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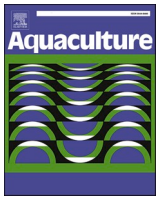

The effect of oxytetracycline treatment on the gut microbiome community dynamics in rainbow trout (*Oncorhynchus mykiss*) over time

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ABSTRACT

Antibiotic compounds play an important role in the control of bacteria disease outbreaks on fish farms. Yet, the impact of commercially licensed antibiotics on the diversity and composition of the gut microbiome in some farmed fish species remains unclear. The following study explored the effect of a low-level oxytetracycline treatment on the gut microbiome community in rainbow trout (*Oncorhynchus mykiss*) (average weight 152.8 ± 8.9 g). In this study, fish were fed diets with or without oxytetracycline (35 mg/kg bodyweight/day) for 7-days, followed by a 14-day withdrawal period. Distal gut digesta samples were collected from individual fish in a time series manner (on days 0, 2, 8, 10, 15 and 22). The microbiome community was profiled from the gut digesta using next generation sequencing of the bacterial 16S rRNA gene. No mortality was observed and all animals remained clinically healthy throughout the study. Furthermore, results showed that oxytetracycline treatment led to significant changes in the gut microbiome of rainbow trout. Oxytetracycline treatment led to a decline in *Mycoplasma* and *Bacillus* in treated fish compared with control fish, accompanied by an increase in *Aeromonas, Deefgea* and *Pseudomonas*. The gut microbiome of treated fish continued to change after antibiotic treatment and was not found to stabilise by the end of the study. After 14-days withdrawal from the antibiotic, treated fish displayed microbiomes with significantly higher microbial richness compared with control fish. Moreover, a number of taxa were found to become enriched in the distal guts of treated fish by day 22 including *Aeromonas, Brevinema* and *Deefgea* as well as diet-associated *Chloroplast_ge*. However, this was accompanied by a decline in the prevalence of *Bacillus* and *Clostridium_sensu_stricto_1*. Further work is required to better understand the long-term impacts of antibiotics and post-antibiotic recovered gut microbiome communities on the health and welfare of fish.

1. Introduction

The fast growth of the global aquaculture industry over the last seven decades has brought the multi-billion-dollar industry to the forefront in the supply of aquatic animal protein and global food security (Metian et al., 2019). Like many farmed fish species, the global production of rainbow trout (*Oncorhynchus mykiss*) has witnessed steep intensification since the 1950's, growing from a production of 4400 t in 1950 to 916,510 t in 2019, worth an estimated USD $4.2 billion (FAO, 2021). However, further intensification of this sector to meet the growing demand for seafood will likely be met with an increased prevalence of disease, as higher stocking densities allow for better transmission of pathogens and are often accompanied by water quality issues and induced stress, which can impact on disease resilience within the fish host. Indeed, infectious disease outbreaks, which cost USD $6 billion annually (Brummett et al., 2014), are currently one of the biggest constraining factors limiting future production and economic growth within the global aquaculture industry. Rainbow trout are known to be susceptible to a number of opportunistic pathogens including the Gram-negative bacterial species *Aeromonas hydrophila, Pseudomonas fluorescens* and *Yersinia ruckeri*, as well as Gram-positive organisms such as *Lactococcus garviae* (Dinçtürk and Tanrıkul, 2021; Duman et al., 2018; Shahi et al., 2018). Despite the significant economic impacts imposed by bacterial diseases in the farmed rainbow trout sector, there are limited

Abbreviation: ANOVA, Analysis of Variance; LEfSe, Linear Discriminant Analysis of Effect Size; OTC, Oxytetracycline; OTU, Operational Taxonomic Unit; PERMANOVA, Permutational Analysis of Variance; UoS, University of Stirling.

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prophylactic measures e.g., vaccines available for this fish species to prevent infectious diseases (Brudeseth et al., 2013). As a result, this sector relies heavily on the use of antibiotics to treat or reduce the severity of bacterial disease outbreaks on farms.

Oxytetracycline (OTC) is one of the most used antibiotic compounds within the global aquaculture industry due to its low cost and broad-spectrum activity (Limbu et al., 2018; Lulijwa et al., 2019). Oxytetracycline is a natural bacteriostatic tetracycline compound produced by *Streptomyces* species, which prevents the growth of both Gram-negative and Gram-positive bacteria through the inhibition of translation and subsequent protein synthesis (Mog et al., 2020; Yang et al., 2019; Zhou et al., 2018a). In rainbow trout, OTC is often prescribed for use in the treatment of furunculosis and enteric red mouth disease caused by atypical *Aeromonas salmonicida* and *Y. ruckeri* (National Office of Animal Health, 2017). Across the global industry, OTC is most often administered orally through feed for 3–15 days, where doses range between 5 and 250 mg/kg bodyweight/day (Leal et al., 2019). Furthermore, in some countries, OTC can also be administered at subtherapeutic levels for long periods of time, where it serves as a growth promotor in farmed fish (Mog et al., 2020; Van Boeckel et al., 2015). Whilst several studies have demonstrated the histopathological, immunosuppressive and genotoxic properties of OTC in farmed fish species including rainbow trout (Lundén et al., 1998; Rodrigues et al., 2017a, 2017b, 2019), further research is necessary to better understand the influence of this antibiotic on gut health in these animals.

