Journal of Fisheries
Volume 9 Issue 3 December 2021
Peer Reviewed | Open Access | Online First

eISSN 2311-3111
pISSN 2311-729X

DOI: https://doi.org/10.17017/j.fish.361

Original Article

Efficacy of oral oxytetracycline therapy against *Aeromonas caviae* infection in Nile tilapia *Oreochromis niloticus* (L.) juveniles

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Manuscript history
Received 5 August 2021 | Revised 4 December 2021 | Accepted 5 December 2021 | Published online 26 December 2021

Citation
Roy A, Abraham TJ, Singha J, Julinta RB, Boda S (2021) Efficacy of oral oxytetracycline therapy against *Aeromonas caviae* infection in Nile tilapia *Oreochromis niloticus* (L.) juveniles. Journal of Fisheries 9(3): 93206. DOI: 10.17017/j.fish.361

Abstract
The present study evaluated the effects of *Aeromonas caviae* infection and oral oxytetracycline (OTC) therapy based on the histopathological alterations, serum creatinine, and antibiotic resistance in the gut-bacteria of *Oreochromis niloticus*. Ten days of OTC therapy at 2 g kg feed^{-1} at 3% body weight reduced the mortalities to 30% compared to 50% in the abrasion-immersion (AI) challenged and untreated group. The feed intake was reduced in challenged fish, which consumed about 42 mg OTC against the recommended minimum therapeutic dose of 55 mg kg biomass^{-1} day^{-1}. The OTC-resistant gut bacteria increased by 5.84 folds in 10 days of OTC feeding. The fish kidney exhibited glomerulopathy, nephropathy including nephritis, and lost its structural integrity within 5 days post-injection. An improved structural organization of glomerulus and renal tubules was noted with OTC therapy. Despite the improved kidney functions, as confirmed by the serum creatinine levels, the fish could not recuperate fully on day 21 post-OTC therapy. The initial high mortalities in *A. caviae* challenged fish suggested that the dose and feed ration used in this study was only partially effective. The findings of the present study may be useful while planning the mitigation strategies for disease management.

Keywords: Abrasion-immersion challenge; *Aeromonas caviae*; antibiotic-resistance; *Oreochromis niloticus*; oxytetracycline-therapy

1 | INTRODUCTION

The world aquaculture production of food fish increased from 32.4 million tonnes in 2000 to about 82.1 million tonnes in 2018 (FAO 2020). Worldwide, tilapia (*Oreochromis* spp.) are one of the intensively reared fishes, with production ranging from backyard ponds to large commercial operations (Jansen *et al.* 2019). It is the third most farmed fish worldwide. Production of Nile tilapia *Oreochromis niloticus* increased from 2.6577 million mt in 2010 to 4.5254 million mt, with a share of 8.3% of the total world aquaculture production in 2018. The Chinese tilapia industry is the largest in the world (FAO 2020). As Indian aquaculture is expanding, tilapia has become an important cultivable species (Menaga and Fitzsimmons 2017). Tilapias are found to be susceptible to various diseases due to the intensification of cultural practices. In tilapia production, bacterial infections are the most serious problem, which often causes mortalities as high as 80-90% (El-Sayed 2006; Pretto-Giordano *et al.* 2010; Towers 2016; Julinta *et al.* 2017a, 2017b). Streptococcal

infections have been reported from a large number of Asian countries, with significant resultant mortalities and losses valued at USD 480 million (Towers 2016).

Motile *Aeromonas* spp. can cause significant mortalities in cultured freshwater finfish when the water quality becomes unfavourable (Austin and Austin 2012). Several drugs and chemicals are used to combat infectious diseases (Romero *et al.* 2012; Lee *et al.* 2020; Love *et al.* 2020). The United States Food and Drug Administration (USFDA) approved the antibiotics like Terramycin® (oxytetracycline (OTC)) and Romet-30® as oral antibacterials for the treatment of specific bacterial diseases in temperate and warm water finfish. The use of medicated feed for temperate food fish is, however, regulated by the FDA in terms of their effectiveness and safety levels (Bondad-Reantaso *et al.* 2012; USFWS 2015; Love *et al.* 2020). Such studies on farmed tilapia in a tropical climate are scarce. In earlier studies, we evaluated the usefulness of OTC in *A. hydrophila* infected *Oreochromis niloticus* (Julinta *et al.* 2017a, 2017b), wound healing in *A. caviae* infected *O. niloticus* (Roy *et al.* 2019), and on the serum biomarkers of *O. niloticus* (Julinta *et al.* 2019). This study evaluated the effect of experimental *A. caviae* infection by abrasion and immersion challenge and oral OTC therapy in *O. niloticus* juveniles in terms of mortality, histopathology, and kidney functioning, and development of OTC-resistant gut bacteria.

