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Effect of Oxytetracycline-dosing on the Growth, Safety and Intestinal Histology of Nile Tilapia, *Oreochromis niloticus* (L.) Juveniles

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A B S T R A C T

The present study was carried out to evaluate the effect of oxytetracycline (OTC)-dosing at five different concentrations, viz., 0 mg (0X), 80 mg (1X), 240 mg (3X), 400 mg (5X) and 800 mg (10X) kg^{-1} biomass day^{-1} for 30 consecutive days on the growth, safety and intestinal histology of Nile tilapia, *Oreochromis niloticus* (L.) juveniles. The OTC-residues in the edible muscle were detected at scheduled intervals by LC-MS/MS. A dose-dependent decline in feed intake, biomass and survival were recorded in OTC-dosed fish. The OTC-residue levels were 0, 204.75±45.75, 318.00±0.00, 778.50±145.50 and 684.00±18.00 ng g^{-1} in 0X, 1X, 3X, 5X and 10X groups, respectively on day 30 OTC-dosing, which reduced subsequently. Relatively mild histopathological lesions including degeneration of epithelial layer, loss of absorptive vacuoles, necrotized intestinal villi, mucinous degeneration, and necrotized absorptive region were observed in the intestine of OTC-dosed fish. Lamina propria swelling was the characteristic change observed in the 10X group on day 15. The observed data revealed that OTC-dosing is reasonably safe at the therapeutic dose of 80 mg kg^{-1} biomass day^{-1}. However, the precise dose for safe usage of OTC is to be determined according to the culture conditions and species cultured.

Introduction

Tilapias (*Oreochromis* spp.) are the most widely grown intensively reared farmed fish with production ranging from extensive backyard ponds to large, commercial operations, world-wide (Jansen *et al.,* 2018). The global tilapia production was about 5.67 million mt in 2015 (FAO, 2017) and is anticipated to reach 7.3 million mt by 2030 (FAO, 2013). The overcrowded situation of fish farming augments the threat of spreading diseases, causing huge economic losses worldwide. As a result, the use of antibiotics and antimicrobial agents is a common practice. Oxytetracycline (OTC) is one of the most widely used antibiotics to treat systemic bacterial infections of fish (Rigos and Troisi, 2005; Jerbi *et al.,* 2011). The antibiotic-diets retain fish healthy and work as a safeguard for disease incidences (Islam *et al.,* 2014). Many studies have also shown the side effects of extended time antibiotic use on fish, which can cause nephrotoxicity and liver damage or malfunctions (Hentschel *et al.,* 2005). The use of antibiotics may also leave antibiotic-residues in fish tissue and products (Samanidou and Evaggelopoulou, 2007). Target animal safety data are a requisite part of the drug registration practice in most of the countries (EU, 2010; USFDA, 2017). The drugs legalized for aquaculture use are scanty in many countries and no such recommendations are available in most of the developing countries including India. The lack of information on the potential effects of OTC-medicated feed administration in fish limited its registration to control susceptible bacterial pathogens. India offers enormous potential for aquaculture progress. Expansive systematic data is mandatory to develop an effectual regulatory mechanism in a variety of aquatic species cultured in Indian farming systems. The regulatory bodies in India have not yet set withdrawal periods for OTC in Indian fish. Systematic reports on the biosafety, tissue-level changes in the vital organs, tissue residues in OTC fed fish, approval and withdrawal period under Indian conditions are, therefore, required. The present study was, thus, conducted to determine the biosafety of dietary supplementation of OTC on the growth, survival, residual depletion in edible tissue and histopathological changes in the intestine of *Oreochromis niloticus* when fed for 30 consecutive days (3X the therapeutic 10-day treatment duration) at the target doses of 0X (control), 1X, 3X, 5X and 10 X the therapeutic dose of 80 mg OTC kg^{-1} biomass day^{-1}.

Materials and Methods

Experimental design

Three sixty healthy Nile tilapia *Oreochromis niloticus* juveniles with an average length and weight of 10.39±0.67 cm and 15.40±0.48 g respectively were used. Fish were kept in FRP tanks of 500-L capacity containing 300-L aerated bore-well water for 15 days to acclimate before starting the experiment. Fish were allocated into polypropylene tanks (L58 × H45 × B45 cm) in six groups (20 fish $tank^{-1}$) in triplicate, viz., 0X group (control); 1X group (80 mg OTC kg^{-1} biomass day^{-1}); 3X group (240 mg OTC kg^{-1} biomass day^{-1}); 5X group (400 mg OTC kg^{-1} biomass day^{-1}) and 10X group (800 mg OTC kg^{-1} biomass day^{-1}).

The fish of all the groups were fed with commercial pellet feed (CP Pvt. Ltd., India) at 2% body weight (BW) thrice daily during the acclimatization period. The physico-chemical characteristics of the water were measured periodically (Boyd, 1979) to maintain the optimal level throughout the experiment. The left-out feed and faecal matter were siphoned out daily and 50% water exchanged in three days interval. During the acclimatization period, the fish showed no signs of lethargy, opercular flaring, abrupt swimming, etc.

Medicated feed preparation

The approved therapeutic dose of OTC is 2.50-3.75 g 100^{-1} pounds biomass day^{-1} (or 55-83 mg kg^{-1} biomass day^{-1}) for 10 consecutive days (USFWS, 2015). The OTC-feeds were prepared by top-coating the commercial feed with appropriate amounts of OTC in vegetable oil (5 ml kg^{-1} feed) to administer doses of 80, 240, 400 and 800 mg kg^{-1} biomass day^{-1}, respectively, when fed at 2% BW day^{-1}. Control feed was top-dressed with vegetable oil only. The feed batches were prepared in order of increasing OTC concentration. The feeds were mixed thoroughly, air-dried for 24 h at room temperature, and stored in airtight containers. The feeds were freshly prepared and used instantly.

