5a). We further compared each treatment for every relevant changed pathway to understand the effects of fat content and antibiotic on functional pathways (Fig. 5b–f). Feeding Nile tilapia on both MFO and HFO diets diminished EIP pathway (Fig. 5d) compared to MF and HF diets, respectively. The Nile tilapia fed on MFO diet reduced MTB pathway (Fig. 5b), but increased CLP pathway (Fig. 5e) compared to MF diet. The Nile tilapia fed on HFO diet had enriched GIP (Fig. 5c) and organismal systems (ORS, Fig. 5f) pathways compared to HF diet. These results suggest that, high fat diet with oxytetracycline alters functional pathways, which might significantly affect specific activities of fish at cellular level.

3.6. Microbiota play distinct functions at medium and high fat diets with oxytetracycline

The alterations of different measured parameters and microbiota across the four experimental groups suggested the presence of widespread associations between the two data types. Therefore, to establish a cause and effect, we examined the correlations between changes in intestinal microbiota and variations in measured parameters for MF vs MFO diets (Fig. 6a) and HF vs HFO diets (Fig. 6b). Several species such as *Hoeflea*, *Rhodobacter*, *Aeromonas* and *Pannonibacter* (Proteobacteria), *Porphyromonas* (Bacteroidetes), *Microbacterium* (Actinobacteria) and *Cetobacterium* (Fusobacteria), mainly played similar adverse functions in Nile tilapia at MF vs MFO diets (Fig. 6a) and HF vs HFO diets (Fig. 6b). However, a few species performed different functions at the two fat levels with oxytetracycline. For example, *Plesiomanas* species (Proteobacteria), which played negative roles in MF vs MFO diets such as reducing growth performance, increasing oxidative stress, ER stress and apoptosis performed beneficial functions in HF vs HFO diets (Table A.4). On the contrary, Bacteroidetes species such *Macellibacteroidetes* that played beneficial functions in MF vs MFO diets, performed adverse functions in HF vs HFO diets, while *Cytophagales*, which did not correlate with any function in the former, performed beneficial functions in the latter treatments (Table A.4). These results indicate that, microbiota play distinct functions at medium and high fat diets with oxytetracycline.

4. Discussion

4.1. The adverse effects of antibiotics are different at medium and high dietary fat contents

We have recently shown that, medium fat with oxytetracycline causes systemic effects in Nile tilapia (Limbu et al., 2018) and affect intestinal health in zebrafish (Zhou et al., 2018a). However, despite the combined use of antibiotics and diets with different fat levels on

