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High carbohydrate diet partially protects Nile tilapia (*Oreochromis niloticus*) from oxytetracycline-induced side effects☆

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ABSTRACT

Antibiotics used in global aquaculture production cause various side effects, which impair fish health. However, the use of dietary composition such as carbohydrate, which is one of the dominant components in fish diets to attenuate the side effects induced by antibiotics, remains unclear. We determined the ability of high carbohydrate diet to protect Nile tilapia (*Oreochromis niloticus*) from oxytetracycline-induced side effects. Triplicate groups of thirty *O. niloticus* (9.50 ± 0.08 g) were fed on medium carbohydrate (MC; 335 g/kg) and high carbohydrate (HC; 455 g/kg) diets without and with 2.00 g/kg diet of oxytetracycline (80 mg/kg body weight/day) hereafter, MCO and HCO for 35 days. Thereafter, we assessed growth performance, hepatic nutrients composition and metabolism, microbiota abundance, immunity, oxidative and cellular stress, hepatotoxicity, lipid peroxidation and apoptosis. To understand the possible mechanism of carbohydrate protection on oxytetracycline, we assessed the binding effects and efficiencies of mixtures of medium and high starch with oxytetracycline as well as the MCO and HCO diets. The *O. niloticus* fed on the MCO and HCO diets had lower growth rate, nutrients utilization and survival rate than those fed on the MC and HC diets, respectively. Dietary HCO increased hepatosomatic index and hepatic protein content of *O. niloticus* than MCO diet. The *O. niloticus* fed on the HCO diet had lower mRNA expression of genes related to protein, glycogen and lipid metabolism compared to those fed on the MCO diet. Feeding *O. niloticus* on the HCO diet increased innate immunity and reduced pathogenic bacteria, pro-inflammation, hepatotoxicity, cellular stress and apoptosis than the MCO diet. The high starch with oxytetracycline and HCO diet had higher-oxytetracycline binding effects and efficiencies than the medium starch with oxytetracycline and MCO diet, respectively. Our study demonstrates that, high carbohydrate partially protects *O. niloticus* from oxytetracycline-induced side effects by binding the antibiotic. Incorporating high carbohydrate in diet formulation for omnivorous fish species alleviates some of the side effects caused by antibiotics.

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1. Introduction

Antibiotics are used abusively not only as human health medicines (Imran et al., 2019), but also in other animals such as in aquaculture for treating fish diseases, posing a serious threat to humans as final consumers (Binh et al., 2018; Limbu et al., 2018). Globally, antibiotics are administered in fish production intended for human consumption mainly through medicated feeds (Romero et al., 2012), most commonly on a daily basis and in the absence of disease problems (Pham et al., 2015; Phu et al., 2015). Consequently, antibiotics are discharged into the aquatic environment (Barreto et al., 2018; Chen et al., 2018) because of their poor absorption in the fish intestine (Zhang et al., 2016). Antibiotics used in aquaculture cause several side effects, which affect fish health. For example, oxytetracycline, which is one of the most commonly used antibiotics in aquaculture reduced growth performance in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Gaikowski et al., 2003) and feed efficiency in Nile tilapia (*Oreochromis niloticus*) (Limbu et al.,

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2018). It also suppressed immunity in gilthead seabream (*Sparus aurata* L.) (Guardiola et al., 2012), reduced antioxidant capacity in adult zebrafish (*Danio rerio*) (Oliveira et al., 2013), increased lipid peroxidation in zebrafish (Zhou et al., 2018a) and elicited liver damages in hybrid striped bass, channel catfish (*Ictalurus punctatus*), *O. niloticus* (Topic Popovic et al., 2012) and Coho salmon (*Oncorhynchus kisutch*) (Nakano et al., 2018). Therefore, oxytetracycline cause several side effects in cultured fish, which ultimately affect production. However, it remains unclear whether nutritional composition can alleviate the side effects induced by antibiotics in fish.

Dietary composition is an important factor, which not only influences growth rate, immunity, nutrients metabolism and general well-being of fish (Li et al., 2012; Oliva-Teles, 2012; Wang et al., 2014; Ye et al., 2016), but also affects the general toxicity of various chemicals in different organisms (Walker, 1996; Yang et al., 1992), environmental pollutants in humans (Hennig et al., 2012), chronic cadmium toxicity in mice (Li et al., 2016), chronic toxicity of sodium pentachlorophenate (Hickie et al., 1989) and methylmercury toxicity in fish (Olsvik et al., 2011). Therefore, in the evaluation of any chemical toxicity, dietary components play important roles in modulation of toxic effects. However, the potential use of dietary composition in modulating antibiotic-induced side effects in fish remains unclear.

Starch is a polysaccharide carbohydrate that is commonly used in fish feeds as the cheapest energy source (Kamalam et al., 2017) to provide protein and lipid-sparing effects (Ma et al., 2019) and improve quality of feeds (Kanmani et al., 2018), particularly when pre-gelatinization is applied due to its binding effect (Lin et al., 2018; Romano et al., 2018). However, from a nutritional point of view, most fish have limited ability to digest, absorb and assimilate starch (Kamalam et al., 2017). Accordingly, feeding fish with high carbohydrate diets caused metabolic disorders such as cellular stress in Wuchang bream *(Megalobrama amblycephala)* (Zhou et al., 2013), hepatic damage in yellow catfish (*Pelteobagrus fulvidraco*) (Wang et al., 2014) and oxidative stress in largemouth bass (*Micropterus salmoides*) (Song et al., 2018). Moreover, high carbohydrate diets suppressed innate immunity (Lin et al., 2018) and induced lipid peroxidation in largemouth bass (Ma et al., 2019; Song et al., 2018). Contrarily, from a toxicological perspective, dietary starch may provide some beneficial effects such as changing the conjugation of drug and metabolites formation (Pantuck et al., 1991) and binding efficiency towards many chemical substrates (Arnesen and Krogdahl, 1993; Kamalam et al., 2017; Lu et al., 2018). Thus, starch can reduce the absorption and accumulation of toxic substrates in fish body. However, currently no study has explored the ability of carbohydrate to bind antibiotics. In the present study, we hypothesized that, high carbohydrate diet may attenuate the side effects induced by antibiotic in fish.

In our previous studies we fed zebrafish on medium starch diet (325 g/kg) supplemented with oxytetracycline at 80 mg/kg body weight per day and found that, the treated fish had suppressed innate immunity and increased lipid peroxidation (Zhou et al., 2018a). To confirm further the side effects induced by oxytetracycline at medium starch, we fed *O. niloticus*, a commercial species, the same amount of antibiotic and found similar adverse effects on innate immunity and lipid peroxidation, together with reduced feed efficiency, disturbed hepatic and intestinal nutrients metabolism and increased oxidative and cellular stress responses (Limbu et al., 2018). Therefore, in the present study, we fed *O. niloticus* on medium and high carbohydrate diets with or without oxytetracycline, a broad-spectrum antibiotic, widely authorized for use in aquaculture production globally, for five weeks. The *O. niloticus* is an omnivorous species, which adapts and uses efficiently high carbohydrate for energy supply (Boonanuntanasarn et al., 2018a; Kamalam et al., 2017; Wang et al., 2005). Using *O. niloticus*, we aimed to explore the ability of high carbohydrate diet to protect fish from oxytetracycline-induced side effects on growth performance, feed utilization, survival rate, hepatosomatic index, microbiota abundance, hepatic nutrients composition and metabolism. We further assessed the protection capability of high carbohydrate on immunity, oxidative stress and lipid peroxidation in the intestine, liver and serum of *O. niloticus*. Additionally, we determined the ability of high carbohydrate to protect *O. niloticus* on oxytetracycline-induced side effects on intestinal and hepatic cellular stress response and apoptosis. Finally, we explored the possible mechanism used by high carbohydrate to protect fish from oxytetracycline-induced side effects. To the best of our knowledge, this is the first study to investigate the ability of dietary carbohydrate levels to ameliorate antibiotic-induced side effects in fish.

2. Materials and methods

2.1. Ethical statement

This study was approved by the Animal Ethics Committee of East China Normal University (Shanghai, China) and all experiments were conducted according to the protocols and procedures for the use of Laboratory Animal Management Ordinance of China.

2.2. The source of oxytetracycline and fish

Oxytetracycline (CAS: 615 3-64-6) was purchased from Yuanmu Biotechnology Co., Ltd (Shanghai, China). About 500 all-male genetically improved tilapia (GIFT) *O. niloticus* fingerlings weighing 5.10 ± 0.67 g (mean ± standard error) and aged about two months were purchased from Yueqiang Fish Farm in Guangzhou (Guangdong, China). We used all male *O. niloticus* because they grow faster and at a uniform size than females. The fish were transported by flight in plastic bags supplied with dissolved oxygen to the Laboratory of Aquaculture Nutrition Environmental Health (LANEH), East China Normal University (Shanghai, China).

2.3. Acclimatization of fish prior to the experiment

All the fish were acclimatized in a 500-L aseptic tank, supplied with dechlorinated water for two weeks. During acclimatization, the fish in the tank were provided with compressed air via airstones 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, 4.8–6.4 mg/L, 7.5 to 7.9 and <0.02 mg/L, respectively. During this period, the fish were hand-fed twice a day using a commercial diet (Chengdu, China) containing ≥ 33% protein and ≥5% lipid.

2.4. Diets formulation and experimental design

The cornstarch used for the formulation of the experimental diets was purchased from Shanghai Yutian Agricultural Science and Technology Co., Ltd (Shanghai, China). Four experimental diets were formulated to contain medium carbohydrate (MC: 334.66 ± 4.67 g/kg) and high carbohydrate (HC: 453.30 ± 2.58 g/kg) without oxytetracycline and the same dietary amounts supplemented with 80 mg oxytetracycline/kg body weight of fish (2.00 g/kg diet), hereafter MCO (334.42 ± 3.09 g/kg) and HCO (454.63 ± 1.61 g/kg) diets, respectively (Table 1). The medium and high starch were chosen based on previous studies (Boonanuntanasarn et al., 2018a; Boonanuntanasarn et al., 2018b; Hickie et al., 1989; Kamalam et al., 2017; Li et al., 2012; Limbu et al., 2019; Limbu et al., 2018; Zhou et al., 2013; Zhou et al., 2018a; Zhou

Table 1
Formulation and composition of the experimental diets (g/kg).