Dose administration

Nile tilapia from all the experimental groups during the pre-dosing period (1-7 days) were fed with control feed. During the dosing period (8-37 days), the Nile tilapia from 1X, 3X, 5X, and 10X groups were fed with respective dosages of 80, 240, 400 and 800 mg kg^{-1} biomass day^{-1}. The control group (0X) was fed with the control feed. After 30 days of the dosing period, i.e., the post-OTC dosing period for 15 days (38-52 days), all the fish groups were fed with control feed. The unconsumed feed, if any, in each tank were removed daily, dried in air and weighed carefully.

Feeding and behavioural changes and growth performance

The feeding activities of the experimental Nile tilapia were noted daily and evaluated by qualitative (numerical) scores as proposed by Bowker *et al.*, (2013), based on the amount of feed consumed. The qualitative scoring ranged from 0 to 4, i.e., 0: no feed consumption, 1: 25% feed consumption 2: 50% feed consumption, 3: 75% feed consumption and 4: 100% feed consumption. Ten fish from each tank were individually weighed on day 0 (initial) and day 52 (final) to assess the growth performance. Behavioural changes in the experimental fish including their position in the water column, gasping for air, flashing, hyperactivity, lethargy, loss of equilibrium, abnormal pigmentation, discolouration, and any other abnormal behaviour or signs and gross lesions including the presence and severity of dermal lesions were observed daily during the experimental period.

Oxytetracycline-residue depletion

The edible fish tissue samples for OTC-residue analysis were collected on the day 0 pre-dosing, day 1 and 30 OTC-dosing and day 15 post-OTC dosing periods. All fish samples were dissected, beheaded, degutted, washed thoroughly and stored at -20 °C. The stored fish tissue samples were analyzed by LC-MS/MS at the ICAR-Central Institute of Fisheries Technology, Kochi as per the standard protocol developed by them.

Histopathology

The intestine of *O. niloticus* from all the groups collected on day 0, day 15 and day 30 OTC-dosing periods were fixed in Bouin's solution for 24 h. The fixed samples were processed by standard techniques and embedded in paraffin wax. Thin (5 µm) sections were prepared and stained with hematoxylin and eosin (Roberts, 2012).

Statistical analyses

The data were expressed as a mean±standard deviation. Feeding behaviour scores were analyzed by Kruskal–Wallis test and' with The feeding behaviour scores, survival and biomass data were analyzed by Kruskal-

Wallis test and one-way ANOVA with Tukey HSD post-hoc for the comparison of means using Statistical Package for Social Sciences (IBM-SPSS) Version: 22.0, considering a probability level of $P<0.05$ for the significance of the collected data.

Results and Discussion

General and feeding behaviour of oxytetracycline-dosed Nile tilapia

The feeding behaviour scores of *O. niloticus* fed with OTC-feeds are presented in Table 1. During the pre-dosing period, the feeding behaviour was normal and the entire feed rations were consumed (score: 4.00). Reduced feed intake was noticed in 1X - 10X groups during the OTC-dosing period, with a mean score in the range of 2.33±0.55 (10X) - 3.62±0.49 (1X; Table 1). During the post-OTC dosing period, an improved feed intake was noted in 1X - 10X groups, with scores ranging from 3.25±0.45 (10X) to 4.00±0.00 (1X). There existed significant differences between the control and 3X, 5X, 10X groups. The feed intake was reduced significantly in a dose-dependent manner from 1X to 10X groups (Table 1). In contrast, Gaikowski *et al.*, (2003a) documented that walleye (*Sander vitreus*) feed consumption appeared to be slightly reduced in the highest OTC-dose (413 mg kg^{-1} bodyweight day^{-1}) group, primarily during the third to fifth dosing days. The reduced feed intake could be due to the fact that OTC is a feeding deterrent that can make feed less palatable in the short term (Toften and Jobling, 1997). Likewise, a statistically significant decrease in feed consumption (62.5% and 55.3% of the feed offered) of tilapia administered with florfenicol (45 and 75 mg kg^{-1} fish) was observed only during the last 10 dosing days from day 10 to day 19 (Gaikowski *et al.*, 2013). Conversely, no dose-related effect on general fish behaviour or feeding behaviour was observed in juvenile sunshine bass Aquaflor-medicated feed at 0, 15, 45, and 75 mg FFC kg^{-1} bodyweight day^{-1} for 20 days (Straus *et al.*, 2012). Upon cessation of OTC-dosing, an improved feed intake was noted in 1X-10X groups, with scores increasing towards the maximum till day 52. Likewise, Atlantic salmon (*Salmo salar*) fed with OTC-medicated feed exhibited numeric reductions in voluntary feed intake for the first few weeks of a 9-week feeding trial and biomass (Toften and Jobling, 1997). The results of the present study supported several earlier works, which affirmed that the acceptability of medicated feeds decreased as drug dose increased (Poe and Wilson, 1989; Robinson *et al.*, 1990; Toften *et al.*, 1995).