2 | METHODOLOGY

2.1 Bacterial strain

An α-haemolytic strain of *Aeromonas caviae* CBT_1K_2 (NCBI GenBank Accession number MH581386), isolated from septicemic Nile tilapia *O. niloticus* (Roy *et al.* 2019), was used in the challenge experiment. The pure culture of *A. caviae* CBT_1K_2 was revived from the glycerol stock and maintained on tryptic soy agar (TSA) slant. *Aeromonas hydrophila* ATCC 49410 was used as a control strain.

2.2 Antibiogram, and determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of oxytetracycline against *Aeromonas caviae*

The sensitivity of *A. caviae* CBT_1K_2 was screened against 10 antibiotic-impregnated discs (HiMedia, India) namely chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), co-trimoxazole (25 µg), sulphafurazole (300 µg), gatifloxacin (5 µg), amoxyclav (30 µg) and erythromycin (15 µg), by agar disc diffusion assay (CLSI 2006a) on Mueller Hinton agar. The broth dilution method was used for the determination of MIC and MBC of OTC in Mueller Hinton broth (CLSI 2006b).

2.3 Feed topdressing and oral oxytetracycline-therapy

The therapeutic dose of OTC is 55–83 mg kg biomass^{-1} day^{-1} for 10 consecutive days for warm water fish (USFWS 2015). The oxytetracycline dihydrate (OTC; HiMedia, India) medicated feed to feed the fish at 3% body weight (BW) was prepared following Roy *et al.* (2019). In brief, OTC feed was prepared by mixing 2 g OTC powder with 5 mL vegetable oil and then admixing with 1 kg commercial floating pellet feed. Similarly, the control feed without OTC was prepared by mixing 5 mL of vegetable oil with 1 kg pellet feed. After thorough mixing and 24-h air-drying, the feeds were stored in airtight plastic containers.

2.4 Evaluation of the efficacy of oral oxytetracycline-therapy

Abrasion-immersion (AI) challenge: The experimental fish (mean ± standard deviation [SD]: 10.89 ± 0.08 g and 8.71 ± 0.51 cm) were introduced into the tanks at 40 numbers tank^{-1} and grouped as the negative control (group 1), positive control (group 2), 0 g OTC kg feed^{-1} (group 3) and 2 g OTC kg feed^{-1} (group 4), in triplicate. After 7 days of acclimatization, the fish of groups 2, 3, and 4 were abraded gently with a scalpel to remove the scales from the caudal peduncle to the pectoral fin. The abraded fish of groups 3 and 4 were immersion challenged with *A. caviae* at a dose of 1.20×10^7 CFU mL^{-1} for 1 h as described in Roy *et al.* (2019) and transferred to their respective tanks. Similarly, the abraded fish of group 2 were immersed in sterile saline for an hour. Group 1 (non-abraded and unchallenged) and group 2 (abraded and saline immersed) served as negative and positive controls, respectively.

Oral oxytetracycline-therapy: On day 8 (i.e., the day of the AI challenge), all fish groups were starved. The groups 1-3 were then fed with control feed at 3% BW twice daily throughout the experiment. To get an effectual therapeutic dose of 60 mg kg biomass^{-1} day^{-1}, group 4 (AI challenged) were fed with OTC-feed at 3% BW for 10 consecutive days (day 9 – 18). Recording of mortality, external signs of infections, and behavioural changes was done daily. Within 2 hours of each feeding, the unconsumed feeds, if any, in each tank were removed carefully and dried overnight. The amount of feed consumed was calculated daily and assigned the numerical score for feeding behaviour ranging from 0 to 4 as per the descriptions of Bowker *et al.* (2015).

2.5 Serum creatinine

Before blood collection, three fish from each group were anaesthetized with clove oil at 25 µL L^{-1} water. The serum was collected on day 0, 1, and 10 OTC therapy, and day 21 post-OTC therapy. The serum creatinine was determined using a creatinine test kit (Span Diagnostics Ltd., India) based on modified Jaffe's reaction initial rate assay in a Photometer (Julinta *et al.* 2019).

2.6 Enumeration of oxytetracycline-resistant gut-bacteria

Before dissection, the fish were euthanized by increasing

the clove oil dose to 50 µL L^{-1} water. The fish were carefully cut and exposed the internal organs. At first, a portion of the gut from the middle region of the intestine (≈0.5 g) was dissected out aseptically, incised longitudinally, and transferred into a sterile tube containing 10 mL physiological saline. The gut-bacterial flora of AI-challenged and OTC-fed *O. niloticus* were enumerated as described in Miranda and Zemelman (2002) with slight modification. The gut tissues from three fish for each group were pooled, macerated along the inner side of the tubes with a sterile glass rod, and vortexed for 5 min. Aliquots (0.1 mL) of the serially diluted samples from unchallenged fish on day 0, challenged fish on day 1 and day 10 OTC-therapy, and day 21 post-OTC therapy were spread plated onto TSA and TSA supplemented with OTC (30 µg mL^{-1} medium) to enumerate the total viable counts (TVC) and antibiotic-resistant bacterial counts (ARBC) respectively. The samples from the control, as well as the challenged and untreated fish, were also processed similarly on each sampling day. The viable colonies on all plates were counted after 2 – 5 days of incubation at 30 ± 2 °C.