Ingredient composition (g kg^{-1})	MC	HC	MCO	HCO
Casein	300	300	300	300
Gelatin	80	80	80	80
Soybean oil	70	70	70	70
Corn starch	335	455	335	455
Vitamin premix	10	10	10	10
Mineral premix	40	40	40	40
Carboxy methyl cellulose (CMC)	30	30	30	30
Cellulose	129.75	9.75	127.75	7.75
Choline chloride	5	5	5	5
Butylated hydroxytoluene (BHT)	0.25	0.25	0.25	0.25
Oxytetracycline (OTC)	0	0	2	2
Total	1000	1000	1000	1000
Proximate composition				
Moisture	106.54 ± 0.95	105.59 ± 0.85	104.23 ± 0.96	108.19 ± 1.17
Protein content	318.51 ± 3.94	318.77 ± 6.72	320.51 ± 2.03	316.83 ± 3.91
Lipid content	53.02 ± 2.12	55.70 ± 2.98	53.25 ± 3.47	55.08 ± 1.03
Ash	44.68 ± 0.57	43.96 ± 0.26	45.35 ± 1.33	44.42 ± 0.84
Crude fiber	142.59 ± 5.14	22.68 ± 0.11	142.24 ± 4.89	22.85 ± 0.33
Nitrogen-free extract	334.66 ± 4.67	453.30 ± 2.58	334.42 ± 3.09	454.63 ± 1.61

et al., 2018b). The concentration of oxytetracyline used in the present study is a commonly administered therapeutic dose for fish treatment worldwide (Limbu et al., 2019; Limbu et al., 2018; Trushenski et al., 2018).

After acclimatization, 360 visually health *O. niloticus* (9.50 ± 0.08 g; mean ± standard error of the mean) were distributed randomly into 12 sterile tanks (90 fish per treatment in three replicate, each 30 fish) filled with dechlorinated water and fed the four experimental diets. The randomization was done by collecting five fish at a time using a scoop net from the acclimatization tank containing all fish. The bulk weight of the five fish was measured before distributing them randomly into each replicate until the 30 fish per treatment were obtained. The fish in all treatments 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 35 days. During each feeding occasion, fish in all treatments were fed by using little amounts until all feeds were consumed completely. During the whole feeding experiment, no any remaining feeds in the culture water were observed. The experimental conditions were similar to those used during the acclimatization period. Half of the water in each tank was replaced with fresh-dechlorinated water every 24 h. The weight of individual fish was measured fortnightly and the feed rations were adjusted accordingly.

2.5. Estimation of growth performance, feed utilization and survival rate

At the end of the experiment, all fish were fasted for 24 h and then they were collected by using a scoop net. All fish were weighed individually for estimation of weight gain as the percentage increase in weight relative to the initial weight. The amount of feed fed on daily basis and the fish weight gained in each treatment were used to calculate feed efficiency as the ratio of total fish weight gained to the total amount of dry feed fed. The protein efficiency ratio was estimated from the total amount of protein fed and the total fish wet weight gained. The percentage survival was calculated as the ratio of the number of fish at the end to that at the start of the experiment multiplied by 100.

2.6. Collection of blood from fish

After weight measurements, two fish per replicate (six fish from each dietary treatment) were sampled randomly, anesthetized individually by using MS-222 (20 mg/L) (tricaine methanesulfonate, Western Chemicals, Inc., Ferndale, Washington) and sacrificed for blood collection. The blood was drawn from the caudal blood vessels of individual fish by using 2 mL syringes (Klmedical, China). Blood samples were centrifuged immediately at 3000 rpm for 10 min at 4 °C, and the resulting serum was pipetted into polypropylene tubes, subsequently stored at −80 °C until needed for biochemical analyis.

2.7. Measurement of hepatosomatic index, biochemical assays and hepatic composition analysis

The remaining fish after blood sampling were dissected to obtain liver and small intestine organs for measurement of hepatosomatic index (HSI), biochemical assays and composition analysis. The liver weights of individual fish were used to determine HSI as the percentage of liver weights to the individual fish weights. After measurement, the livers from each of the six fish per treatment were stored at −80 °C for further analysis. To obtain the samples for biochemical assays, the small intestines and livers from the six *O. niloticus* were homogenized in glass beds with 9 vol (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. During assays, one whole small intestine, liver and the same serum from each fish were used for all analyses.

The activities of acid phosphatase (ACP), alkaline phosphatase (AKP), glutathione S-transferase (GST) and superoxide dismutase (SOD) and the contents of malondialdehyde (MDA), alanine transaminase (ALT), aspartate transaminase (AST), lysozyme (LZM) and glycogen were measured by using specific commercial assay kits (Nanjing Jiancheng Institute, China), with their results recorded on a microplate reader (Epoch, BioTek, USA). The protein and lipid contents in the liver were determined by Kjeldahl method (AOAC, 1995) and the chloroform-methanol method (Bligh and Dyer, 1959), respectively.

2.8. 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). The DNA quantity and quality were measured by using a NanoDrop 2000 Spectrophotometer (Thermo, USA). The OD260/280 ranged from 1.98 to 2.12 showing good quality. The quality of the extracted DNA was further confirmed by using agarose gel electrophoresis.

2.9. 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 by using Illumina HiSeq 2500 (Illumina, San Diego, USA).

2.10. 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.

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

Total RNA from six small intestine and liver tissues from each treatment were isolated by using a Tri Pure Reagent (Aidlab, China) for genes expression analysis. The quality and quantity of total RNA were tested by using a NanoDrop 2000 Spectrophotometer (Thermo, USA). Only samples with OD260/280 values ranging from 1.86 to 2.01 were used for real-time polymerase chain reaction (qRT-PCR). We synthesized first strand cDNAs by using a PrimerScript™ RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Takara, Japan) in the S1000TM Thermal Cycler (Bio-Rad, USA). The 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).

We evaluated the nutrients metabolism, cellular stress response, immunity, pro-inflammation and apoptosis by using mRNA expression of genes in the intestine and liver. During the analysis, 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 2. During the study, the qRT-PCR efficiency was between 99% and 101% 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 included. The relative expressions of genes were calculated by using the $2^{-\Delta Ct}$ method, thereof, $\Delta Ct = Ct_{target} - (Ct_{EF1\alpha} + Ct_{\beta\text{-actin}})/2$.

2.12. Detection of oxytetracycline concentration in fish muscle, starch, mixed diet and evaluation of human health risk

Muscles tissues from six fish samples per treatment were collected for analysis of oxytetracycline concentrations by using oxytetracycline enzyme-linked immune sorbent assay (ELISA) (REAGEN LLC, Moorestown, New Jersey, USA). The potential human health risk of consuming *O. niloticus* treated with oxytetracycline was assessed based on target hazard quotient (THQ) according to our previous study (Limbu et al., 2018) by using the following equation:-

$$THQ = \left(\frac{E_F \times E_D \times F_{IR} \times C}{R_{FD} \times W_{AB} \times T_A}\right) x10^{-3}$$

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 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 oxytetracycline based on U.S. Environmental Protection Agency (U.S. EPA, 2017). 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 oxytetracycline. A THQ ≤1 means the exposure population is unlikely to experience obvious adverse health risk.

2.13. Assessment of the binding effect and efficiency of starch and diets

The ability of starch to bind the oxytetracycline was assessed by mixing 335 g of starch with 2 g of oxytetracycline (MSO) and 455 g of starch with 2 g of oxytetracycline (HSO) in clean-sterilized glass beakers, to represent the contents of the medium and high starches and oxytetracycline used in the present study. We further mixed separately the ingredients used to produce the oxytetracycline diets (MCO and HCO) using the same amounts of starches and oxytetracycline in order to assess the possible binding effect of the carbohydrate diets on oxytetracycline. The resulting mixtures of starches were dissolved in a litre of distilled water, while the dietary were dissolved into 4.5 L of distilled water. A control medium on which only water and the 2 g of oxytetracycline (WO) was also included as a control. The mixed contents were wrapped by using aluminum foils and incubated in a dark room at 26–28.2 °C for 24 h. This temperature was appropriate for chemical reactions to take place in order to understand the differences in binding effects among the treatments. On the other hand, 24 h was sufficient time to allow the reactions to proceed and produce differences in binding effects among the treatments before the analysis was done. The pH of the mixtures ranged from 6.63 to 6.88. The ability of the starch and carbohydrate diets to bind oxytetracycline was assessed

Table 2
The primers sequences used in the present study.

Type	Gene	Primer sequence (5′- 3′)	GenBank number
Protein metabolism	*GDH-1*	F: GGTTCCAGGACAAGACCTTCGT	XM_003457465.3
	APN	R: TCTCCGACTCCGACGCACTT	XM_005449270.3
		F: CCGCCTGGATGAAGAGCAACAA	
		R: TCCTTCTCACCAGCAGCCACAG	
Glycogen metabolism	*PGM-1*	F: CAGCCAACGACGCAGTCACAA	XM_005458066.4
	GP	R: ATGGCAGAACTGGCAGGACAAC	XM_003442862.4
		F: AGGCAGATGACTGGCTGAGACA	
		R: GGCATTGCGAGAACCACCTGAG	
Lipogenesis	*FAS*	F: TCATCCAGCAGTTCACACTGGCATT	XM_013276808.2
		R: TGATTAGGTCCACGGCCACA	
	ACCα	F: TAGCTGAAGAGGAGGGTGCAAGA	XM_005471970
		R: AACCTCTGGATTGGCTTGAACA	
Lipid catabolism	*ATGL*	F: AAAACGTCCTGGTGACCCAGT	XM_003440346
	HSL	R: TAGGAGGAATGATGCCACAGTACA	XM_005463936.3
		F: AACCTGGATGTCCATTTCTGGAAG	
	CPT-1	R: TCGGTTTACCTTGACTTGAGTGGA	XM_003440355.4
		F: ATCTTGGCAGACAAGTCGGAGC	
		R: TTTGCCCACGGTGTTCTATCC	
Cellular stress	*HSP70*	F: TGCCTTTGTCCAGACCGTAG	XM_019357557.1
	XBP1	R: GTGTCCAACGCTGTCATCAC	XM_013276886.1
		F: GCAGCACACCGACTTGGAGA	
		R: ACCACGACCTCCGTAACAGGAA	
Apoptosis	*Caspase - 9*	F: ATACTTGAGGAAAACGCTGCCACT	XM_003455320.4
		R: GAACCAGGCATTTGTTTGTAGAGC	
Immunity	*MyD88*	F: TTTACGCTTCCTCACCATTTGT	NM_001311322.1
		R: CCGCCTGCTCCACAGTTAT	
	NF-κB P65	F: CGACCACTACCTACACGCTC	XM_019363515.2
		R: GATGTCGTTTGAGGCATCGC	
	IL-1β	F: GAGCACAGAATTCCAGGATGAAAG	XM_019365841.1
		R: TGAACTGAGGTGGTCCAGCTGT	
	TNF-α	F: CAGAAGCACTAAAGGCGAAGAACA	NM_001279533
		R: TTCTAGATGGATGGCTGCCTTG	
Internal reference	*β-actin*	F: AGCCTTCCTTCCTTGGTATGGAAT	KJ126772.1
	EF1a	R: TGTTGGCGTACAGGTCCTTACG	KJ123689.1
		F: CTACGTGACCATCATTGATGCC	
		R: AACACCAGCAGCAACGATCA	