The fish fed the highest concentration of OTC showed abnormal movement, darkening of the body colour, mucus associated gills, and some unusual feeding behaviour like spitting out the feed once the drug was sensed during the dosing period. Trushenski *et al.*, (2018) specified that the frequency of skin/body surface abnormalities was elevated among Nile tilapia fed the OTC-medicated feed (80 mg kg^{-1} bodyweight day^{-1}), so also in our study with the increase in OTC-dosing. The freshly dead fish were subjected to necropsy in the present study. Internally, pale kidney and liver, discolouration and liquefaction of internal organs were observed in fish fed the higher concentrations of OTC [3X, 5X, and 10X groups]. Few studies elucidated the side effects of long term antibiotic use in fish, which induce nephrotoxicity and liver damage (Horsberg and Berge, 1986; Hentschel *et al.*, 2005; Kori-Siakpere *et al.*, 2010). The present study also recorded significant changes in the liver and kidney tissues of OTC-dosed Nile tilapia upon dissection as well as histological observations (data not shown), thereby confirming nephrotoxicity and liver damage. Administration of higher OTC dosages greatly impair the liver regeneration and decrease the mitochondrial protein synthesis after causing a deficiency in cytochrome oxidase C and ATP

synthetase enzymes (Den Bogert *et al.*, 1983) and immune functions (Sanchez-Martinez *et al.,* 2008). These changes may make the fish more susceptible to diseases after antibiotic residual periods.

Survival and biomass in oxytetracycline-dosed fish

The results on the survival and biomass of OTC-dosed Nile tilapia during the initial and final periods are presented in Table 2. The control group recorded cent percent survival with highest growth rate, i.e., 1.6 times the initial weight. The 1X, 3X and 5X groups recorded 95.00, 93.35 and 88.35% survival, respectively on day 52. The 10X group (800 mg OTC kg^{-1} fish) recorded the least survival (86.65%) and growth rate, i.e., 1.45 times the initial weight. There existed significant differences in the survival of Nile tilapia among the groups ($P<0.05$). Similarly, Roy (2017) noted Nile tilapia survival percentage of 100, 100, 95, 93.35 in 10 days and 98.33, 95.00, 93.33, 91.64 in 20 days OTC-dosing trials in 1X (80 mg OTC kg^{-1} fish day^{-1}), 3X, 5X and 10X groups, respectively.

Likewise, 97% of survival was recorded in Nile tilapia fed with 80 mg OTC kg^{-1} fish day^{-1} for 10 days (Trushenski *et al.*, 2018). Also, survival in lake trout *Salvelinus namaycush* injected intraperitoneally (IP) with OTC at 275 mg kg^{-1} body weight was 93.40% and 80.20% on 2nd and 3rd week of injection, respectively (Marking *et al.,* 1988). Conversely, cent per cent survival was observed in Channel catfish, *Ictalurus punctatus* (Gaikowski *et al.*, 2003b) and juvenile sunshine bass, female white bass *Morone chrysops* × male striped bass *M. saxatilis* (Straus *et al.,* 2012) fed with aquaflor (Florfenicol, 50% Type A medicated article) at 50 and 75 mg kg^{-1} bodyweight day^{-1} for 20 days. The results of the present study on the decreased survival with the increase in OTC-dosing corroborate the observations of Hentschel *et al.*, (2005), who reported that any antibiotic/drug at a higher concentration than its permissible limit turns out to be toxic to the host organism and results in toxicity reaction.

The biomass (in g) of OTC-dosed Nile tilapia increased from 170 to 275 in control group, 170 to 265 in the 1X group, 160 to 255 in 3X group, 170 to 250 in 5X group, 175 to 255 in 10X group on the 52nd day of observation. The control group recorded the highest growth rate, about 1.6 times the initial weight. Addition of OTC at higher doses (5X and 10X) in feed resulted in poor feed consumption and reduced growth. Similarly, the mean initial and terminal weight of hybrid striped bass (striped bass *M. saxatilis* × white bass *M. chrysops*) increased from 123.80 to 144.40 g, 127.50 to 143.40 and 122.20 g to 129.80 g at terminal sampling when fed with OTC-medicated feed at a nominal daily dose of 0, 248 and 413 mg kg^{-1} bodyweight day^{-1} for 10 days, respectively (Gaikowski *et al.*, 2003a). Conversely, a significant increase of weight gain in *O. niloticus* (Reda *et al.*, 2013) and channel catfish, *Ictalurus punctatus* (Sanchez-Martinez *et al.,* 2008) was reported when fed OTC at a lower dose (100 mg kg^{-1} feed). In another study, OTC did not promote growth or efficiency at the therapeutic (80 mg kg^{-1} fish day^{-1}) and sub-therapeutic (16 mg kg^{-1} fish day^{-1}) doses in Nile tilapia (Trushenski *et al.*, 2018), which corroborate the findings of the present study in the 1X group. Further, many previous studies have shown the negative effects of antibiotics on the immune system of fish (Lunden and Bylund, 2000; Guardiola *et al.*, 2012), possibly by interfering with humoral innate immune parameters as well as cellular parameters. The present study noted increased biomass in the 1X and 3X groups compared to the 5X and 10X groups, but not on par with control. Similarly, Lawal *et al.* (2012) stated that the average final weight gain of fish was greater at the lower

dose (0.2 g OTC 100^{-1} g feed) than the higher dose (0.4 g OTC 100^{-1} g feed). It is likely that at higher concentrations, antibiotics may eliminate the beneficial bacteria in the gastrointestinal (GI) ecosystem (WHO, 2006), thus leading to a reduction in nutrient utilization in such animal and concurrently reduced growth. Long-term feeding of OTC reportedly impairs the feed digestibility (Toften and Jobling, 1997) and reduces the feed palatability in some finfish, which affect the growth performance indirectly (Trushenski *et al.*, 2018).