2.7 Histopathology

After the bacteriological sampling, the kidneys were removed from the normal and *A. caviae* challenged *O. niloticus* of OTC treated as well as untreated groups and immersed in Bouin's solution for 24 h for fixation. The kidney samples were dehydrated in ethanol of ascending concentrations, embedded in paraffin wax, sectioned at 5-µm thickness, processed, and stained with haematoxylin and eosin (Roberts 2012). Besides, the freshly dead fish during the treatment period were also processed as above.

2.8 Statistical analyses

The data are expressed as the mean ± SD. The Statistical Package for Social Sciences (IBM-SPSS, version 22.0) was used for the data analyses, considering a probability level of $P < 0.05$. The feeding behaviour scores were analysed by non-parametric Kruskal Wallis test with pair-wise comparisons. The differences in fish mortalities and serum creatinine levels among the treatment groups and/or days were tested by repeated-measures ANOVA with Greenhouse-Geisser correction and Bonferroni correction for pair-wise comparison. The differences in TVCs, ARBCs, and proportion of ARBCs in the TVCs among the treatment groups and/or days were explored by one-way ANOVA and post-hoc Duncan multiple range test (DMRT).

3 | RESULTS

3.1 Antibiotic sensitivity and determination of MIC and MBC of oxytetracycline

Aeromonas caviae CBT_1K_2 was sensitive to the majority of the tested antibiotics except to amoxyclav and erythromycin. The MIC and MBC of OTC were 0.78 µg mL^{-1} and 1.56 µg mL^{-1} respectively. The respective MIC and MBC of OTC for the control strain *A. hydrophila* ATCC 49410 were 0.39 µg mL^{-1} and 1.56 µg mL^{-1}.

3.2 Evaluation of the efficacy of oral oxytetracycline-therapy

The AI-challenged *O. niloticus* juveniles were lethargic, hanging and/or positioned at the tank bottom, and displayed erratic movement initially. On 1-day post-abrasion (dpa), the fish had peeled skin with the haemorrhagic lesion, swelled and softened tissue, and loss of scales at the site of abrasion, pale gills, tail rot, and darkened body colour. As shown in Figure 1a, no mortalities were noticed in fish on 2 dpa. Mortalities increased with time, reaching a level of 30% in OTC treated and 40% in untreated groups on day 10. The treated group recorded no mortalities upon cessation of OTC therapy. A significant increase in mortalities ($P < 0.05$) was noted in challenged and untreated *O. niloticus*. The differences in the mortalities between the treated and untreated fish on day 10 OTC therapy as well as on day 21 post-OTC therapy were significant ($P < 0.05$). As seen in Table 1, the feeding behaviour was normal during the pre-treatment period. The feed intake was low during the disease progression and treatment period, with the mean score of 2.78 ± 0.42 in treated ($P = 0.05$) and 2.85 ± 0.36 in untreated ($P = 0.06$) fish. The feed intake became almost normal during the post-treatment period in all the experimental groups (score: 3.97).

TABLE 1 Effect of oral oxytetracycline (OTC)-therapy on the feeding behaviour of abraded and immersion challenged *Oreochromis niloticus* with *Aeromonas caviae**

Period	Feeding behaviour scores (Range and Mean ± SD)		
	Positive control	0 g OTC kg $feed^{-1}$	2 g OTC kg $feed^{-1\#}$
Pre-treatment	4.00 (4.00±0.00)[a]	4.00 (4.00±0.00)[a]	4.00 (4.00±0.00)[a]
Disease progression and treatment	3.00-4.00 (3.15±0.19)[b]	2.00-3.00 (2.85±0.36)[b]	2.00-3.00 (2.78±0.42)[b]
Post-treatment	4.00 (4.00±0.00)[a]	3.00-4.00 (3.97±0.03)[a]	3.00-4.00 (3.97±0.03)[a]

*, Fish were abraded and immersed in *Aeromonas caviae* suspension (1.20×10^7 CFU mL^{-1}); #, fish were fed with OTC feed (2 g OTC kg $feed^{-1}$) at 3% BW twice daily for 10 days. Positive control: Abraded, non-challenged, and fed control feed. Feeding behaviour score: 0, no feed consumed; 1, 25% feed consumed; 2, 50% feed consumed; 3, 75% feed consumed; 4, 100% feed consumed. The feeding behaviour score for the negative control, i.e., unchallenged and control fed feed group was 4.00 ± 0.00 throughout the experimental period. [a – b], values sharing uncommon alphabetical superscript differed significantly at $P < 0.05$.