Notes; *GDH-1* = glutamate dehydrogenase 1, *APN* = aminopeptidase N, *PGM-1* = phosphoglucomutase−1, *GP* = glycogen phosphorylase, *FAS* = fatty acid synthase, *ACCα* = acetyl-coA carboxylase alpha, *ATGL* = adipose triglyceride lipase, *HSL* = hormone-sensitive lipase *CPT-1* = carnitine palmitoyltransferase 1, *HSP70* = heat shock protein 70, *XBP1* = X-box binding protein 1, *Caspase − 9* = cysteine-aspartic acid protease 9, *MyD88* = myeloid differentiation primary response gene 88, *NF-κB P65* = nuclear factor kappa-light-chain-enhancer of activated B cells protein 65, *IL-1β* = interleukin 1 beta, *TNF-α* = tumor necrosis factor alpha and *EF1a* = elongation factor 1 alpha.

by analyzing the amount of oxytetracycline in the surface water contained in the WO, MSO, HSO, MCO and HCO mixtures by using oxytetracycline ELISA (REAGEN LLC, Moorestown, New Jersey, USA) kit as described above. The binding efficiency of starch and carbohydrate diets was determined as the percentage-difference of the amount of oxytetracycline detected relative to the initial amount added.

2.14. 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 (SPSS version 20, IBM, Armonk, NY, USA) for Windows. Statistical comparisons were made for the mean values between dietary MC and MCO; HC and HCO; MC and HC; and MCO and HCO. For the binding effect, statistical comparisons were done between WO and MSO; WO and HSO; MSO and HSO; WO and MCO; WO and HCO; and MCO and HCO. Lastly, binding efficiency between MSO and HSO; and MCO and HCO was also statistically compared. 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. Significant differences were judged at a probability level of $p \leq 0.05$ for all analyzes.

3. Results and discussion

3.1. Dietary carbohydrate content does not affect oxytetracycline-induced side effects on growth performance, feed utilization and survival rate of fish

Growth performance, feed utilization and survival rate are important production attributes, which determine the cost of production and profitability of aquaculture. In order to evaluate the ability of high carbohydrate to protect *O. niloticus* from oxytetracycline-induced side effects, we measured the growth performance, feed utilization and survival rate of fish. Results showed reduced weight gain (Fig. 1a), feed efficiency (Fig. 1b), protein efficiency ratio (Fig. 1c) and survival rate (Fig. 1d) for *O. niloticus* fed on MCO and HCO compared to those fed on MC and HC, respectively. Reduced growth performance in *O. niloticus* fed on MCO diet is consistent with results reported by Gaikowski et al. (2003) in hybrid striped bass (*Morone saxatilis* x *M. chrysops*), which is coherent with a recent study that, oxytetracycline does not cause growth promotion in finfish species including channel catfish (*Ictalurus punctatus*), hybrid striped bass (*Morone chrysops* × *M. saxatilis*), *O. niloticus*, or rainbow trout (*Oncorhynchus*

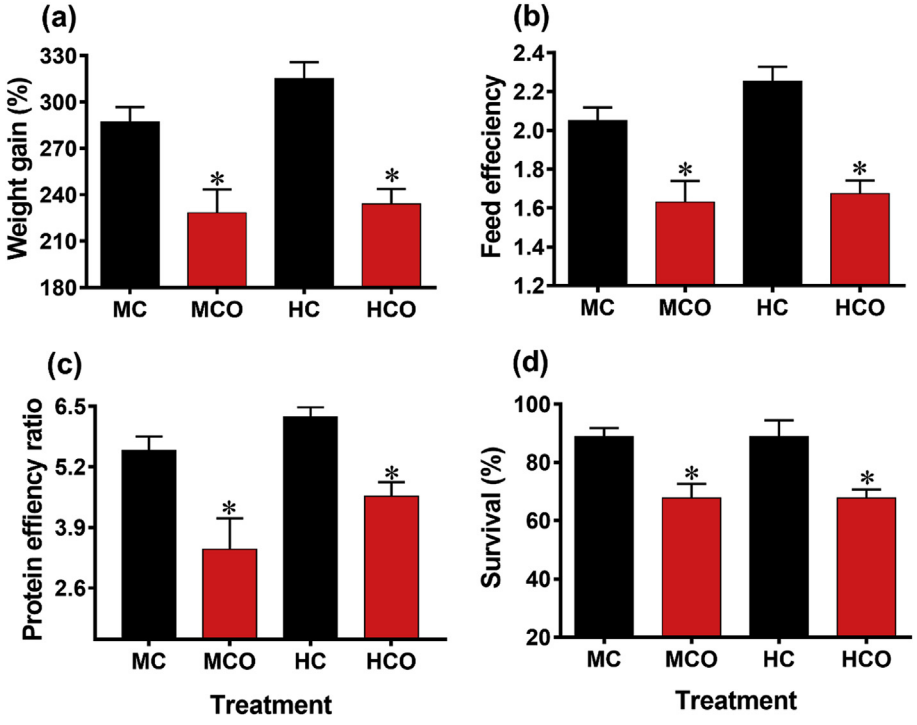


Fig. 1. Growth performance, feed efficiency and survival of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) weight gain, (b) feed efficiency, (c) protein efficiency ratio (PER), and (d) survival (%). Weight gain = [(Final − initial weights)/initial weight] x 100, Feed efficiency = Total fish wet weight gain/Total amount of dry feed fed, Protein efficiency ratio = Total fish wet weight gain/Total amount of dry feed protein fed, and survival = (Final number of fish/initial number of fish) x 100. Values are means ± SEM for three replicates [n = 18–30]. The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets ($p < 0.05$).

mykiss) (Trushenski et al., 2018). The reduced growth performance obtained in the *O. niloticus* fed on the MCO and HCO diets is attributed to lower feed efficiency and protein efficiency ratio, which determine the ability of fish to convert feed into the desired weight and accumulate protein in the body, respectively. Feed efficiency has been shown to account for about 40% of body weight gain in *O. niloticus* (de Verdal et al., 2017), thus its reduction definitely decreased the growth performance. Moreover, protein efficiency ratio determines the deposition of protein in the body, which ultimately affect growth performance in fish (Canada et al., 2017). Subsequently, its reduction in fish fed on the MCO and HCO diets decreased the growth performance. The reduced survival rate of *O. niloticus* in the MCO and HCO diets might be caused by the sustained side effects due oxytetracycline, which the high carbohydrate diet was not able to ameliorate. These results indicate that, high carbohydrate diet does not protect *O. niloticus* from side effects induced by oxytetracycline on growth performance, feed utilization and survival rate.

3.2. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by increasing hepatosomatic index and hepatic protein content

Liver is one of the main target organs for side effects induced by antibiotics in *O. niloticus* (Limbu et al., 2018) and it is known to accumulate oxytetracycline in fish (Yuan et al., 2014). The HSI is a quick indicator of the liver health status. We determined the HSI after feeding *O. niloticus* with medium and high carbohydrate diets with or without oxytetracycline. Feeding *O. niloticus* with HC diet increased significantly the HSI (Fig. 2a) compared to those fed on MC diet, similar to results obtained by Boonanuntanasarn et al. (2018b). The MCO diet reduced significantly the HSI of fish compared to the MC diet (Fig. 2a), similar to our previous results on the same species (Limbu et al., 2018) and those reported by Refstie et al. (2006) in Atlantic salmon (*Salmo salar*) fed on diets containing 32–90 g/kg starch compared to those supplemented with 3 g/kg oxytetracycline. However, feeding *O. niloticus* with the HCO diet increased significantly the HSI (Fig. 2a) compared to those fed on the HC and MCO diets. To understand further the liver health status in *O. niloticus* fed on the HCO, we assessed the hepatic protein, lipid and glycogen contents. Results showed that, feeding *O. niloticus* with the MCO diet decreased the protein content compared to the MC diet (Fig. 2b). However, the HCO diet increased the protein content of *O. niloticus* compared to the MCO diet. Contrary to the decreased protein content, the MCO and HCO diets increased the lipid (Fig. 2c) and glycogen (Fig. 2d) contents compared to the MC and HC diets, respectively. The HC diet increased the glycogen content compared to the MC diet (Fig. 2d), similar to results obtained by Boonanuntanasarn et al. (2018a) in *O. niloticus*.

The reduced HSI and protein content in *O. niloticus* fed on the MCO diet compared to the MC are the typical oxytetracycline-induced toxicity in fish (Limbu et al., 2018; Refstie et al., 2006). We postulate that, liver cells degenerations (Reda et al., 2013; Refstie et al., 2006) and energy deficits (Thoolen et al., 2010) due to side effects induced by oxytetracycline, possibly caused the reduced HSI and protein content. However, the HCO diet provided protective effects in recovering HSI and protein content for *O. niloticus* fed on this diet. Consequently, the protein content in the liver increased (Fig. 2b). This is not surprising given the ability of *O. niloticus* to adapt effectively to long period of high carbohydrate intake up to 500 g/kg (Boonanuntanasarn et al., 2018a). Since lipid and glycogen contents were statistically comparable between *O. niloticus* fed on MCO and HCO diets, it is reasonable to suggest that, the high carbohydrate diet protects *O. niloticus* from oxytetracycline-induced side effects by increasing HSI and hepatic protein content. Therefore, under similar experimental conditions,