Water quality parameters

The results of the water quality parameters are shown in Table 3. The mean water temperature was in the range of 27.49±1.98 - 27.98±1.91 ºC. The dissolved oxygen levels were from 4.00±0.30 to 4.90±0.21 mg L^{-1}. The water pH levels were in the range of 7.80±0.21 - 8.90±0.28. The nitrate and nitrite levels of the experimental tank water were in the range of 0.14±0.27 - 0.83±0.23 mg L^{-1} and 0.14±0.28 - 0.84±0.26 mg L^{-1}, respectively. The ammonia levels ranged from 0.002±0.001 to 0.006±0.002 mg L^{-1}. The hardness of rearing water was in the range of 715.00±25.92 - 793.00±16.81 mg L^{-1}. The water quality parameters, except hardness, were maintained optimally well within the tolerable ranges required for the normal growth of fish (Boyd, 1979), thus excluded as stressful factors for the observed mortalities during the experimental period.

Table.1 Feeding behaviour of *Oreochromis niloticus* fed with oxytetracycline feeds at 0-10 times the approved dose of 80 mg kg^{-1} $biomass^{-1}$ day (1X) for 30 consecutive days

Period	Feeding behaviour. Scores are in range (Mean±SD)				
	0 mg (0X)	80 mg (1X)	240 mg (3X)	400 mg (5X)	800 mg (10X)
Pre-dosing (0-7 days)	4.00 (4.00±0.00)	4.00 (4.00±0.00)	4.00 (4.00±0.00)^{1}	4.00 (4.00±0.00)^{1}	4.00 (4.00±0.00)^{1}
OTC-dosing (8-37 days)	4.00 (4.00±0.00)^{a}	3.00 - 4.00 (3.62±0.49)^{ab}	3.00 - 4.00 (3.23±0.45)^{b2}	2.00 - 4.00 (2.60±0.56)^{c2}	2.00 - 4.00 (2.33±0.55)^{c2}
Post- OTC dosing (38-52 days)	4.00 (4.00±0.00)^{a}	4.00 (4.00±0.00)^{a}	3.00-4.00 (3.92±0.31)^{a1}	3.00-4.00 (3.40±0.51)^{b1}	3.00-4.00 (3.25±0.45)^{b1}

a-c: Values sharing uncommon alphabets within the row differed significantly (P<0.05).
1-3: Values sharing uncommon numerals within the column differed significantly (P<0.05).

Table.2 Survival (%) and biomass (g) of *Oreochromis niloticus* fed with oxytetracycline (OTC) feeds at 0-10 times the approved dose of 80 mg kg^{-1} biomass day^{-1} (1X) for 30 consecutive days

Concentration of OTC	Survival (%)		Biomass (g)	
	Initial	Final	Initial	Final
0 mg (0X)	100.00	100.00^{abcd}	170	275^{abcd}
80 mg (1X)	100.00	95.00^{aef}	170	265^{aefg}
240 mg (3X)	100.00	93.35^{bgh}	160	255^{be}
400 mg (5X)	100.00	88.35^{ceg}	170	250^{cf}
800 mg (10X)	100.00	86.65^{dfh}	175	255^{dg}

a-h: Values sharing common alphabets differ significantly (P<0.05).

Table.3 Physico-chemical characteristics of water from different experimental tanks

Parameter	**OTC ration: mg kg^{-1} biomass day^{-1}**				
	0 mg (0X)	80 mg (1X)	240 mg (3X)	400 mg (5X)	800 mg (10X)
	Range Mean±SD	Range Mean±SD	Range Mean±SD	Range Mean±SD	Range Mean±SD
Temperature (^{0} C)	23.50-32.00 (27.82±1.92)	23.50-32.00 (27.49±1.98)	23.50-32.00 (27.98±1.91)	23.50-32.00 (27.98±1.91)	23.00-32.00 (27.59±1.96)
Dissolved oxygen (mg L^{-1})	4.40-4.80 (4.54±0.17)	4.40-4.90 (4.64±0.21)	4.00-4.80 (4.46±0.32)	4.10-4.80 (4.48±0.26)	4.20-4.90 (4.56±0.30)
pH	7.90-8.90 (8.20±0.28)	7.80-8.60 (8.09±0.21)	7.90-8.90 (8.21±0.28)	7.90-8.90 (8.21±0.28)	7.90-8.60 (8.09±0.21)
Hardness (mg L^{-1})	725.00-784.00 (753.00±24.24)	718.00-793.00 (763.60±32.44)	721.00-787.00 (743.00±28.04)	715.00-774.00 (747.60±25.92)	721.00-783.00 (743.20±24.60)
Ammonia (mg L-1)	0.002-0.005 (0.003±0.001)	0.002-0.006 (0.004±0.001)	0.002-0.005 (0.003±0.001)	0.002-0.006 (0.003±0.001)	0.002-0.006 (0.003±0.001)
Nitrate (mg L^{-1})	0.14-0.75 (0.44±0.25)	0.17-0.65 (0.40±0.23)	0.14-0.75 (0.44±0.27)	0.34-0.75 (0.54±0.15)	0.25-0.83 (0.58±0.23)
Nitrite (mg L^{-1})	0.18-0.71 (0.43±0.21)	0.18-0.76 (0.46±0.25)	0.15-0.84 (0.42±0.26)	0.14-0.82 (0.40±0.28)	0.22-0.71 (0.51±0.20)

Table.4 Residues of oxytetracycline (OTC) in *Oreochromis niloticus* fed with OTC feeds at 0-10 times the approved dose of 80 mg kg^{-1} biomass day^{-1} (1X) for 30 consecutive days