The fish gut is colonised by a diverse and specialised microbiome community (Colston and Jackson, 2016). The membership of this microbial community is influenced by a range of endogenous and external factors including host genetics, temperature, salinity and diet (Huyben et al., 2018; Li et al., 2014; Naviner et al., 2006; Sullam et al., 2012). In addition, antimicrobial compounds have also been demonstrated to induce changes in the diversity and composition of the gut microbiome in several farmed fish species, including Atlantic salmon (*Salmo salar*) (Gupta et al., 2019) and Nile tilapia (*Oreochromis niloticus*) (Limbu et al., 2018), whilst also promoting the potential development of antibiotic resistance in other fish species through the transmission and exchange of resistance genes and other mobile genetic elements (Sáenz et al., 2019). Any potential side-effects on this community through antibiotic treatment is a concern for farmed fish, as members within the microbiome have been demonstrated to serve numerous microbial-mediated functions involved in the behaviour, growth, immunity and metabolism of the fish host (Borrelli et al., 2016; Gioacchini et al., 2018; Kelly and Salinas, 2017; Ni et al., 2014; Weiss et al., 2017). Therefore, any antibiotic-mediated disruption in the gut microbiome, through routine antibiotic treatment, may have detrimental consequences for the health and welfare of the farmed fish. Whilst the gut microbiome of rainbow trout has been reported shift in response to OTC (Roy Choudhury et al., 2021), the microbial community dynamics in this farmed fish species following antibiotic treatment remain unknown. Therefore, the aim of this study was to determine the community-level changes in the gut microbiome of rainbow trout in response to and following OTC treatment. To achieve this, a 16S rRNA amplicon-sequencing approach was applied to profile the changes in microbiome community diversity and composition in the distal gut of fish before, during and after antibiotic treatment.

2. Materials and methods

2.1. Fish and experimental design

The effects of OTC exposure on the distal gut microbiome in rainbow trout was performed over a 27-day time series feeding study. Ninety adult rainbow trout (average weight 152.8 ± 8.9 g) were obtained from a local trout farm and transferred to the freshwater aquarium facilities at the University of Stirling (UoS; Stirling, UK). All fish had been previously vaccinated against enteric red mouth using the AquaVac® RELERA vaccine (MSD Animal Health, Buckinghamshire, UK), but had not received any antibiotic treatment within 9 months prior to the start of the study. All fish were held in a single tank upon arrival at the aquaria where they received a salt water (Instant Ocean; Aquarium Systems®, France) treatment at 2 g/L for 1 h, followed by two separate Halamid (Tosylchloramide Sodium) (Axcentive SARL®, France) treatments, both at 5 ppm for 1 h. This was to treat a low protozoan parasite infection which was observed in the gills and dorsal fin of fish sampled during the pre-transfer health check. Fish were then randomly allocated into six 300 L tanks ($n = 15$ per tank) which were maintained on a flow through system, under a 12:12 h light:dark cycle and an ambient water temperature of 2.85 ± 0.9 °C. Fish were maintained in these conditions throughout the entire study.

Following a six-day acclimation period, tanks were randomly allocated into two experimental groups with three replicate tanks per group. Fish in the antibiotic treated experimental group, were fed an antibiotic diet surface coated with OTC at a dose of 35 mg/kg bodyweight/day, whereas fish in the control experimental group were fed a non-medicated diet. Both diets were delivered into respective tanks at a feeding rate of 0.7% bodyweight/day for 7 days. Oxytetracycline treatment was stopped after 7 days and fish in both treatment groups were fed the control diet at a feeding rate of 0.2% bodyweight/day for 14 days, after which time the experiment was terminated. All feed was delivered into tanks using an automatic feeder, operated between 08:00 and 16:00 daily. The study was conducted according to the guidelines of the UK Home Office Animals (Scientific Procedures) Act 1986 and was approved by the Animal Welfare and Ethical Review Body at the University of Stirling (AWERB (17 18) 006 New ASPA (B)).