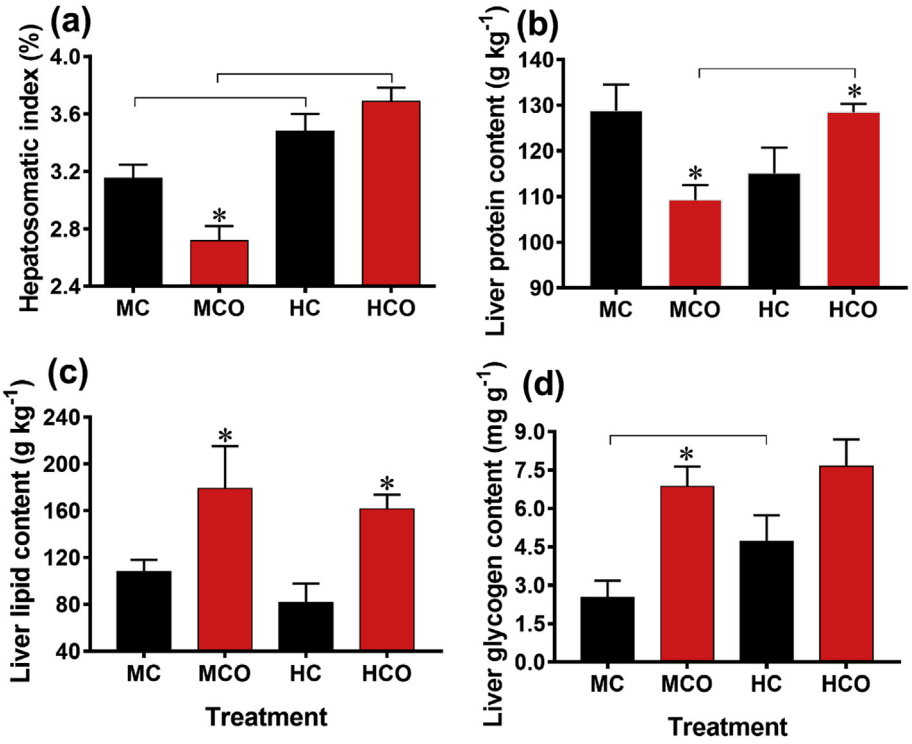


Fig. 2. Hepatosomatic index and hepatic protein, lipid, and glycogen contents of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) hepatosomatic index, (b) hepatic protein content, (c) hepatic lipid content, and (d) hepatic glycogen content. Hepatosomatic index = (Liver weight/fish body weight) x 100. Values are means ± SEM for three replicates (n = 6). The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets, while a line above the bars shows significant differences between MC vs HC and MCO vs HCO diets ($p < 0.05$).

feeding *O. niloticus* with high carbohydrate diet protects fish from impaired liver function.

3.3. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by lowering hepatic protein, glycogen and lipid metabolism

Liver plays critical roles in nutritional metabolism of carbohydrates (Graham and Adamo, 1999). Considering nutrients metabolic response provides energy for expenditure in various activities including stress in the body, we determined the hepatic protein, glycogen and lipid metabolism. We first detected the mRNA expression of glutamate dehydrogenase 1 (*GDH-1*), a gene responsible for reversible synthesis of L-glutamate for use in protein synthesis or alternatively transamination reactions (Vázquez-Martínez et al., 2017) and aminopeptidase N (*APN*) gene, which plays an essential role in the metabolic balance of proteins in caloric synthesis (Tang et al., 2016). We hypothesized that, *GDH-1* and *APN* are critical enzymes for synthesis and catabolism of protein in fish subjected to oxytetracycline-induced side effects and high carbohydrate may protect *O. niloticus* from the resulting adverse effects. Results showed that, the *O. niloticus* fed on the MCO diet had higher mRNA expression of *GDH-1* (Fig. 3a) and *APN* (Fig. 3b) genes than those fed on the MC diet. Similarly, the HCO diet had higher mRNA expression of *APN* gene than those fed on the HC diet. These results suggest that, the dietary oxytetracycline increased the catabolism of protein in the fish body in order to provide more energy under oxytetracycline-induced side effects. However, the HCO diet reduced the mRNA expression of *GDH-1* (Fig. 3a) and *APN* (Fig. 3b) in *O. niloticus* compared to those fed on the MCO diet, which confirmed our hypothesis set earlier that, high carbohydrate can protect fish from increased protein synthesis and catabolism. Apparently, the fish fed on the MCO increased *GDH-1* and the proteolytic *APN* genes in order to synthesis and catabolise more protein so as to generate additional energy under sustained oxytetracycline-induced side effects. However, when Nile fish were fed on the HCO diet, the side effects were attenuated, which resulted in the downregulation of *GDH-1* and *APN* genes.

Secondly, we detected the mRNA expression of phosphoglucomutase - 1 (*PGM-1*), a key enzyme in gluconeogenesis and glycogenesis, which catalyses the bidirectional transfer of phosphate from position 1 to 6 on glucose (Morava, 2014) and glycogen phosphorylase (*GP*) gene, which acts on the reaction at the initiation of glycogen degradation. We found that, the MCO diet also upregulated the mRNA expression of *PGM-1* (Fig. 3c) and *GP* (Fig. 3d) genes in *O. niloticus*, but the expression was only significant for *GP* gene. Interestingly, the HCO diet downregulated both the *PGM-1* (Fig. 3c) and *GP* (Fig. 3d) genes expression in *O. niloticus* compared to the MCO diet. The increased mRNA expressions of *PGM-1* and *GP* in *O. niloticus* fed on the MCO is not surprising because the liver is involved in monitoring and maintaining glucose homeostasis in the bloodstream by controlling gluconeogenesis, glycogenesis and glycogenolysis. Since *PGM-1* is a key enzyme in gluconeogenesis and glycogenesis, the body of *O. niloticus* fed on the MCO diet needed to elevate this gene in order to balance the amount of glucose for energy supply under oxytetracycline-induced side effects. Notably, the higher expression of *GP* gene in *O. niloticus* fed on the MCO diet intended to accumulate glycogen as a local carbohydrate reserve. This was necessary in order to avoid glycogen reserves depletion and increase sensitivity to subsequent stress (Hickie et al., 1989) caused by oxytetracycline. The glycogen

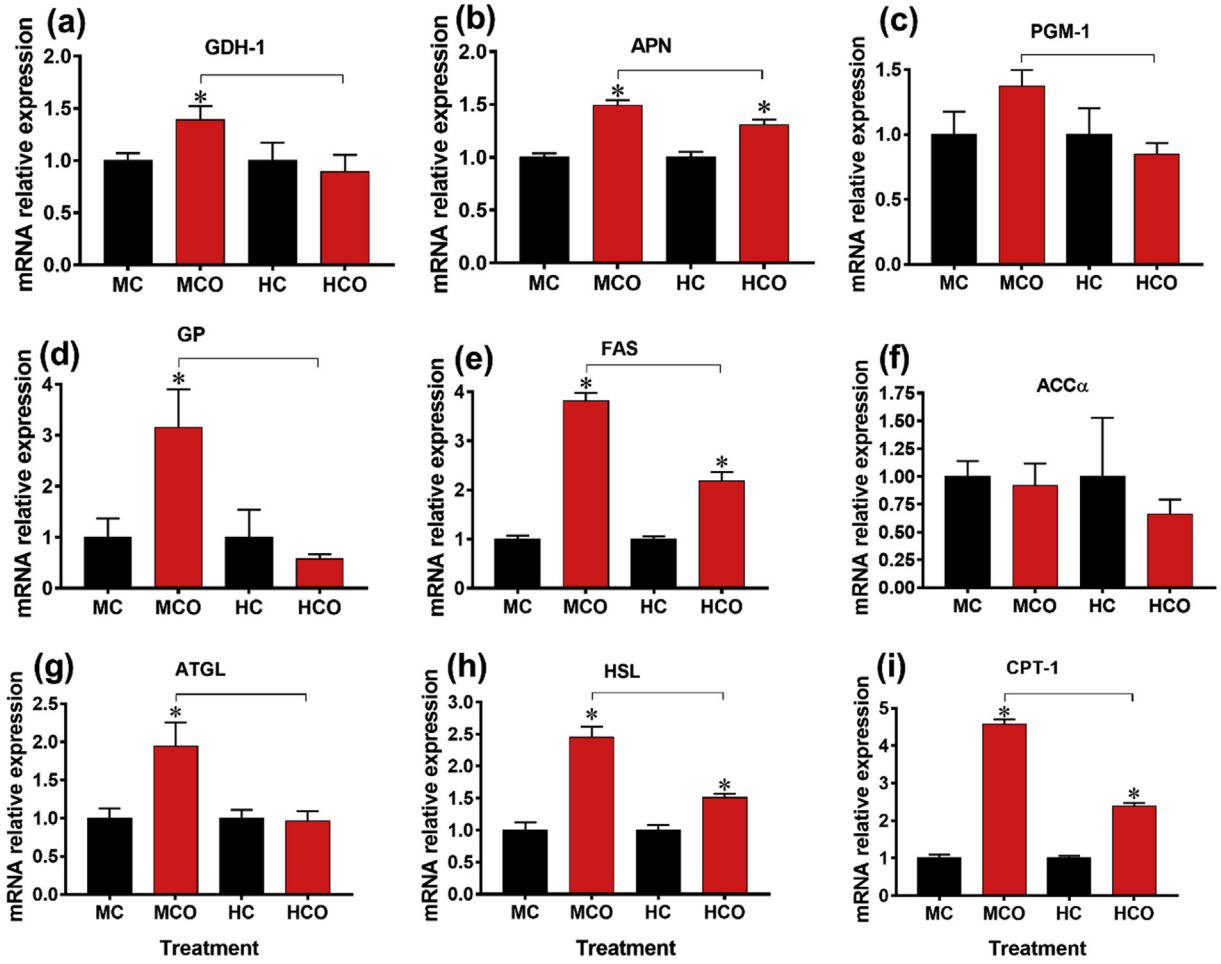


Fig. 3. The hepatic mRNA relative expression of genes involved in protein, glycogen and lipid metabolism of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) glutamate dehydrogenase-1 (*GDH-1*), (b) aminopeptidases N (*APN*), (c) phosphoglucomutase - 1 (*PGM-1*), (d) glycogen phosyporylase (*GP*), (e) fatty acid synthase (*FAS*), (f) acetyl-coA carboxylase alpha (*ACCα*), (g) adipose triglyceride lipase (*ATGL*), (h) hormone-sensitive lipase (*HSL*), and (i) carnitine palmitoyltransferase 1 (*CPT-1*). Values are means ± SEM for three replicates (n = 6). The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets, while a line above the bars shows significant differences between MCO vs HCO diets ($p < 0.05$).

reserves accumulated were degraded to provide a prompt emergency energy supply for fish to survive under oxytetracycline-induced stress. Interestingly, feeding *O. niloticus* on the HCO diet alleviated the oxytetracycline-induced side effects, subsequently returned the *PGM-1* and *GP* mRNA genes expression similar to control fish.