Period	Residues of OTC in ng g^{-1}				
	0 mg (0X)	80 mg (1X)	240 mg (3X)	400 mg (5X)	800 mg (10X)
Pre-dosing (0-7 days)	0.00	0.00	0.00	0.00	0.00
OTC-dosing (8-37 days)	0.00	204.75±45.75	318.00±0.00	778.50±145.50	684.00±18.00
Post- OTC dosing (38-52 days)	0.00	80.19±12.37	124.71±48.31	110.20±20.76	108.30±17.19

Fig.1 Intestinal tissue of juvenile Nile tilapia fed with non-medicated feed showing epithelial layer (E), Absorptive vacuoles (AV) mucus-secreting or goblet cells (GC) and lamina propria (LP), X100 H&E staining

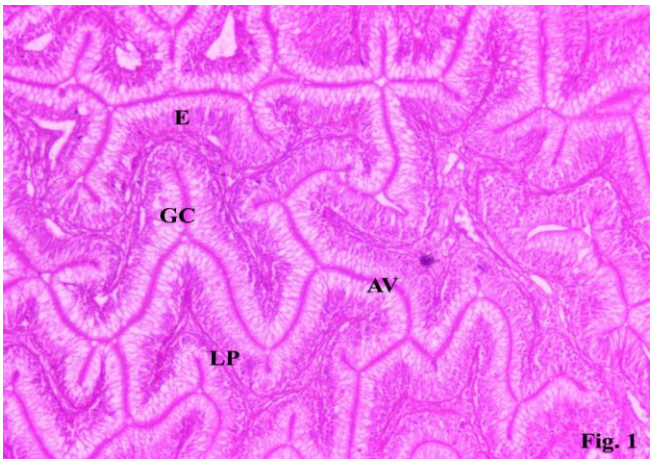


Fig.2 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 80 mg OTC kg^{-1} fish day^{-1} (1X group) for 30 consecutive days on day 15 OTC-dosing showing necrosis in the intestinal villi (NIV), mucinous degeneration (MD) and degenerated epithelial layer (DE), X200 H&E staining

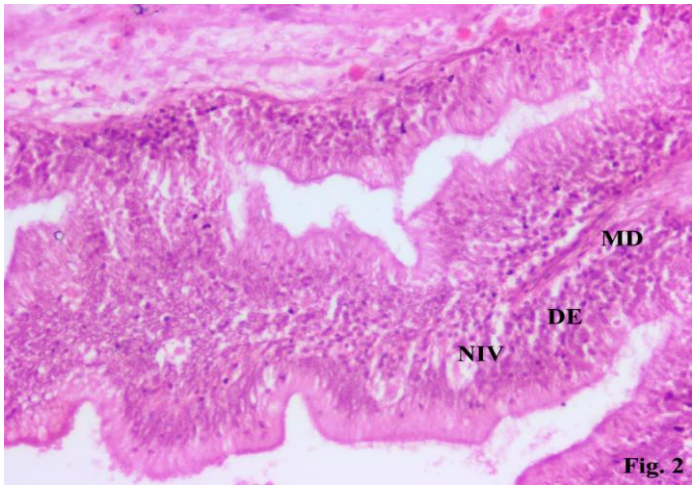


Fig.3 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 80 mg OTC kg^{-1} fish day^{-1} (1X group) for 30 consecutive days on day 30 OTC-dosing showing necrosis in the intestinal villi (NIV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X400 H&E staining

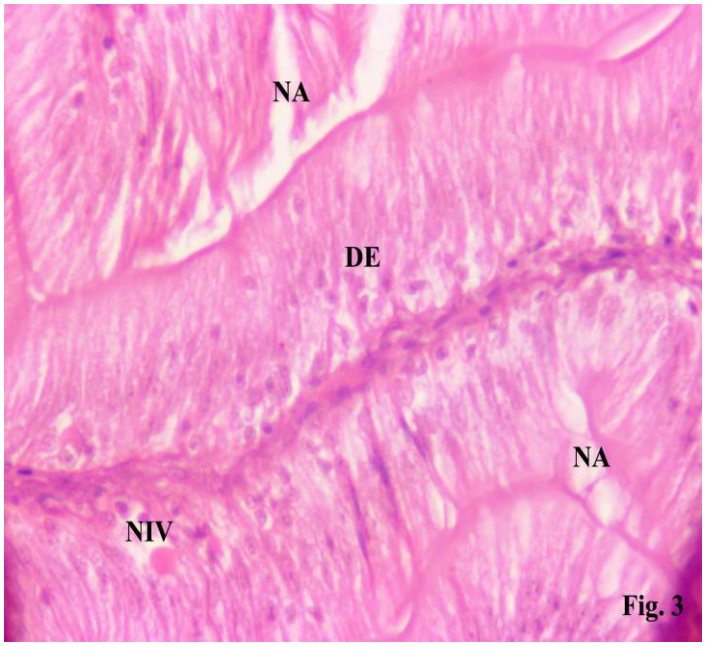


Fig.4 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 240 mg OTC kg^{-1} fish day^{-1} (3X group) for 30 consecutive days on day 15 OTC-dosing showing necrosis in the intestinal villi (NIV), mucinous degeneration (MD), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining

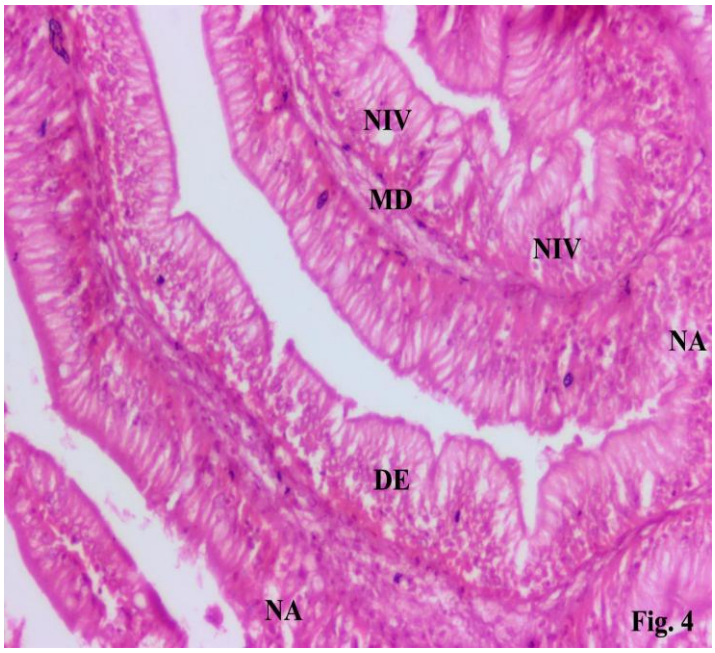


Fig.5 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 240 mg OTC kg^{-1} fish day^{-1} (3X group) for 30 consecutive days on day 30 OTC-dosing showing mucinous degeneration (MD), necrosis in the intestinal villi (NIV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining

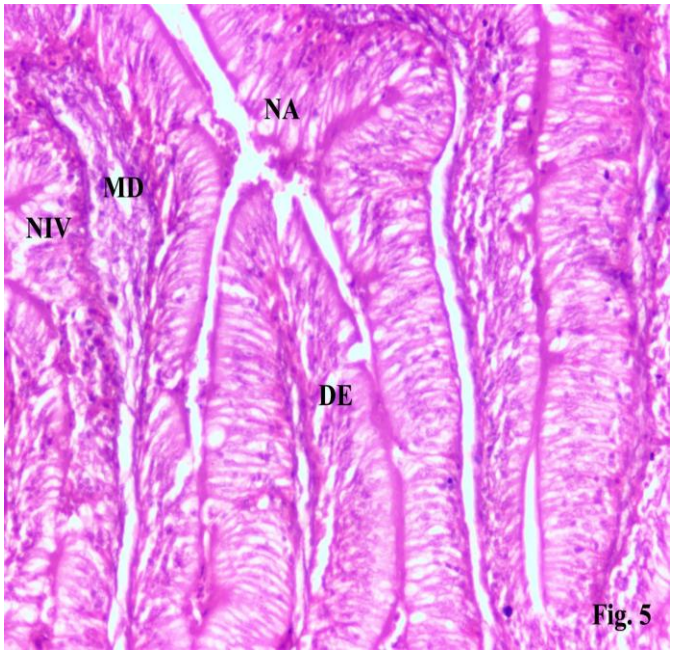


Fig.6 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 400 mg OTC kg^{-1} fish day^{-1} (5X group) for 30 consecutive days on day 15 OTC-dosing showing necrosis in the intestinal villi (NIV), mucinous degeneration (MD), loss of absorptive vacuoles (LAV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining

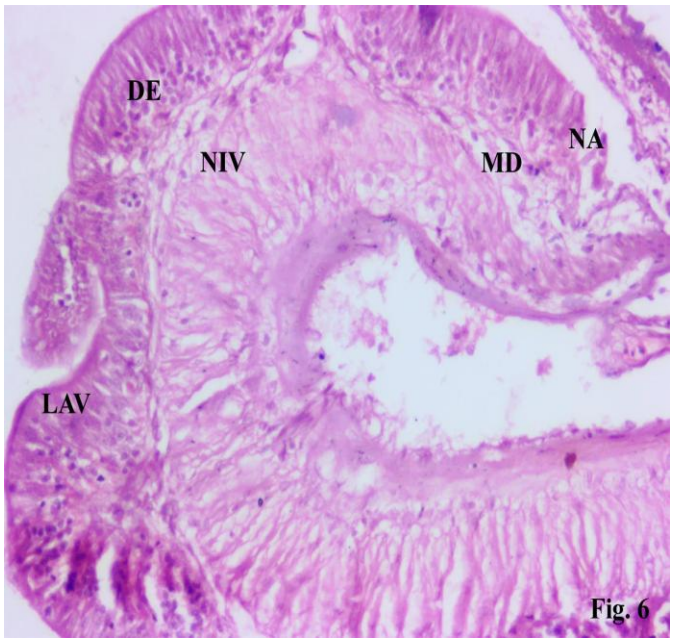


Fig.7 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 400 mg OTC kg^{-1} fish day^{-1} (5X group) for 30 consecutive days on day 30 OTC-dosing showing mucinous degeneration (MD), necrosis in the intestinal villi (NIV), loss of absorptive vacuoles (LAV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining