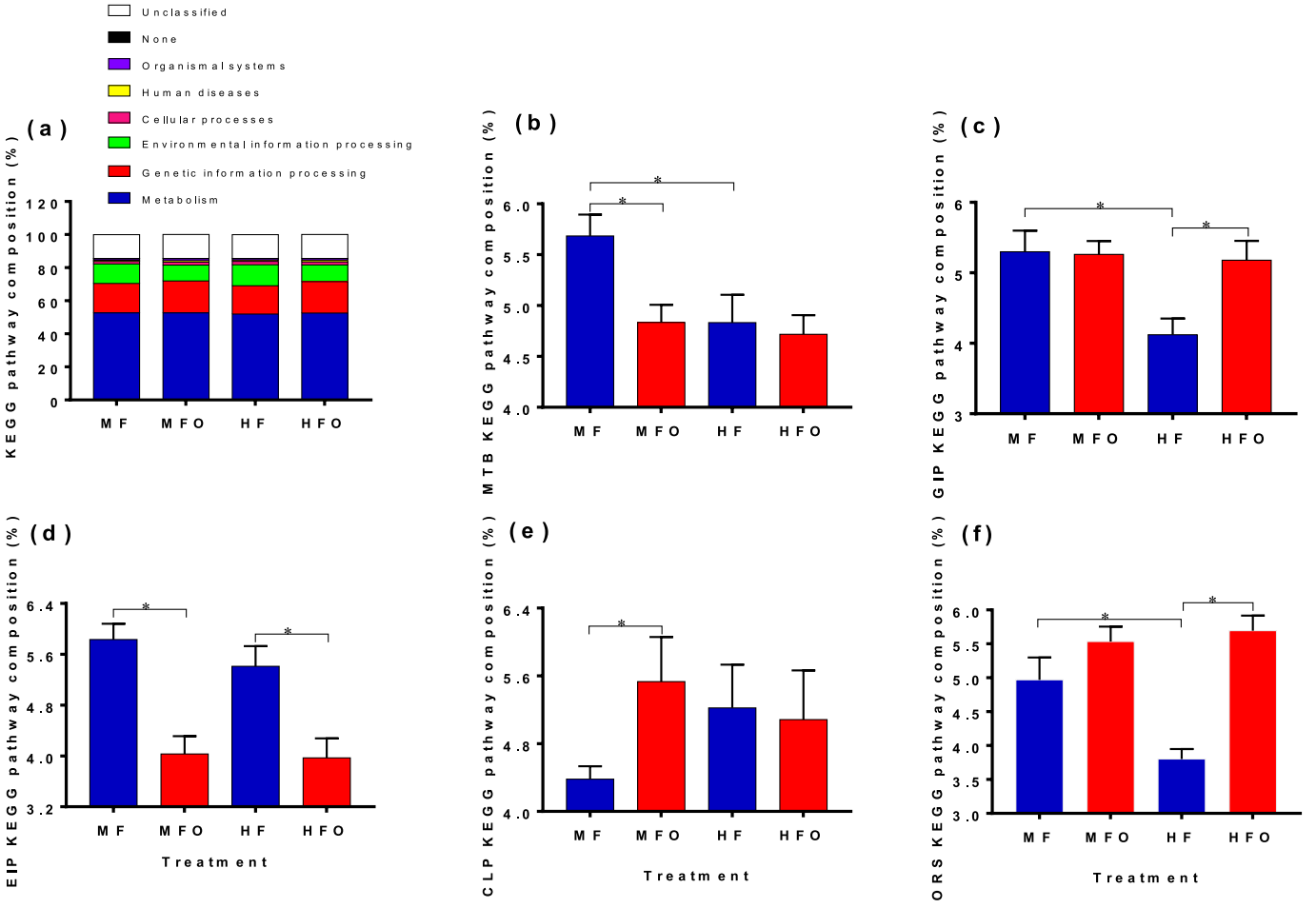


Fig. 5. Relative altered KEGG pathway compositions of Nile tilapia (*Oreochromis niloticus*) fed medium and high fat diets with or without oxytetracycline; (a) Composition of all KEGG, (b) Metabolism (MTB) pathway, (c) Genetic information processing (GIP) pathway, (d) Environmental information processing (EIP) pathway, (e) Cellular processes (CLP) pathway, and (f) Organismal systems (ORS) pathway. Values are means ± SEM (n = 5). The asterisk (*) indicates significant differences between fish fed on medium fat (MF) and medium fat with oxytetracycline (MFO), high fat (HF) and high fat with oxytetracycline (HFO), and MF vs HF diets ($p < 0.05$).