To sum up the nutrients metabolism required for energy generation, we measured the mRNA expression of genes involved in lipid metabolism. Similar to protein and glycogen genes, feeding *O. niloticus* on the MCO diet upregulated fatty acid synthase (*FAS*) gene, which is used in lipogenesis (Fig. 3e) and adipose triglyceride lipase (*ATGL*; Fig. 3g), hormone-sensitive lipase (*HSL*; Fig. 3h) and carnitine palmitoyltransferase 1 (*CPT-1*; Fig. 3i) genes, which are responsible for lipid catabolism, similar to results reported by Limbu et al. (2018). However, the expression of these genes were downregulated when the fish were fed on the HCO diet compared to the MCO diet. These results highlight the need for higher energy demand in *O. niloticus* fed on the MCO diet due to the oxytetracycline-induced side effects because it is generally accepted that, lipids are the favored source of metabolic energy in fish via β-oxidation (Sargent et al., 2003) and they provide more energy per unit weight than carbohydrate and protein (Tacon, 1990). Accordingly, the *O. niloticus* fed on the MCO diet elevated lipogenesis and lipid catabolism in order to generate higher energy under the oxytetracycline-induced side effects. On the contrary, *O. niloticus* fed on the HCO diet protected them from the oxytetracycline-induced side effects, which resulted in down-regulation of lipogenesis and lipid catabolism. Taken together, the high carbohydrate diet protects *O. niloticus* from oxytetracycline-induced high-energy demand by lowering the protein, glycogen and lipid metabolism.

3.4. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by reducing hepatotoxicity but not oxidative stress and lipid peroxidation

Oxytetracycline commonly causes disturbance in antioxidant capacity and increases hepatotoxicity and lipid peroxidation; thereof, we assessed these parameters in the intestine, liver and serum in the present study. The *O. niloticus* fed on the HCO diet increased SOD activity (Fig. 4a) and MDA content (Fig. 4c) compared to the HC diet in the intestine. The *O. niloticus* fed on the MCO increased the GST activity (Fig. 4b) in intestine than the MC diet. The HC diet decreased the MDA content compared to the MC diet (Fig. 4c). In the liver, the MCO diet reduced the SOD (Fig. 4d) GST (Fig. 4e) activities and MDA content compared to the MC diet, while the HCO diet increased the MDA content compared to the MCO diet (Fig. 4f). In the serum, the HCO diet decreased ALT content

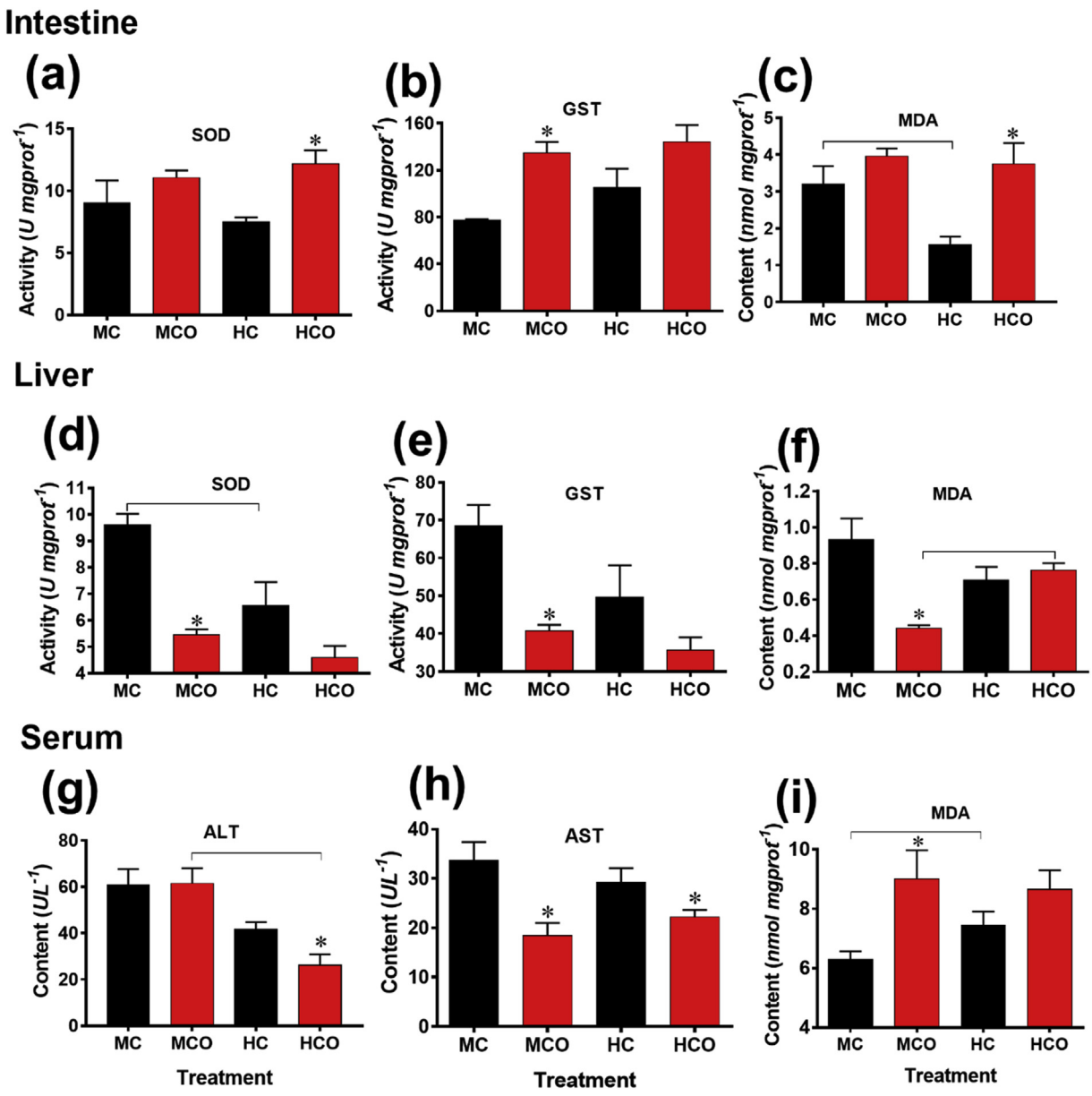


Fig. 4. The antioxidant capacity and hepatotoxicity of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) superoxide dismutase (SOD) activity in intestine, (b) glutathione S transferase (GST) activity in intestine (c) malondialdehyde (MDA) content in intestine, (d) SOD in the liver, (e) GST activity in the liver, (j) MDA content in the liver, (g) alanine transaminase (ALT) content in the serum, (h) aspartate transaminase (AST) content in the serum, and (i) MDA content in the serum. Values are means ± SEM for three replicates (n = 6). The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets, while a line above the bars shows significant differences between MC vs HC and MCO vs HCO diets ($p < 0.05$).

(Fig. 4g), an indicator of hepatotoxicity compared to the MCO diet in the serum. Both the MCO and HCO diets decreased the AST content, another indicator of hepatotoxicity compared to the MC and HC diets, respectively in the serum (Fig. 4h). The *O. niloticus* fed on the MCO diet had increased MDA content than those fed on the MC diet (Fig. 4i) and those fed on the HC diet had higher MDA content than the MC diet in the serum.

The results on antioxidant capacity, hepatotoxicity and lipid peroxidation obtained in the present study are threefold. First, oxytetracycline induces tissue-specific side effects on antioxidant capacity and lipid peroxidation on intestine and liver between fish fed on the MCO and MC diets. For instance, while the *O. niloticus* fed on the MCO diet had higher GST activity in the intestine (Fig. 4b), those fed on the same diet had lower activity of the same enzyme compared to those fed on the MC diet in the liver (Fig. 4e). Moreover, whereas the *O. niloticus* fed on the MCO had lower MDA content in liver (Fig. 4f), those fed on the same diet had higher MDA content in the serum (Fig. 4i). This is similar to results reported by Nakano et al. (2018) in coho salmon fed on 240 g/kg carbohydrate diet supplemented with 100 mg oxytetracycline/kg body weight of fish. The differences in antioxidant capacity and lipid peroxidation between the intestine and liver may be related to tissue-sensitivity variations on oxytetracycline-induced oxidative stress, which is related to their ability to accumulate the antibiotic. The liver of fish is known to accumulate more oxytetracycline than the intestine (Yuan et al., 2014), which exhausted the antioxidant capacity resulting in elevated MDA content for fish fed on the HCO diet compared to those fed on the MCO diet in the liver (Fig. 4f).

Secondly, the high carbohydrate diet is unable to protect *O. niloticus* from oxytetracycline-induced oxidative stress and lipid peroxidation because both the MCO and HCO diets had no differences in SOD and GST activities and MDA content in the intestine and serum. Lastly, oxytetracycline exerts parameter-specific response on hepatotoxicity and lipid peroxidation among tissues. Accordingly, although MDA content increased in the liver of fish fed on the HCO diet compared to those fed on the MCO diet (Fig. 4f), the ALT content was reduced in the serum (Fig. 4g), consistent with results obtained by Jayanthi and Subash (2010) in albino rats. This implies increased lipid peroxidation in the liver and hence lack of protection from the high carbohydrate diet, contrary to the reduced ALT content in the serum, suggesting the ability of the latter to protect fish from oxytetracycline-induced hepatotoxicity. Concisely, these results suggest that, high carbohydrate diet partially protects *O. niloticus* from oxytetracycline-induced side effects by reducing hepatotoxicity, while it does not protect them from oxidative stress and lipid peroxidation.

3.5. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by reducing pathogenic bacteria abundance in the intestine

The use of oxytetracycline in cultured fish affects both pathogenic and symbiotic microbiota. Therefore, we analyzed the richness, diversity and abundance of microbiota in *O. niloticus* after feeding with medium and high carbohydrate diets with or without oxytetracycline. Feeding *O. niloticus* with the experimental diets did not affect the species richness abundance based coverage estimator (ACE) and Chao1 index and the diversity indices (Shannon and Simpson) of microbiota among treatments (Table S1). We further analyzed the abundance of dominant phyla representing >1% of the total bacterial count. The results of independent *t*-test depicted 44 OTUs that were significant different among treatments and these were selected for drawing a heatmap (Fig. 5a). Feeding *O. niloticus* with the MC and MCO diets caused 33 microbiota dysbiosis. The MCO diet increased the abundance of 27 OTUs compared to MC diet, which only increased six OTUs. Interestingly, only one OTU was increased when *O. niloticus* were fed with the HC and HCO diets (Fig. 5a). On the contrary, a total of 15 OTUs were increased in *O. niloticus* fed the MCO diet compared to the HCO diet. Feeding the MC diet increased three OTUs compared to the HC diet. A further classification of the increased OTUs in MCO diet indicated that, most of them belong to Actinobacteria, Chlamydiae, Firmicutes, Planctomycetes and Proteobacteria phyla (Fig. 5b).