3.3 Serum creatinine

The serum creatinine level of group 2 (abraded and saline immersed) was in the range of 0.88 ± 0.02 – 0.99 ± 0.03 mg dL^{-1}. A significant rise in creatinine was observed on 1 dpa (1.91 ± 0.05 – 1.94 ± 0.06 mg dL^{-1}) compared to control ($P < 0.05$). On 10 dpa, the creatinine levels increased further both in untreated (2.71 ± 0.05 mg dL^{-1}) and OTC-treated (2.12 ± 0.06 mg dL^{-1}) fish. On day 21 post-OTC-therapy, its level reduced significantly to 1.58 ± 0.04 mg dL^{-1} ($P < 0.05$). While in the untreated group, it was significantly higher (2.28 ± 0.06 mg dL^{-1}) than in the OTC-treated group ($P < 0.05$). The creatinine levels in all the experimental groups differed significantly on day 10 OTC therapy as well as on day 21 post-OTC therapy. Also, on day 21 post-OTC therapy, the creatinine levels of the challenged groups were significantly higher ($P < 0.05$) than on day 0 (Figure 1b).

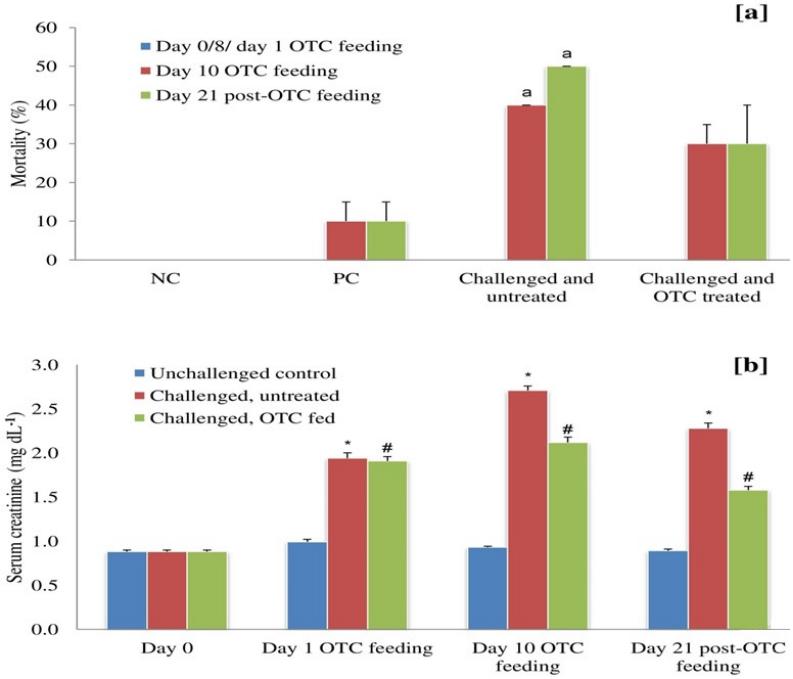

FIGURE 1 [a] Mortalities and **[b]** serum creatinine levels in *Oreochromis niloticus* challenged with *Aeromonas caviae* by abrasion-immersion and subsequently fed oxytetracycline (2 g $kg\ feed^{-1}$) for consecutive 10 days. NC, negative control (non-abraded); PC, positive control (abraded); day 1 – 7, pre-treatment period; day 8, bacterial challenge by abrasion-immersion; no feeding day; day 9 – 18, treatment period (oral OTC-therapy); day 19 – 39, post-treatment period. No mortalities were observed on day 0, day 8, and day 1 OTC feeding. Significant differences existed in mortalities with days among the treatment groups of *A. caviae* challenged *O. niloticus* ($P < 0.05$); a, bars sharing a common alphabet within a particular group differed significantly ($P < 0.05$). Bars with an asterisk (*) and hash (#) differed significantly ($P < 0.05$) from the respective control (day 0). Serum creatinine levels differed significantly among the treatment groups and days ($P < 0.05$).