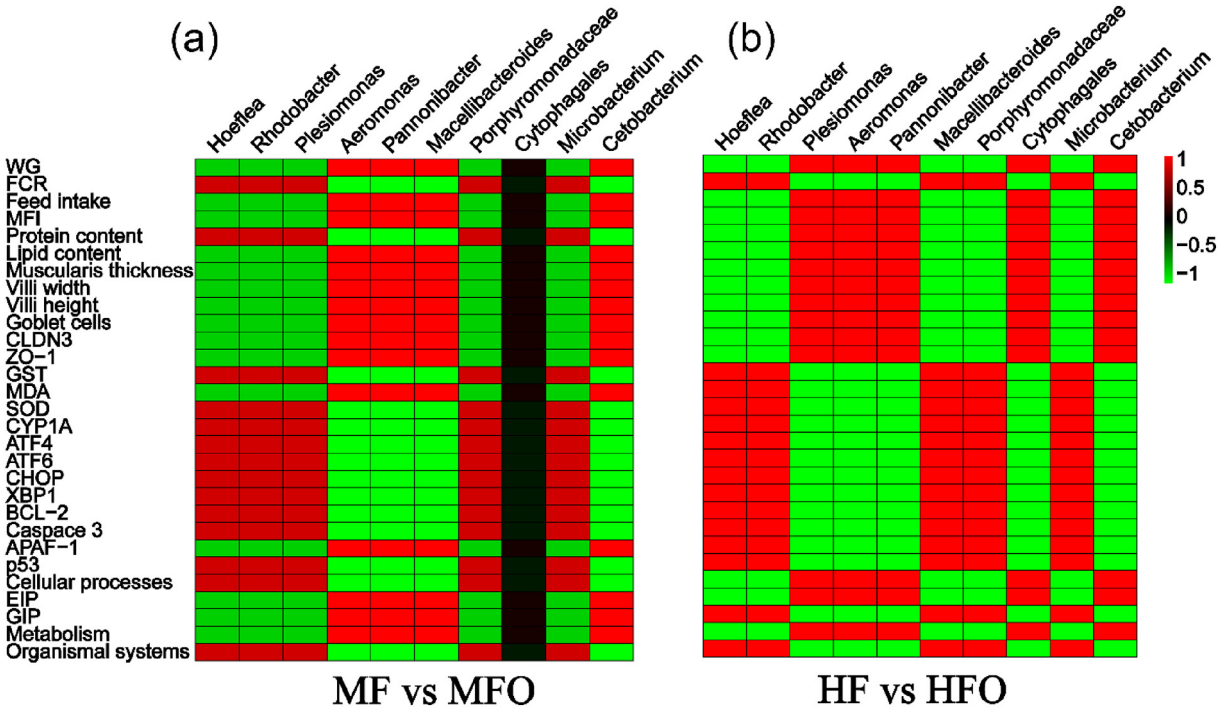


Fig. 6. Significant correlations between measured fish parameters and intestinal microbiota, (a) fish fed on medium fat (MF) and medium fat with oxytetracycline (MFO), and (b) fish fed on high fat (HF) and high fat with oxytetracycline (HFO). The abbreviated fish parameters are weight gain (WG), feed conversion ratio (FCR), mesenteric fat index (MFI), Claudin-3 (*CLDN3*), zonula occludens-1 (*ZO-1*), glutathione S-transferase (GST), malondialdehyde (MDA), superoxide dismutase (SOD), cytochrome P450 1A (*CYP1A*), B-cell lymphoma 2 (*BCL-2*), activating transcription factor 4 (*ATF4*), activating transcription factor 6 (*ATF6*), C/EBP homologous protein (*CHOP*), X-box binding protein 1 (*XBP1*), cysteine-aspartic acid protease 3 (*caspase-3*), apoptotic peptidase activating factor 1 (*APAF-1*), environmental information processing (EIP) pathway, and genetic information processing (GIP) pathway.

cultured fish, no study in the literature has attempted to elaborate critically their variations in toxicological effects. In this study, we compared the differences in adverse effects between medium and high fat with oxytetracycline. We found that, although both diets affected the general body growth, feed efficiency and intestine health of fish, the high fat diet with oxytetracycline generally induced distinct toxicological effects from those of medium fat with oxytetracycline (Table 1). In fact, in most parameters on which both MFO and HFO diets caused adverse effects compared to MF and HF diets, respectively, HFO diet worsened them. Noticeable, the Nile tilapia fed on HFO diet had decreased growth performance, feed efficiency and body protein content compared to those fed on MFO diet. This is undesirable in aquaculture because it affects production, profitability and quality of fish produced for human consumption. Furthermore, Nile tilapia fed on HFO diet had distorted intestinal histomorphology and increased permeability compared to MFO diet. These results highlight the adverse effects induced by feeding fish on high fat diets with antibiotics because increased intestinal permeability might affect absorption of dietary nutrients and allow easy entrance of intestinal pathogens, allergens and toxins, which may lead to disease eruption, consequently affect fish health and lower aquaculture production. Moreover, the Nile tilapia fed on HFO diet had reduced SOD antioxidant enzyme activity and increased drug metabolism response compared to those fed on MFO diet. The increased oxidative stress characterized by reduction in the antioxidant enzymes activities may contribute to the additional progression of adverse effects of HFO in fish. The induction in the expression of CYP1A gene, which is a well-established biomarker of exposure in response to environmental xenobiotics (Mihailović et al., 2006) affirms the worsened adverse effects induced by feeding Nile tilapia on HFO diet. Since CYP1A plays significant roles in mediated-oxidative detoxification of xenobiotics (Dračínská et al., 2016), its stimulation suggests aggravated adverse effects in Nile tilapia fed on HFO diet.