The results on microbiota indicate that, the MCO diet provided a chance for proliferation of pathogenic bacteria. For instance, the MCO diet increased the OTU 1408 (*Microbacterium* species), which is an Actinobacteria that causes mortality in *O. niloticus* (Lara-Flores et al., 2014), while OTUs 1507 and 1577 both belonging to *Candidatus* and OTU 329 (*Parachlamydiaceae* species) are Chlamydiae responsible for causing diseases in cultured fish (Pawlikowska-

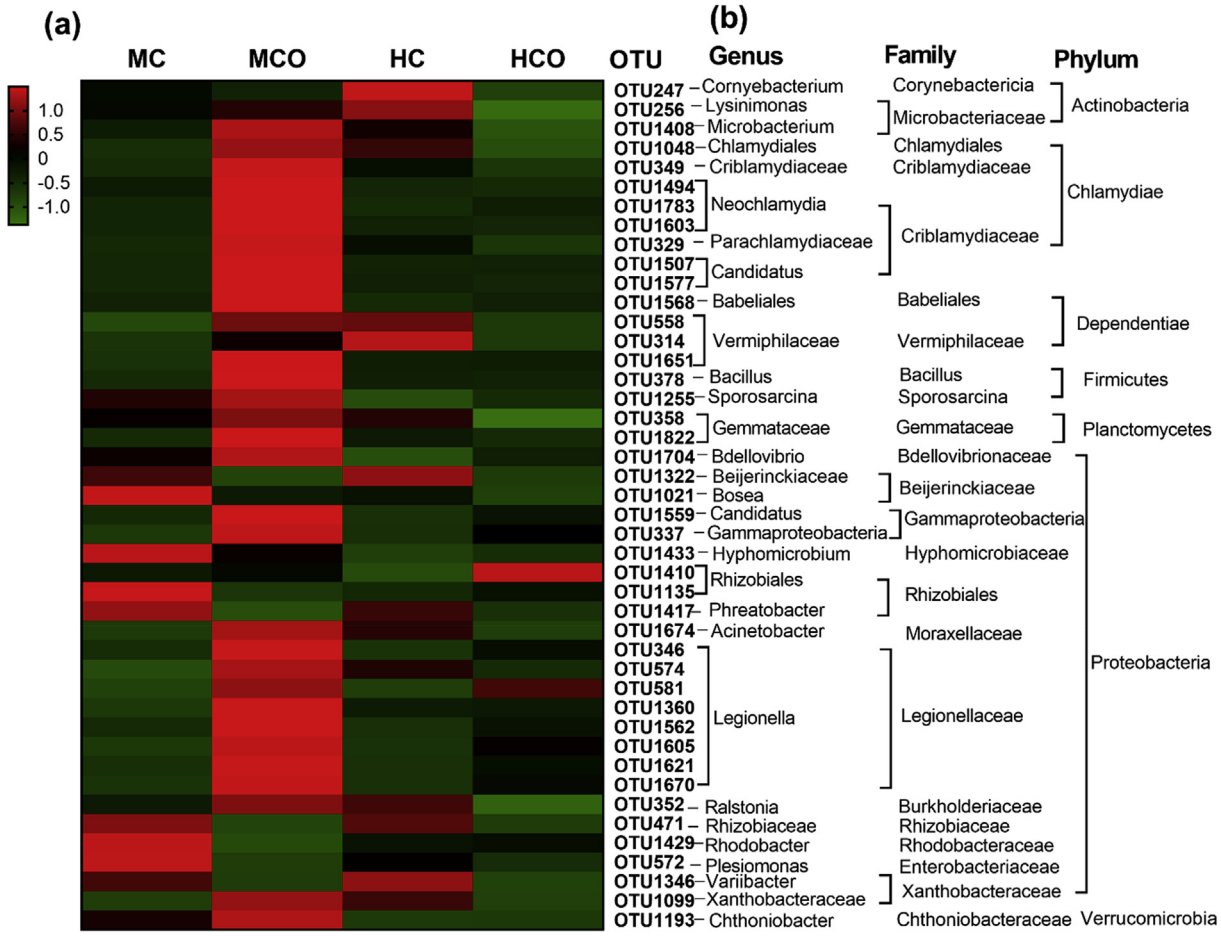


Fig. 5. The microbiota abundance of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) heatmap of the changed 44 operational taxonomic units (OTUs) and (b) the classification of the changed OTUs. Red, green and black colors indicate the abundance of OTUs that were increased, decreased and not changed, respectively (n = 5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Warych and Deptuła, 2016). Moreover, the increased Proteobacteria such as OTU 572 (*Plesiomonas* species) is pathogenic to *O. niloticus* (Yilmaz, 2019), whereas OTU 1021 (*Bosea* species) has been related to antibiotic-resistance bacteria in freshwater environment (Zothanpuia et al., 2016). Furthermore, *Acinetobacter* and *Legionella* species are potential pathogens to cultured *O. niloticus* and humans (Grande Burgos et al., 2018). Interestingly, the HCO diet decreased most of the above OTUs and increased OTUs 1410 and 1135 belonging to *Rhizobiales* species (Proteobacteria), which are well-known for their beneficial effects such as immunity stimulators (Ikeda-Ohtsubo et al., 2018) and nutritional benefits in fish (Gaikwad et al., 2017). It is more likely that, the increased pathogenic bacteria for *O. niloticus* fed on MCO stimulated the abundance of *Bacillus* species (a Firmicute) in order to enhance the fish immunity (Abarike et al., 2018). The ability of high carbohydrate to reduce pathogenic bacteria is due to the capability of the carbohydrate-binding molecules to inhibit pathogenic organisms such as viruses fusion and entry by crosslinking membrane glycoproteins (Leikina et al., 2005). In general, our results suggest that, the high carbohydrate diet protects *O. niloticus* from oxytetracycline-induced side effects by reducing pathogenic and increasing beneficial bacteria abundance in the intestine.

3.6. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by increasing innate immunity and reducing pro-inflammatory response

Oxytetracycline impairs immunity and induces inflammation response (Limbu et al., 2018). Therefore, we detected the immunity and pro-inflammation response in the present study. Results indicated that, the *O. niloticus* fed on the MCO diet decreased the ACP (Fig. 6a) and AKP (Fig. 6b) activities, but increased the mRNA expression of myeloid differentiation primary response gene 88 (*MyD88*; Fig. 6c), nuclear factor kappa-light-chain-enhancer of activated B cells protein 65 (*NF-κB P65*; Fig. 6d), interleukin 1 beta (IL-1β; Fig. 6e) and tumor necrosis factor alpha (*TNF-α*; Fig. 6f) genes in intestine than the MC diet. However, feeding *O. niloticus* on the HCO diet increased the ACP and AKP activities and reduced the mRNA expression of *MyD88*, *IL-1β* and *TNF-α* genes compared to the MCO diet in the intestine. In the liver, the MCO diet equally reduced the ACP (Fig. 6g) and AKP (Fig. 6h) activities, but increased the mRNA expression *MyD88* (Fig. 6i), *NF-κB P65* (Fig. 6j), *IL-1β* (Fig. 6k) and *TNF-α*; Fig. 6l). Like in the intestine, feeding *O. niloticus* with HCO diet increased ACP and AKP activities and reduced the mRNA expression of *MyD88*, *NF-κB P65*, *IL-1β* and *TNF-α* genes. In the serum, the MCO diet decreased the ACP (Fig. 6m) and AKP (Fig. 6n) activities compared to the MC diet, while feeding the *O. niloticus* with HCO diet increased ACP activity, similar to the intestine and liver organs. However, the MCO diet increased the LZM content (Fig. 6o). The HC diet increased ACP, but decreased AKP activities compared to the MC diet.

The results on immunity response show clear that, feeding *O. niloticus* on the MCO diet reduce innate immunity (ACP and AKP activities) than those fed on the MC in the three tissues, consistent to our previous results on *O. niloticus* (Limbu et al., 2018) and zebrafish (Zhou et al., 2018a), both fed on medium carbohydrate diets (325 g/kg) with and without oxytetracycline. Interestingly, feeding *O. niloticus* on the HCO diet improved the innate immunity,