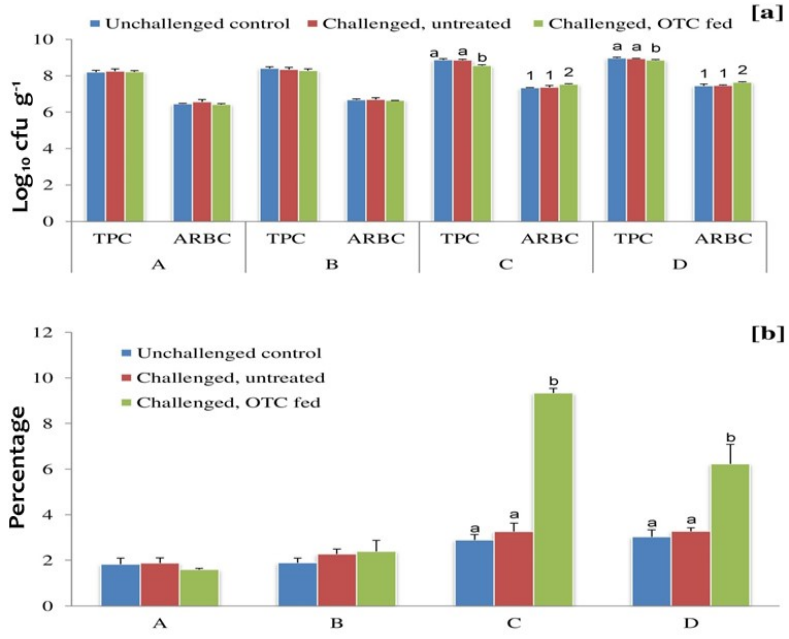

FIGURE 2 [a] Total viable counts (TVC) and antibiotic-resistant bacterial counts (ARBC), and **[b]** the proportion of antibiotic-resistant bacteria in the gut of *Oreochromis niloticus* challenged with *Aeromonas caviae* by abrasion-immersion and fed subsequently oxytetracycline (2 g $kg\ feed^{-1}$) for consecutive 10 days. A, unchallenged fish on day 0; B, challenged fish on day 1 OTC feeding; C, challenged fish on day 10 OTC feeding; D, challenged fish on day 21 post-OTC feeding. Counts of bacteria (TVC and ARBC) increased significantly with days ($P < 0.05$); a – b, bars sharing uncommon alphabets within a particular group (C or D) differed significantly ($P < 0.05$); 1 – 2, bars sharing uncommon numerical within a particular group (C or D) differed significantly ($p < 0.05$). The proportion of antibiotic-resistant bacteria in the TVC among the treatment groups increased significantly with days ($P < 0.05$).

3.4 Oxytetracycline-resistant gut-bacteria in abrasion-immersion challenged fish

The unchallenged fish recorded a significant increase in TVCs ($P < 0.05$) from 8.20 to 8.97 log_{10} CFU g^{-1} gut with days of feeding. The ARBCs were initially in the range of 6.42 – 6.57 log_{10} CFU g^{-1}, which rose significantly ($P < 0.05$) to 7.34 – 7.53 log_{10} CFU g^{-1} on day 10 OTC-therapy and then to 7.45 – 7.65 log_{10} CFU g^{-1} on day 21 post-OTC

therapy (Figure 2a). In the control group, the ARBCs were 1.82 ± 0.27% of the TVCs, which rose to 3.03 ± 0.30% on the last day of the experiment (Figure 2b). A similar trend in the TVCs (8.26 – 8.94 $\log_{10}$ CFU g^{-1}), ARBCs (6.57 – 7.46 $\log_{10}$ CFU g^{-1}), and the proportion of OTC-resistant bacteria (1.87 ± 0.24 – 3.27 ± 0.16%) in challenged and untreated fish were noticed. In challenged and OTC-treated fish, the recorded TVCs and ARBCs during the experimental period were 8.22 – 8.86 $\log_{10}$ CFU g^{-1} and 6.42 – 7.65 $\log_{10}$ CFU g^{-1} respectively (Figure 2a). The proportion of OTC-resistant bacteria in the TVCs was enhanced significantly from 1.60 ± 0.05% to 9.34 ± 0.21% on day 10 OTC therapy ($P < 0.05$). The OTC-resistant gut-bacterial populations were, however, reduced ($P < 0.05$) to 6.23 ± 0.85% of the TVCs on day 21 post-OTC therapy (Figure 2b).

3.5 Histopathology

Histologically, the kidney tissue of normal *O. niloticus* showed the typical structural organization of renal tubules, glomerulus, and haematopoietic tissue (Figure 3A). The changes observed in *O. niloticus* on day 1 were severe glomerulopathy with dilated Bowman's space and extensive nephropathy with the loss of tubular epithelial cells, widen lumen, inflamed and obliterated renal tubules, and necrosis (Figure 3B). The changes observed in the untreated *O. niloticus* on day 5 were extensively necrotised haematopoietic tissue and melanised area (Figure 3C). The alterations observed in treated juveniles on day 3 OTC therapy were glomerulopathy with dilated Bowman's space, necrotised haematopoietic area, and hypoplastic haematopoietic tissue (Figure 3D). The fish on day 6 OTC therapy had a diminution in glomerulopathy and Bowman's space dilation and improved structural organization of the renal tubules. They also exhibited renal tubules with a constricted lumen and melanised area (Figure 3E).