Interferences with the optimal functions of the ER cause sustained cell stress response and can eventually activate apoptosis if the ER dysfunction is severe or prolonged (Xu et al., 2005). In the present study, feeding Nile tilapia with HFO diet induced ER stress and caused apoptosis compared with those fed on MFO diet. Considering the important roles played by ER in lipid biosynthesis, calcium storage, and protein folding and processing, the ER stress sustained by Nile tilapia fed on HFO diet points into severe impairment in protein folding,

Table 1
Toxicity of oxytetracycline varies between different dietary lipid contents.

Type of effect	Toxicological effect	Comparison between MFO vs HFO		Reference figure
Growth performance	Weight gain	MFO⇧	HFO⬇	Fig. 1a
Feed efficiency	Feed intake	MFO⇧	HFO⬇	Fig. 1b
	Feed conversion ratio	MFO⬇	HFO⇧	Fig. 1c
Body composition	Protein content	MFO⇧	HFO⬇	Fig. 1e
Intestinal histomophology	Muscularis thickness	MFO⇧	HFO⬇	Fig. 2i
	Villi height	MFO⬇	HFO⇧	Fig. 2j
	Goblet cells	MFO⬇	HFO⇧	Fig. 2l
Intestinal permeability	CLDN3	MFO⇧	HFO⬇	Fig. 2m
	ZO-1	MFO⇧	HFO⬇	Fig. 2n
Oxidative stress	SOD	MFO⇧	HFO⬇	Fig. 3a
Drug metabolism	CYP1A	MFO⬇	HFO⇧	Fig. 3d
Endoplasmic reticulum stress	ATF4	MFO⬇	HFO⇧	Fig. 3e
	ATF6	MFO⬇	HFO⇧	Fig. 3f
	XBP1	MFO⬇	HFO⇧	Fig. 3h
Apoptosis	APAF-1	MFO⬇	HFO⇧	Fig. 3k
Microbiota dysbiosis	Macellibacteroides	MFO⬇	HFO⇧	Fig. 4c
	Porphyromonadaceae	MFO⇧	HFO⬇	Fig. 4c
	Cytophagales	MFO⇧	HFO⬇	Fig. 4c

The fish parameters abbreviated are claudin-3 (*CLDN3*) mRNA expression, zonula occludens-1 (*ZO-1*) mRNA expression, superoxide dismutase (SOD) activity, cytochrome P450 1A (*CYP1A*) mRNA expression, activating transcription factor 4 (*ATF4*) mRNA expression, activating transcription factor 6 (*ATF6*) mRNA expression, X-box binding protein 1 (*XBP1*) mRNA expression, and apoptotic peptidase activating factor 1 (*APAF-1*) mRNA expression.

suggesting increased proteotoxicity risk. This phenomenon, coupled with the observed apoptosis for Nile tilapia fed on HFO diet, weakens the immunity, which may cause cell death and finally affects fish production. To escalate the adverse effects, feeding Nile tilapia with HFO diet induced microbiota dysbiosis compared with MFO diet, in line with the results obtained in mice (Cani et al., 2008). Such results may impair the beneficial functions performed by microbiota on nutritional, physiological, immunity and metabolic processes of the host fish. Taken together, these results demonstrate that, the adverse effects of antibiotics are different at medium and high dietary fat contents. Apparently, feeding fish with high fat diets with antibiotics aggravate the adverse effects. Practically, since the concentration used is legally authorized for aquaculture production, fish farmers and environmentalists should be warned on using oxytetracycline and high fat diets together. Oxytetracycline should be more strictly limited in aquaculture production and mechanisms to revert the adverse effects in fish are required.