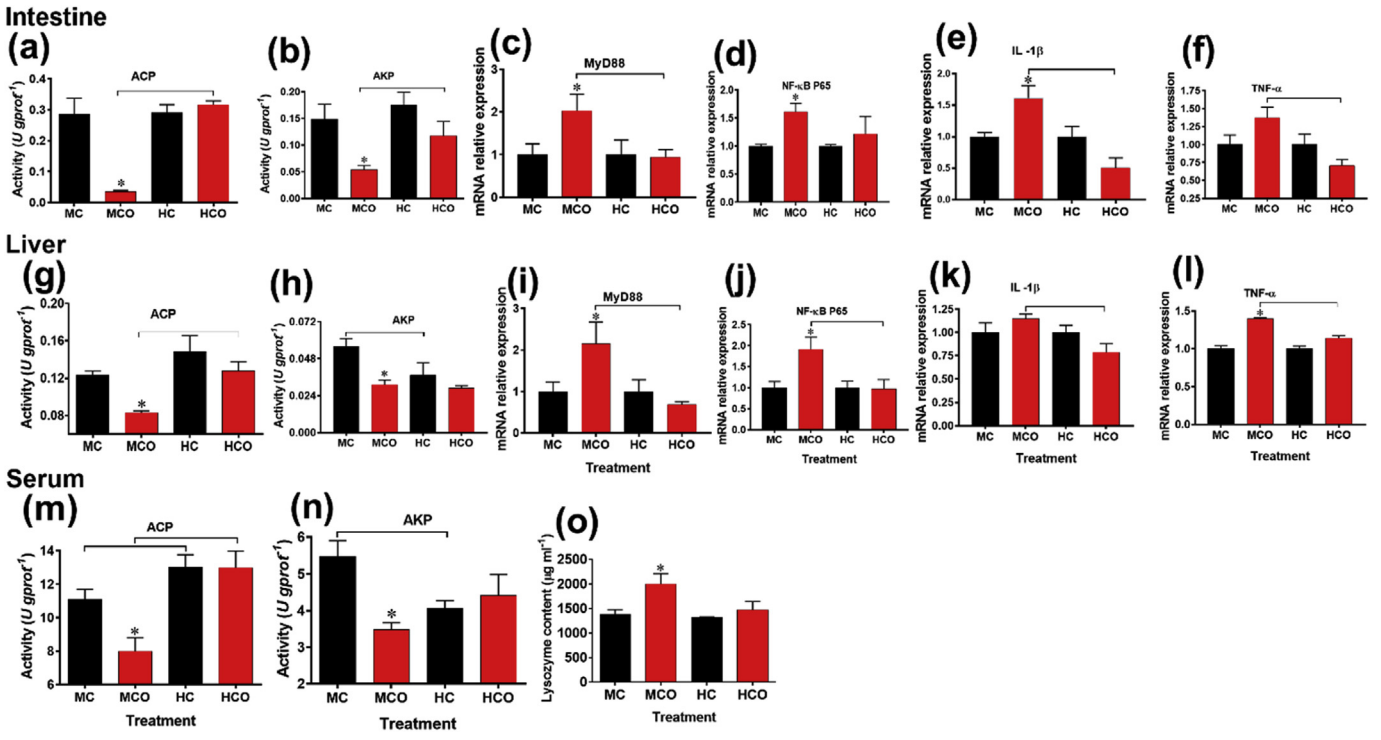


Fig. 6. The innate immunity and pro-inflammatory response of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) acid phosphatase (ACP) activity in intestine, (b) alkaline phosphatase (AKP) activity in the intestine, (c) the mRNA expression of myeloid differentiation primary response gene 88 (*MyD88*) in the intestine, (d) the mRNA expression of nuclear factor kappa-light-chain-enhancer of activated B cells protein 65 (*NF-κB P65*) in the intestine, (e) the mRNA expression of interleukin 1 beta (*IL-1β*) in the intestine, (f) the mRNA expression of tumor necrosis factor alpha (*TNF-α*) in intestine, (g) ACP activity in liver, (h) AKP activity in liver, (i) the mRNA expression of *MyD88* in the liver, (j) the mRNA expression of *NF-κB P65* in the intestine, (k) the mRNA expression of *IL-1β* in the liver, (l) the mRNA expression of *TNF-α* in liver, (m) ACP activity in the serum, (n) AKP activity in the serum and (o) the content of lysozyme (LZM) in the serum. Values are means ± SEM for three replicates (n = 6). The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets, while a line above the bars shows significant differences between MC vs HC and MCO vs HCO diets ($p < 0.05$).

indicating the ability of high carbohydrate diet to protect fish from oxytetracycline-compromised innate immunity effects in the three tissues. This may be due to reduced pathogenic bacteria and increased useful microbiota as indicated in the previous section. The *O. niloticus* fed on MCO diet increased adaptive immunity (*MyD88* and *NF-κB P65*) and LZM due to reduced innate immunity in order to protect the fish body. Increased LZM in *O. niloticus* fed on oxytetracycline diet has also been obtained in a previous study (Reda et al., 2013). Potentially, the MCO diet stimulated the *MyD88*, which is the canonical adaptor for inflammatory signaling pathways (Deguine and Barton, 2014) to elicit downstream signaling events, resulting in *NF-κB* activation and the induction pro-inflammatory cytokines (Bu et al., 2019). This resulted in the production of *IL-1β* and *TNF-α* as pro-inflammatory response. Interestingly, most of these parameters were reduced when *O. niloticus* were fed on HCO diet, indicating the ability of high carbohydrate diet to protect fish from oxytetracycline-induced side effects by increasing innate immunity and reducing inflammation response.

3.7. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by reducing cellular stress response and apoptosis

In the previous section we have shown that, oxytetracycline induced oxidative stress and lipid peroxidation in *O. niloticus*, were not attenuated by high carbohydrate diet. Therefore, we postulated that, the oxidative stress and lipid peroxidation might induce cellular stress and apoptosis in the *O. niloticus*. Accordingly, we measured the mRNA expression of genes related to cellular stress response and apoptosis. Expectedly, the MCO diet elevated the mRNA expression of heat shock protein 70 (*HSP70*; Fig. 7a) in the intestine, which is involved in cellular stress response. On the contrary, both the MCO and HCO diets decreased the mRNA expression of X-box binding protein 1 (*XPB1*; Fig. 7b) gene in the intestine, which is also responsible for cellular stress response compared to the MC and HC diets, respectively. However, both the MCO and HCO diets increased the mRNA relative expression of cysteine-aspartic acid protease 9 (*caspase-9*; Fig. 7c) in the intestine, which is an apoptotic gene. Interestingly, the HCO diet depressed the expression of *HSP70* (Fig. 7a) and *caspase-9* (Fig. 7b) genes in the intestine of *O. niloticus* compared to the MCO diet. Similar to the intestine, feeding *O. niloticus* on the MCO diet increased the expression of *XPB1* (Fig. 7e) gene in the liver and both the MCO and HCO diets increased the *caspase-9* (Fig. 7f) gene in the liver compared to the MC and HC diets, respectively. The HCO diet decreased the expression of *HSP70* (Fig. 7d) and *XBP1* (Fig. 7e) genes compared to the MCO diet in the liver, suggesting reduced cellular stress response.

Our data on increased mRNA expression for *HSP70* in *O. niloticus* fed on the MCO diet compared to the MC corroborate with previous results in Wuchang bream (Zhou et al., 2013) and our previous study on the same species (Limbu et al., 2018). In general, feeding *O. niloticus* with the MCO sustained the oxidative stress and lipid peroxidation, which increased cellular stress in intestine (Fig. 7a) and liver (Fig. 7d and e) and caused apoptosis in both tissues (Fig. 7c and f). However, when the fish were fed on the HCO diet, the cellular stress (Fig. 7a, d and e) in both tissues and apoptosis in the intestine (Fig. 7c) were attenuated. It is possible that, the sustained apoptosis in the liver was responsible for the lower survival rate of the fish obtained in the previous section. It is clear from these results that, feeding *O. niloticus* with high carbohydrate diet protects the fish from oxytetracycline-induced side effects by decreasing

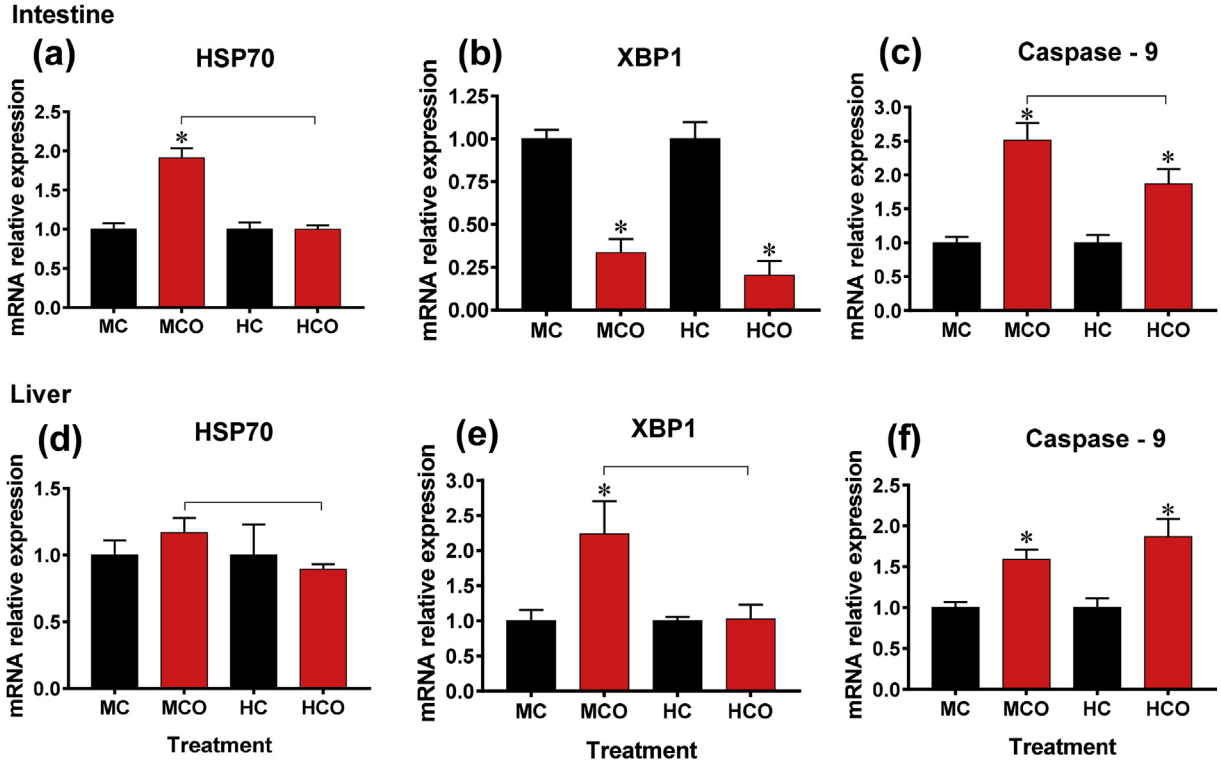


Fig. 7. The intestinal and hepatic mRNA relative expression of genes involved in cellular stress response and apoptosis of Nile tilapia (*Oreochromis niloticus*) fed on medium and high carbohydrate diets with or without oxytetracycline; (a) heat shock protein 70 (*HSP70*) in intestine, (b) X-box binding protein 1 (*XPB1*) in intestine, (c) cysteine-aspartic acid protease 9 (*caspase-9*) in intestine, (d) *HSP70* in liver, (e) *XPB1*) in liver, and (f) *caspase-9* in liver. Values are means ± SEM for three replicates (n = 6). The asterisk (*) indicates significant differences between medium carbohydrate (MC) and medium carbohydrate with oxytetracycline (MCO), high carbohydrate (HC) and high carbohydrate with oxytetracycline (HCO) diets, while a line above the bars shows significant differences between MC vs HC and MCO vs HCO diets ($p < 0.05$).

cellular stress response and apoptosis.

3.8. High carbohydrate diet protects O. niloticus from oxytetracycline-induced side effects by reducing the antibiotic in the muscles, starch and diet through binding

We have reported the ability of high carbohydrate diet to protect *O. niloticus* from oxytetracycline impaired liver function; increased energy demand; reduced innate immunity and increased pro-inflammation and hepatotoxicity; and increased cellular stress and apoptosis. However, the mechanism used by high carbohydrate in the protection are currently unknown. To explore the possible mechanism used by high carbohydrate in protecting *O. niloticus* from oxytetracycline-induced side effects, we measured the accumulation of the oxytetracycline in the *O. niloticus* muscles, the binding effects of starch and the mixture of the ingredients used to produce the antibiotic diets. Expectedly, the levels of oxytetracycline in muscles of *O. niloticus* fed on MCO and HCO diets were higher than fish fed on MC and HC diets (Fig. 8a), respectively. The *O. niloticus* fed the HCO diet had relatively lower oxytetracycline concentration than MCO diet, although not significant. The surface water in the MSO and HSO had lower concentrations of oxytetracycline than in the WO (Fig. 8b). Similarly, the surface water in the MCO and HCO diets had lower concentrations of oxytetracycline than in the WO (Fig. 8c).