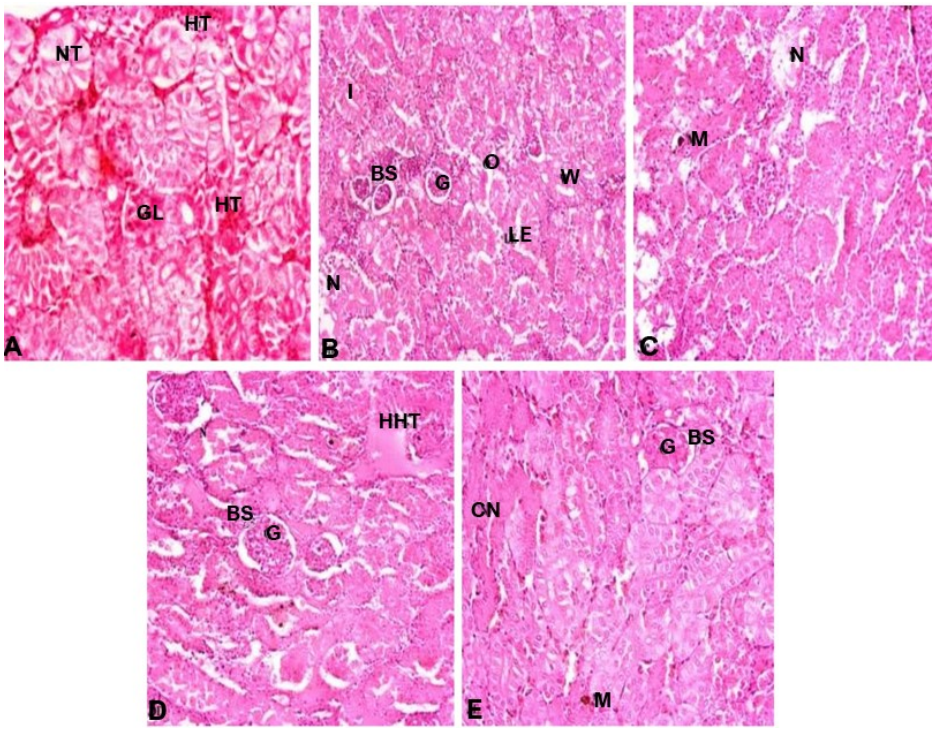

FIGURE 3 [A] Photomicrography of the kidney tissues of healthy *Oreochromis niloticus* showing the typical structural organization of renal tubules (NT), glomerulus (GL) and haematopoietic tissue (HT) X200; **[B]** the kidney tissues of fish challenged with *Aeromonas caviae* CBT_1K_2 on day 1 showing glomerulopathy (G) with dilated Bowman's space (BS) and nephropathy with the loss of tubular epithelial cells (LE), widen lumen (W), inflamed (I) and obliterated renal tubules (O) and necrosis (N), X100; **[C]** the kidney tissues of challenged and untreated fish on day 5 showing extensively necrotised haematopoietic tissue (N) and melanised area (M), X200; **[D]** the kidney tissues of challenged fish on day 3 OTC-therapy (2 g kg $feed^{-1}$) showing glomerulopathy (G) with dilated Bowman's space (BS), necrosis (N) and hypoplastic haematopoietic tissue (HHT) X200; **[E]** the kidney tissues of challenged fish on day 6 OTC-therapy (2 g kg $feed^{-1}$) showing reduction in glomerulopathy (G) and Bowman's space dilation (BS), renal tubule with constricted lumen (CN), melanised area (M) and improved structural organization of renal tubules X200; H&E staining.

4 | DISCUSSION

An understanding of the antibiotic susceptibility of bacterial pathogens based on in-vitro tests may help to make treatment decisions on disease control. The test strain *Aeromonas caviae* was resistant to amoxyclav and erythromycin and sensitive to broad-spectrum antibiotics including OTC, which contradict other earlier observations (Schmidt *et al.* 2000; Hatha *et al.* 2005; Dias *et al.* 2012).

Resistance to ciprofloxacin and gentamycin was also noted among the fish-borne *A. caviae* strains from Egypt and Malaysia (Afizi *et al.* 2013). *Aeromonas* spp. of Indian freshwater fish were reportedly least resistant to chloramphenicol, gentamycin, and nalidixic acid (Hatha *et al.* 2005). The MIC and MBC of OTC against *A. caviae* CBT_1K_2 were 0.78 µg mL^{-1} and 1.56 µg mL^{-1} respectively, which were comparable to the levels (MIC: 0.39 µg mL^{-1}; MBC: 1.56 µg mL^{-1}) recorded in the control strain *A. hydrophila* ATCC 49410. Alike, Wei and Musa (2008) recorded low MIC values (0.02 – 0.39 µg mL^{-1}) for tetracycline against *Aeromonas* spp. In contrast, high MIC values (50-600 µg mL^{-1}) of OTC against *Aeromonas* spp. have been reported (Singh *et al.* 2009). The higher MICs of antibiotics would mean the requirement of higher doses of antibiotics to conflict with bacterial infection, which may cause problems for future chemotherapy.