4.2. Mechanisms of the worsened adverse effects induced by high fat diet with antibiotic

Various mechanisms explain the worsened adverse effects caused by feeding Nile tilapia on high fat diet with oxytetracycline. The decreased growth performance obtained in Nile tilapia fed on HFO diet is due to reduced feed efficiency as evidenced by lower feed intake and higher FCR because feed intake is the major determinant of fish growth (Tran-Duy et al., 2012). The reduced feed efficiency consequently lowered body protein content in the Nile tilapia fed on HFO diet due to limited uptake of protein from the diet. The decreased growth performance is further reflected by the worsened histomorphological changes obtained in the intestine of Nile tilapia fed on HFO diet. The Nile tilapia fed on HFO diet had reduced muscularis thickness as an attempt to minimize the nutrients diffusion distance and increase efficient in absorption. Accordingly, Nile tilapia fed on HFO diet increased the villi height to provide large surface area for nutrients absorption in intestine. Furthermore, they also increased goblet cells, which are involved in the absorption of easily digested nutrients in cichlid fishes (Leknes, 2010; Osman and Caceci, 1991). Interestingly, we also found that Nile tilapia fed on high fat diets (HF vs HFO) turned *Plesiomonas shigelloides* categorized as a facultative and opportunistic pathogen (Pakingking et al., 2015) and *Cyphagales* species which did not correlate with any function in the MF vs MFO to perform beneficial nutritional-related functions. Notably, the Bacteroidetes species which increased in MFO and HFO diets at phylum level compared to MF and HF diets, respectively, was mainly contributed by Poryphyromonads species (87%), which performed adverse effects, while the beneficial Macellibacteroides species was only (13%) of the total microbiota abundance. Although the exact mechanisms of the obtained changes in microbiota functions at the two fat contents are subject to further detailed studies, it is apparent that, Nile tilapia fed on HFO diet devised some mechanisms to improve nutrients absorption and enhance growth performance due to worsened adverse effects caused by feeding them with high fat diet with oxytetracycline. Finally, the reduced growth performance of Nile tilapia fed on HFO diet is related to distorted tight junction proteins, which caused malabsorption of nutrients (Zhang et al., 2015). Antibiotics and high fat diets trigger inflammation (Cani et al., 2008), which irritates the intestine tissues, subsequently breakdown tight junction proteins. These results suggest that, the lower growth performance obtained in Nile tilapia fed on HFO diet was caused by reduced feed efficiency, which affected body protein content and the distorted tight junction proteins coupled with the obtained microbiota dysbiosis.

We further showed multiple worsened oxidative stress, drug detoxification response, ER stress and apoptosis in Nile tilapia fed on HFO diet. We relate these adverse effects to oxidative stress induced by HFO diet. Previous studies have shown that, oxidative stress proceeds metabolic syndromes induced by high fat diet in mice (Matsuzawa-Nagata et al., 2008) and rats (Milagro et al., 2006; Noeman et al., 2011), and caused mitochondrial damage and apoptosis in rats (Du et al., 2012). Similarly, oxytetracycline caused oxidative stress, which elevated *CYP1A* gene expression in zebrafish (Barros-Becker et al., 2012), while various antibiotics induced ER stress, which preceded oxidative stress in human hepatocytes (Burban et al., 2018) and salinomycin induced apoptosis via ER stress mediated by reactive oxygen species (ROS) in human glioma cells (Yu et al., 2017). It therefore follows that, since oxytetracycline is sparingly soluble in fats (Maddison et al., 2008) and is poorly absorbed through the intestinal tract (Romero et al., 2012), it is more likely that when fish were fed on high fat diet with oxytetracycline, they were subjected to duo adverse effects resulting from the high fat diet and the oxytetracycline. Therefore, feeding fish with HFO diet induced oxidative stress, which stimulated drug detoxification response. However the detoxification response was deficient to clear the oxidative stress, subsequently elevated the mRNA expressions of *ATF4* and *ATF6* genes, which are oxidative stress-inducible factors (Lange et al., 2008), indicating ER stress. The Nile tilapia under sustained oxidative stress, the *ATF4* and *ATF6* genes promoted mRNA up regulation of *XBP1* gene, which functions as a sensor and transducer of ER stress (Yoshida et al., 2006) as a signal for cell damage (De Felice and Lourenco, 2015). The activation of *XBP1* mRNA expression led to the initiation of apoptotic cascade as indicated by the higher expression of *APAF-1* gene. Clearly, these results imply that, feeding fish with high fat diets supplemented with antibiotics cause oxidative stress, which induce ER stress and apoptosis. The induced stresses may affect fish health, prompting continued use of antibiotics, which may cause antibiotics resistance genes and human health risk upon fish consumption. In fact, the continued use of antibiotics aimed at improving the compromised fish health may cause direct human health risks as we have reported recently (Limbu et al., 2018).

5. Conclusions

The new findings from our study demonstrate that, the adverse effects of antibiotics on fish are different at medium and high dietary fat contents. Generally, although medium and high fat diets with antibiotics cause several similar adverse effects compared to diets without antibiotics, feeding fish with high fat diets with antibiotics worsen the adverse effects on fish intestinal health. This enlightens our understanding on the risks of antibiotics misuse and also warns that antibiotics should be more strictly limited in aquaculture, in which high fat diets are currently used widely for fish production worldwide.

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Conflict of interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.05.067.

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