These results confirmed our hypothesis that, binding effect of carbohydrate on oxytetracycline was responsible for the observed protection of the high carbohydrate diet on various measured parameters. Although carbohydrates especially, starch are known to possess binding effects (Arnesen and Krogdahl, 1993; Lu et al., 2018), no study in the literature has assessed their effect on antibiotics from a toxicological point of view. Binding effects have been described in several types of carbohydrate-derived materials (Lin et al., 2009; Peng et al., 2014; Yamaguchi et al., 2012), which contain starch-active enzymes (Christiansen et al., 2009) capable of binding different substances. Some of the carbohydrate-binding molecules inhibit pathogenic viruses fusion and entry by cross-linking membrane glycoproteins, which provide broad-spectrum antiviral activity of the innate immune system (Leikina et al., 2005), similar to results obtained on innate immunity in the intestine, liver and serum and intestinal microbiota in the present study.

Noticeably, in both cases, the HSO mixture (Fig. 8b) and HCO diet (Fig. 8c) had lower concentrations of oxytetracycline, suggesting that the binding affinities of starch to oxytetracycline depends on the amount of the starch included in the diets. It is possible that, the higher amount of starch increases the carbohydrate-carbohydrate interactions, consequently the binding affinity also increases, as previous reported (Ferrara et al., 2011). Evidently, the HSO mixture (Fig. 8d) and HCO diet (Fig. 8e) had higher binding efficiencies than the MSO mixture and MCO diet, respectively, confirming that, the binding effect carbohydrate on oxytetracycline increases as the amount of starch increases. Therefore, it is reasonable to suggest that, the binding effect of high carbohydrate diet protected *O. niloticus* from oxytetracycline-induced side effects on various measured parameters. We further noticed that, the MCO and HCO diets had higher binding efficiencies than the MSO and HSO starch mixtures. Indeed, the binding efficiency in the HCO diet was more than 90%, suggesting the bulk mixtures of different ingredients increase the binding efficiency compared to starch alone. Possibly, the binding efficiency of starch on oxytetracycline increases in the presence of mixed ingredients, which provide beneficial-binding interactions (Yamaguchi et al., 2012), an aspect, which requires further studies to verify. Since the feeds were prepared by mixing different ingredients, the binding efficiency increased, which provided protection on various measured parameters. These results suggest that, high carbohydrate diet protects *O. niloticus* from oxytetracycline-induced side effects by binding the antibiotic.

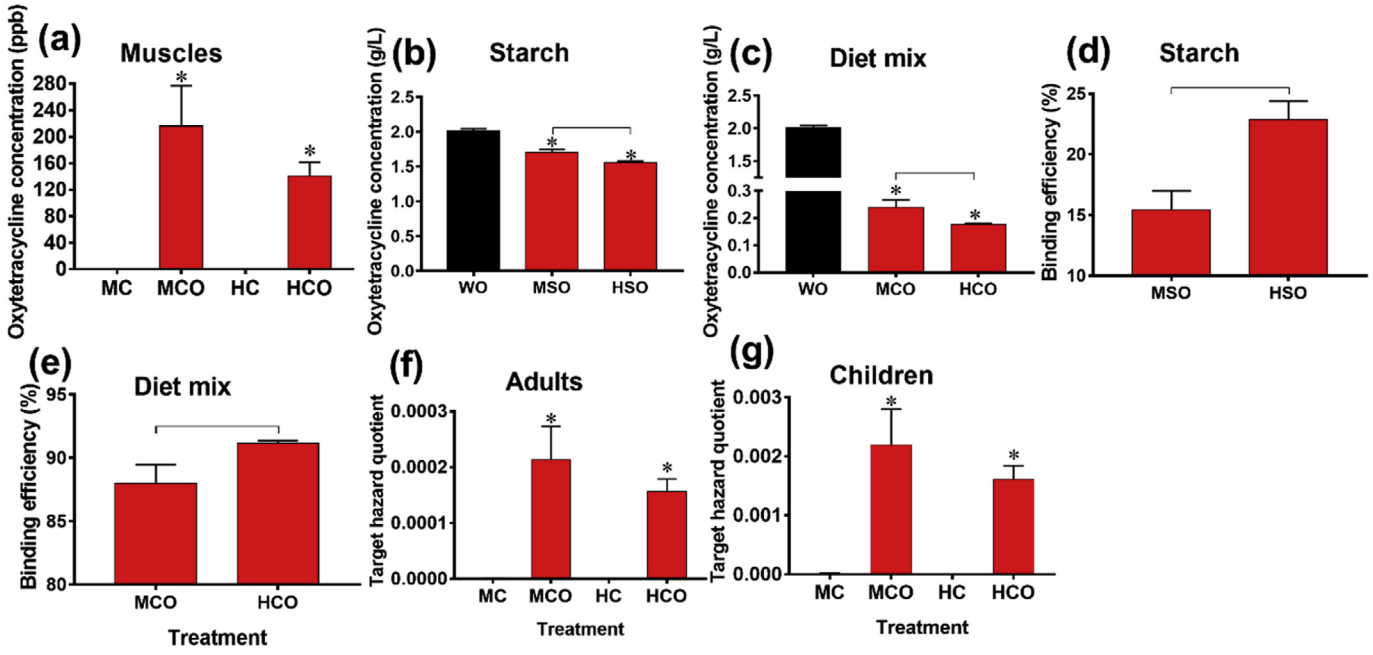


Fig. 8. The concentration of oxytetracycline (OTC) and binding efficiency of starch, medium and high carbohydrate diets; (a) OTC concentration in fish muscles, (b) OTC concentration in water with oxytetracycline (WO), medium starch with oxytetracycline (MSO) and high starch with oxytetracycline (HSO), (c) OTC concentration in water with oxytetracycline (WO), medium carbohydrate diet with oxytetracycline (MCO) and high carbohydrate diet with oxytetracycline (HCO) diet, (d) the OTC binding efficiency of MSO and HSO, (e) the OTC binding efficiency of MCO and HCO diets, (f) Target hazard quotient for adults, and (g) Target hazard quotient for children. Binding efficiency = [(Amount of OTC added − amount of OTC detected)/Amount of OTC added] x 100. Values are means ± SEM (n = 6). The asterisk (*) indicates significant differences between MC and MCO; HC and HCO; WO and MSO; WO and HSO; WO and MCO and WO and HCO, while a line above the bars shows significant differences between MSO vs HSO, and MCO vs HCO ($p < 0.05$).

Although the detailed mechanisms used by starch to bind antibiotics are not yet available, our study generated important scientific knowledge on the ability of high carbohydrate to protect fish from oxytetracycline-induced side effects. It is possible that, binding alleviated the side effects of oxytetracycline by; 1) reducing the bioavailability of oxytetracycline absorbed by fish intestine. The starch reduced the amount of oxytetracycline bioavailability in the intestine of fish for absorption by adsorbing them onto their surface. This reduced the interaction between cells and oxytetracycline, thus minimized the possibilities of causing toxicity; and (2) slowing down the absorption speed of oxytetracycline and transport to target organs. By binding the oxytetracycline, the starch slowed down its absorption speed and transport to target organs, which also alleviated the toxicity of oxytetracycline.

We then used the concentration of oxytetracycline in the muscle to assess the potential human health risk for adults and children after consuming *O. niloticus* fed on the experimental diets. The results showed that, the target hazard quotients for *O. niloticus* fed on MCO and HCO diets were higher than MC and HC diets, respectively, both for adults (Fig. 8f) and children (Fig. 8g). In both cases, the hazard quotient for *O. niloticus* fed on HCO diet was relatively lower than those fed on MCO diet. However, the target hazard quotients for both MCO and HCO diets in adults and children were <1 indicating lack of human health risk for consuming the *O. niloticus* treated with oxytetracycline.

In general, these results further highlight the potential benefit of HCO diet in reducing the side effects induced by oxytetracycline due to its lower hazard quotient and human health risk compared to MCO diet. The lack of human risk for consuming the *O. niloticus* treated with oxytetracycline in the present study is contrary to Limbu et al. (2018) who indicated human health risk in children after consuming the treated fish. More likely, variations in experimental period might explain the observed differences. Limbu et al. (2018) fed the *O. niloticus* with oxytetracyline for 12 weeks as opposed to 35 days in the present study, suggesting that the accumulation of antibiotics in the muscles and thus human health risk is time-dependent. Since antibiotics are abusively used by fish farmers on daily basis and in absence of diseases (Pham et al., 2015; Phu et al., 2015), the lack of risk obtained in our study should be interpreted based on the experimental conditions and duration of the study.

Despite the important scientific knowledge generated in our study, we wish to inform that, our results should be conceived in the context of the following limitations. First, although we used *O. niloticus* fingerlings, which might have different physiological and metabolic mechanisms to detoxify oxytetracycline than adult fish, our results still provide novel knowledge on the possibility of using dietary composition approach to reduce oxytetracycline-induced side effects in fish. Secondly, despite the fact that we analyzed the binding effect of carbohydrate in the starch and ingredients mixture *in vitro*, our results can be applied for *in vivo* studies. Therefore, to expand further our understanding on nutritional modulation of toxicity, future studies are required.

4. Conclusion

Our study for the first time indicates the ability of dietary composition to attenuate toxicological effects caused by antibiotics in fish. We show that, high carbohydrate diet partially protects fish from oxytetracycline-induced side effects. Apparently, it does not protect fish from oxytetracycline-reduced growth performance, survival rate, oxidative stress and lipid peroxidation. However, high carbohydrate diet protects fish from oxytetracycline-impaired liver function, increased energy expenditure, reduced innate immunity and increased pro-inflammation, hepatotoxicity, cellular stress and apoptosis by binding the antibiotic, which reduce bioavailability or slow down the absorption of antibiotic in the intestine. These findings open our understanding on the efficacy of high carbohydrate to attenuate the antibiotic-induced side effects and contribute to the overall knowledge on biological functions of dietary starch in the feed industry.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113508.

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