The fish can overcome the disease upon antibiotic therapy, as antibiotics prevent/retard the growth of a pathogen and reduce the level of infection. Though OTC is one of the therapeutic antibiotics for aquaculture use, it is permitted for use in certain types of aquatic animals and only to treat certain diseases (Bondad-Reantaso *et al.* 2012; USFWS 2015; Love *et al.* 2020). The administered dose of OTC in the present study though differed from some of the previous studies (Bruun *et al.* 2003; Julinta *et al.* 2017a), the decisive results were fairly similar. On day 10 of OTC therapy, 30% mortalities were noted in AI challenged fish as against 40% in untreated fish. To some extent, the results indicated the usefulness of antibiotics as per the therapeutic dose and dosage (USFWS 2015). Different challenge models have been attempted by several earlier authors with varying degrees of success (Russo *et al.* 2006; Pretto-Giordano *et al.* 2010). In this study, skin abrasions enhanced the success of disease establishment similar to those of Ventura and Grizzle (1987), who created similar systemic infections among channel catfish *Ictalurus punctatus* before the bacterial challenge. No role of water quality parameters could be attributed to these mortalities as they were maintained well within the optimal range. Also, tilapia may exhibit varying degrees of resistance to *A. caviae* infection (Schlotfeldt and Alderman 1995).

Lack of appetite or off-feed behaviour is one of the signals of disease in the culture system. The feeding rate was the minimum during the disease progression and treatment period. Upon *A. caviae* challenge, the fish became anorectic and the OTC feed intake was reduced by about 30%. The mean feeding behaviour scores of 2.78 – 2.81 during the treatment period indicated that the fish had consumed only 70% of the OTC incorporated into the feed. It indicated that approximately 42 mg OTC kg biomass^{-1} day^{-1} has been consumed against the minimum daily recommended therapeutic dose of 55 mg kg biomass^{-1} day^{-1} (USFWS 2015). This observably resulted in high mortalities even in OTC-treated groups, i.e., 30% during the OTC-therapy regime. Thus, oral therapy with 2 g OTC kg feed^{-1} at 3% BW twice daily for 10 days controlled the *A. caviae* infection in Nile tilapia only partially. Nonetheless, the effectiveness of OTC at 2 g kg feed^{-1} was demonstrated in fish under artificial culture conditions (Haque *et al.* 2014). The use of OTC medicated feeds is one of the easiest ways to treat fish. However, failure to achieve the right dose of antibiotic in the tissues of diseased fish during the treatment period may result in high mortalities. Therefore, caution must be exercised in providing the right dose of antibiotic at the recommended level during the treatment period.

The TVCs recorded in all the treatment groups were always above 8.20 $\log_{10}$ CFU g^{-1}. Their counts increased significantly by 0.64 – 0.77 log units with days of culture and/or feeding. The results conform with earlier reports (Ringo *et al.* 1995; Abraham *et al.* 2007), which reported as many as 8.00 $\log_{10}$ CFU g^{-1} gut. However, the observations of Abraham *et al.* (2007) revealed significant differences in the gut TVCs with days of culture and OTC therapy. As in TVCs, the ARBCs increased with the period of culture. The ARBCs of all the groups were always above 6.42 $\log_{10}$ CFU g^{-1}. The increment in their counts by 0.89 – 1.23 log units or ≥90% from the initial counts was significant with days of culture and/or OTC therapy. The OTC therapy, thus, led to an enhanced level of OTC-resistant gut bacteria. The share of ARB increased significantly by 5.84 folds from the initial levels in fish on day 10 OTC therapy, possibly indicating a shift in the bacterial population with OTC therapy similar to those of certain earlier studies (Austin and Al-Zahrani 1988; DePaola *et al.* 1995; Navarrete *et al.* 2008; Zhou *et al.* 2018). The results of Zhou *et al.* (2018) also indicated OTC-diet fed zebrafish had a lower bacterial richness in the intestine compared to control. The bacterial shift especially the eradication of normal microbiota may affect the drug metabolism and absorption as the intestinal microbiota reportedly play important roles in drug metabolism (Choi *et al.* 2013). The results corroborate several earlier studies that indicated a significant increase in antibiotic-resistant fish gut-bacteria (Austin and Al-Zahrani 1988; Abraham *et al.* 2007; Navarrete *et al.* 2008) and aquatic bacteria (DePaola *et al.* 1995; Miranda and Zemelman 2002; Watts *et al.* 2017).

On day 21 post-OTC therapy, though the number of ARB reduced slightly, it was about 3.89 folds higher than the initial level. These results suggested the persistence of the OTC-resistant gut bacterial population even after the cessation of therapy. The release of such gut bacteria in the environment through the faeces may spread the ARB. Similarly, DePaola *et al.* (1995) noted a decline in OTC-resistant intestinal bacteria after treatment, but they remained higher than pretreatment levels for at least 21 days in the spring season. In contrast, the intestine and aquatic environment recorded levels similar to the pre-

treatment period within 21 days after treatment in the fall season. They also noted a rapid increase in OTC resistance in aquatic bacteria of medicated ponds, at times exceeding 90%. Further, the proportion of ARB also increased significantly by 1.66 – 1.75 folds in untreated fish. The increment in the ARB population in the control with days of culture could be attributed to the use of grain-based pellet feed. It is noteworthy that the intestinal tract of fish absorbs the OTC very poorly and 70 – 80% of OTC is excreted in intact form through faeces (Ellingsen *et al.* 2002; Samuelsen 2006). Therefore, Romero *et al.* (2012) suggested the administration of OTC at a higher dose (100–150 mg kg biomass^{-1} day^{-1}) for 10 – 15 days. The slow excretion of this antibiotic in large amounts may exert selective pressure, leading to the selection of and spread of OTC-resistant gut bacteria (Austin and Al-Zahrani 1988; Navarrete *et al.* 2008). The poor intake of OTC feed together with the failure to achieve the right therapeutic OTC dose during the treatment period and perhaps the poor absorption of OTC in the challenged fish gut might have exerted selective pressure in the selection of OTC-resistant gut-bacteria. The increase in OTC-resistant bacteria during the oral OTC therapy perhaps limited the effectiveness of the treatment to control *A. caviae* infection in challenged fish.