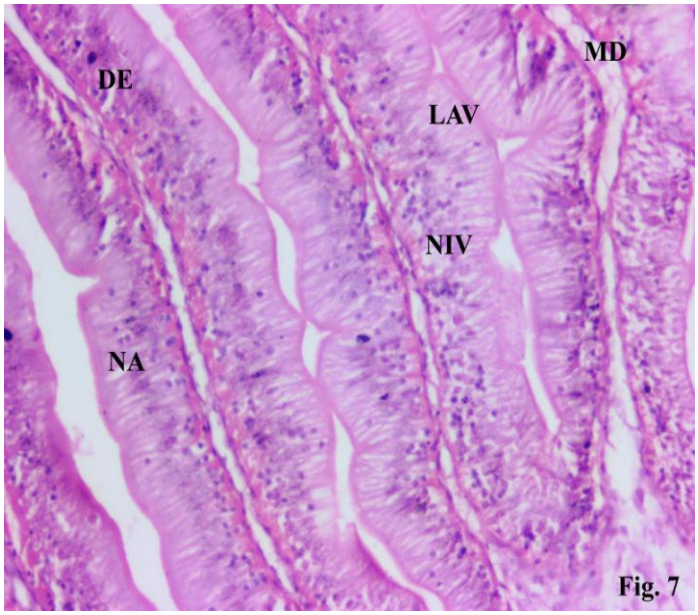


Fig.8 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 800 mg OTC kg^{-1} fish day^{-1} (10X group) for 30 consecutive days on day 15 OTC-dosing showing mucinous degeneration (MD), necrosis in the intestinal villi (NIV), swelling of lamina propria (SLP), loss of absorptive vacuoles (LAV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining

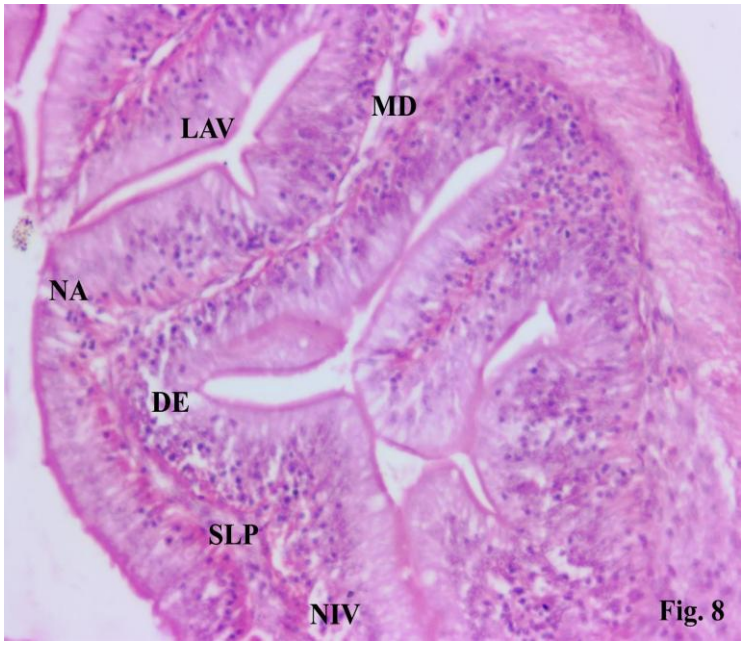
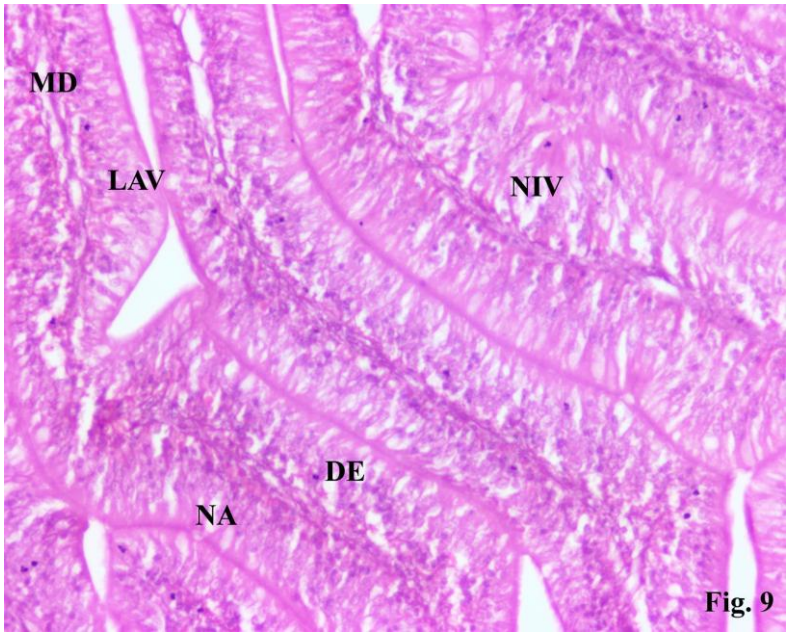


Fig.9 Histological changes in the intestine of *Oreochromis niloticus* juveniles fed 800 mg OTC kg^{-1} fish day^{-1} (10X group) for 30 consecutive days on day 30 OTC-dosing showing mucinous degeneration (MD), necrosis in the intestinal villi (NIV), loss of absorptive vacuoles (LAV), necrotised absorptive region (NA) and degenerated epithelial layer (DE), X200 H&E staining



Oxytetracycline-residue analysis

Drug-residues in the edible muscles of OTC-dosed Nile tilapia for 30 days as analyzed by LC-MS/MS are presented in Table 4. The whole fish devoid of head and entrails were taken for the analysis of OTC-residues from all the groups. No OTC residues were found in the edible muscles of control and all dosing groups during the pre-dosing period. The level of OTC-residues present were 0, 204.75±45.75, 318.00±0.00, 778.50±145.50 and 684.00± 18.00 ng g^{-1} in 0X, 1X, 3X, 5X and 10X groups, respectively on day 30 OTC-dosing. On day 15 post-OTC dosing, the OTC-residue levels were 0, 80.19±12.37, 124.71±48.31, 110.20±20.76 and 108.30±17.19 ng g^{-1} in 0X, 1X, 3X, 5X and 10X groups, respectively.