The kidney is one of the vital organs most affected by *A. caviae* in several fish species (Thomas *et al.* 2013; Baldissera *et al.* 2017) and hence targeted for the histopathological changes in this study. The challenged fish lost its structural integrity of the kidney tissues and well-defined histopathological changes were observed on day 1, which indicated extensive changes in the tissues of this vital organ and disease progression. The inflammation of the renal tubules (nephritis) and glomerulopathy with dilated Bowman's space are the indications of a defective glomerular filtration of blood and removal of excess wastes and fluids from the kidney. The extent of kidney damage was more in untreated fish on day 5 compared to day 1. The cytosolic creatine kinase and Na^+, K^+-ATPase activities, which are active in the kidney epithelial cells, were inhibited by *A. caviae* infection in catfish (Baldissera *et al.* 2017). According to them, the reduced activities harmed the renal energy homeostasis by depleting the ATP levels and thus, contributed to disease pathogenesis. The observations on inflammation and loss of structural integrity of the renal tubules in *A. caviae* infected fish possibly suggested the reduced availability of ATP and energy supply. This indicated the ability of *A. caviae* in causing systemic infection in fish at a high dose despite its low virulence potential. These observations were, more or less, similar to those observed by Julinta *et al.* (2017a) in *O. niloticus* challenged with *A. hydrophila*. Paul *et al.* (2015) in their study reported similar changes in the kidney of *Clarias batrachus* naturally infected with motile *Aeromonas* spp.

A reduction in glomerulopathy and improvement in the structural organization of the renal tubules was noted in OTC treated fish possibly due to the inhibition of bacteria and toxin production. On day 6 of OTC therapy, comparatively minimal kidney damages were seen in fish, suggesting an improvement in the renal energy homeostasis with the progression of OTC therapy. The results of the serum creatinine levels also confirmed improved kidney functioning. The recorded serum creatinine levels in control corroborate the results of Julinta *et al.* (2019). The AI challenge elevated the serum creatinine levels significantly in both treated (2.41 times) and untreated (3.08 times) groups compared to control. However, the increment was significantly low in the OTC-treated group than in the untreated group. The creatinine levels were about 1.80 and 2.59 times higher in treated and untreated fish, respectively on day 21 post-OTC-therapy than on day 0. It suggested an improvement in the kidney functions, yet the fish did not get well fully. Nonetheless, Roy *et al.* (2019) found that wound healing was more prominent in AI-challenged fish during the OTC treatment periods. Also, the combination of dietary β-glucan and OTC was reportedly exerted synergistic immunostimulating effects on *Epinephelus fuscoguttatus* × *Epinephelus lanceolatus* hybrids from *Vibrio alginolyticus* infection (Lee *et al.* 2020), which may be explored during the medicated fish feed formulation and disease treatment.

5 | CONCLUSIONS

In general, our observations on the initial high mortalities in *A. caviae* challenged fish and the feeding behaviour scores implied that the dose (2 g OTC kg feed^{-1}), dosage and feed ration (10 days feeding at 3% BW twice daily) used in this study has failed to achieve the right dose of therapeutic OTC. Yet, this study demonstrated some encouraging effects of oral OTC therapy to overpower the bacterial challenge and improve kidney architecture and functioning. The observations of this study particularly on the reduced OTC-feed intake and the selection of OTC-resistant gut bacteria during the treatment period are important for the aquaculture industry and human health while planning the mitigation strategies.

ACKNOWLEDGEMENTS

The authors thank the Vice-Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata for providing the necessary infrastructure facility to carry out the work. The research work was supported by the Indian Council of Agricultural Research, Government of India, New Delhi under the All India Network Project (AINP) on Fish Health (Grant F. No. CIBA/AINP-FH/2015-16 dated 02.06.2015).

ETHICAL APPROVAL

All the experimental protocols were as per the guidelines

of the Government of India and approved by the Indian Council of Agricultural Research, Government of India, New Delhi (F.No. CIBA/AINP-FH/2015-16 dated 16.7.2015) under the AINP on Fish Health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

AR, **JS**, and **RBJ** performed the wet laboratory experiments, laboratory analyses, and generated data. **SB** performed the statistical analyses. **TJA** conceived the study, designed and supervised the experiments, drafted and finalized the manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request to the corresponding author.

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