Oxytetracycline remains one of the most common antibiotic residues found in animal tissues (Granados-Chinchilla and Rodríguez, 2017) and is an easily degradable drug, but can stay in muscle as metabolites forms and in the sediment of culture environment for a certain period (Burridge *et al.*, 2010; Muhammad *et al.*, 2011). Controlled studies are, therefore, needed to make a conclusion about safe OTC-dose and dosage for treatment purpose. The present study recorded a dose-dependent residual increment on day 30 OTC-dosing. Yet, the observed residue levels on day 30 OTC-dosing were lower than the maximum residue limit (MRL) set by the United States Food and Drug Administration (USFDA), i.e., 2 µg g^{-1} (Chen *et al.*, 2005). Approved withdrawal periods for FDA approved drugs used in food animals are only legal for the specified species, dose, route, and dosage regimen (Riviere and Sundlof, 2001). The withdrawal period varies depending on the fish species and the experimental conditions (Wang *et al.*, 2004). Thus, data about the residue depletion can be useful in establishing either the optimal

dosages or the safe withdrawal periods in farmed fish (Zhang and Li, 2007). The number of dosages and frequency of application, application methods have impacts on efficiency and residual periods (Cabello *et al.,* 2013). Withdrawal periods of 5–20 days are recommended before slaughter, depending on the species and the nature of the food products (Blanchflower *et al.*, 1997). In our study, detectable levels of residues are present from 1X to 10X groups even after 15 days of cessation of OTC-dosing. The results indicated that the OTC-residue levels recorded in the 1X group (80.19±12.37 ng g^{-1}) are less than the MRL (100 ng g^{-1}) of the European Commission (EC) regulation (EU, 2010) within 15 days of cessation of OTC-dosing at the therapeutic dose (IX) at 23-32 °C. It is worth to mention here that the absorption and elimination period are greatly influenced by temperature (Bjorklund *et al.*, 1992). Likewise, Reda *et al.,* (2013) detected OTC and florfenicol-residues in the fish muscle at a level of 0.05 and 0.04 µg g^{-1} muscle, respectively after 15 days of feeding cessation. It was also noted that the serum residue of OTC in *Barbonymus gonionotus* (4 g kg^{-1} rate for 45 days) did not decline until 8 weeks (Islam *et al.,* 2015). Improper withdrawal periods may pose an increased risk of the possible transfer of drug residues and antibiotic-resistant bacterial infection to consumers. DePaola *et al.,* (1995) opined that oral administration of OTC to catfish by the recommended protocol and consumption after 21 days of OTC treatment do not appear to pose an increased risk of antibiotic-resistant bacterial infection to consumers. Our results corroborate the observations of Elia *et al.*, (2014), who recorded significantly lower OTC-residue levels in muscle 10 days after the 75 mg kg^{-1} antibiotic withdrawal than the MRL permitted by EEC regulation (EU, 2010). The official withdrawal period is 30 days, which is sufficiently conservative for human safety (EEC Regulation No. 2377/90).

Histopathology

The normal architecture of the intestine characterized by the presence of epithelial layer comprising mainly absorptive vacuoles and mucus-secreting or goblet cells and lamina propria were noted in the control group (Fig. 1). The histopathological alterations in the intestine of Nile tilapia subjected to 30 days of OTC-dosing are represented in Figs. 2-9. Exposure of freshwater fish to OTC drug may result in adaptive alterations, changes in tissue and cellular damages (Nunes *et al.,* 2015). The major histopathological changes found in the intestine were degeneration of epithelial layer, necrotised intestinal villi, mucinous degeneration and necrotised absorptive region (Figs. 2-5) in 1X and 3X groups, together with loss of absorptive vacuoles in 5X and 10X groups (Figs. 6-9) on day 30 OTC-dosing. The histological changes and impairments observed in the intestine tissues due to prolonged OTC-dosing subsisted in varying degrees in a dose-dependent manner. The histopathological results displayed the toxic consequences of different OTC-doses on the different layers of the intestine. These damages were the response of fish to the direct effect of higher doses of antibiotics for an extended period as was described earlier (Pachcco and Stantos, 2002). Epithelial layer degeneration was observed in all the OTC groups throughout the dosing and post-dosing period possibly due to the drug rejection by the intestinal lining of the exposed fish, which was more prominent at the higher doses. Swelling of lamina propria was the characteristic change observed in the intestine of the 10X group on day 15 (Fig. 8), which indicated the indigestibility factors associated with the higher concentration. All the noted histopathological changes in the intestine were of meagre to mild (≥ 5% affected tissue) and recorded throughout the experimental period.

The results of the present study demonstrated that feeding OTC-medicated diets at the higher doses (240, 400 and 800 mg kg^{-1} biomass day^{-1}) for an extended duration (30 days) may cause reduced feed intake, biomass and survival, and residue build-up in the muscle in a dose-dependent manner. Oxytetracycline-dosing in apparently healthy Nile tilapia induced a meagre to mild dose-dependent histological changes in the intestine. Though the OTC at the therapeutic dose caused 3.33% mortality, 9.5% reduced feed intake, 3.65% reduction in biomass on day 30 dosing, it is unlikely to produce such adverse effects on the tested parameters of Nile tilapia at the therapeutic dose (80 mg kg^{-1} biomass day^{-1}) and dosage (10 days). The results of the current study would offer a useful reference for the policymakers, investigators, and aquaculturists to evaluate the safety of OTC-dosing in cultured Nile tilapia.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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