2.2. Diet preparation and in vitro antimicrobial testing

A commercial trout feed (Skretting, France) (20% oil content, 39% protein content) was used throughout the study. The OTC diet was prepared as described by Payne et al. (2021). Briefly, a pre-weighted volume of pellets was surface coated with OTC hydrochloride (98.2% purity) (Duchefa Biochemie®, Haarlem, the Netherlands), which was homogenised by hand mixing for 5 min. Cod liver oil (Vitarenew®; Principle Healthcare International Limited, Skipton, UK) was then applied as a binding agent at a rate of 20 mL/kg diet. The control diet was similar in composition to the OTC diet, except it lacked OTC hydrochloride. Both diets were prepared 24 h prior to starting the antibiotic treatment period. Both diets were distributed into sterile universal tubes according to the required daily volume of feed per tank, which were stored at 4 °C until use. Prior to starting the antibiotic treatment, the OTC diet was tested for antimicrobial activity against the OTC-sensitive *Y. ruckeri* NCIMB 2194, following the method described by Payne et al. (2021). Briefly, one colony of *Y. ruckeri* was incubated in 30 mL sterile tryptone soy broth (Oxoid®, UK) for 18 h at 28 °C, before being centrifuged at 2600 ×*g* for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in sterile phosphate buffered saline (pH 7.2) to a MacFarland standard equivalent of 5.0, as judged by the naked eye. Then 100 μL of the bacterial suspension was inoculated onto sterile tryptone soy agar (Oxoid®, UK) as a bacterial lawn. After 5 min, three pellets from the OTC diet were then aseptically placed onto individual sections of the agar plate. The agar plate was incubated at 28 °C for 48 h. The agar plate was checked every 24 h for bacterial growth and the presence of inhibition zones around the diet pellets. The control diet was also tested for comparison, and to confirm this diet was free of any antimicrobial compounds.

2.3. Sample collection

The intestinal digesta was aseptically collected from the distal gut of individual fish at six time points: immediately before antibiotic treatment (day 0), 2 days after receiving antibiotic treatment (day 2), immediately after the end of the antibiotic treatment (day 8), 2 days

post-treatment withdrawal (day 10), 1-week post-treatment withdrawal (day 15) and at the end of the 2-week withdrawal period (day 22). At each timepoint, two fish were randomly sampled from each tank giving $n = 6$ fish per treatment group and per sampling time point. Following euthanasia using a lethal dose of the anaesthetic tricaine methanesulfonate (Tricaine 1000 mg/g powder; Pharmaq®, UK), each fish was weighed, and the total length (snout to caudal fin) recorded. Then, digesta from the distal gut was collected as described by Lyons et al. (2017). No intact feed pellets were observed within the digesta material of any fish sampled. All tubes were held on dry ice during sampling, before being stored at −80 °C and processed within 6 months. A total of ten pellets from each diet and a 10 mL sample of tank water from a random tank for each treatment group was also collected at each sampling point, stored at 4 °C and processed within 6 months. The same tank was sampled at each sampling point to allow for comparison of the tank water over time.

2.4. Library preparation and illumina MiSeq sequencing

A total of 150 mg of digesta was processed for genomic DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen®, Hilden, Germany), which followed that described by Lyons et al. (2017), except final DNA was eluted in 50 μL EB buffer (10 mM Tris-HCl, pH 8.5; Qiagen®, Hilden, Germany). Genomic DNA was also extracted from diet (two pellets) and tank water (1 mL) samples using the same commercial DNA extraction kit and method described previously. Lastly, genomic DNA was extracted for a negative sequencing control to track all sources of microbial DNA contamination in DNA libraries. No sample or DNA was added to this sample, instead ASL lysis buffer (Qiagen®, Hilden, Germany) was used as the starting material. Final eluted DNA samples were stored at −20 °C until required.

Prior to preparing DNA libraries, the bacterial DNA yield was quantified by real-time qPCR using the primer pair 341F/805R (Huang et al., 2018) (Table 1), which target the V3–4 region of the bacterial 16S rRNA gene. Real-time qPCR was performed on a Stratagene Mx3005P QPCR System (Agilent Technologies LDS UK Ltd., Cheshire, UK). All qPCR reactions were prepared in triplicate for each DNA sample to a total volume of 20 μL and contained 10 μL 2× Luminaris Color HiGreen qPCR Master Mix (ThermoFisher Scientific, Basingstoke, UK), 2 μL (20 ng) template DNA, 0.5 μL of each primer at 10 pM concentration and 7 μL nuclease free water. To confirm reagents and qPCR reactions were free from microbial DNA contamination; duplicate no DNA template control reactions were included in every qPCR run. Following an initial denaturation at 95 °C for 3 min, absolute quantification was conducted over 40 x cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 20 s. All qPCR reactions underwent dissociation melt curve analysis at the following conditions: 95 °C for ten seconds, 65 °C for ten seconds and 95 °C for 30 s. The number of 16S rRNA genes per microlitre of DNA sample, was calculated from the final Ct values in each qPCR reaction from a standard curve. The standard curve contained plasmid DNA with the V3–4 region of interest at concentrations ranging from 9.86×10^8–9.86×10^4 copies/μL. Plasma DNA standards were generated using the pGEM-T Easy Vector system (Promega Corporation, Madison, USA) and transformed into *E. coli* XL1-Blue cells (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]) (Agilent Technologies Inc., Cheshire, UK), following the manufacturer's protocol. Real-time qPCR runs achieved correlation coefficients and efficiencies >0.95 and 73%, respectively. Following 16S rRNA quantification, DNA samples that were identified to be of poor quality and fall below a minimum threshold of 1×10^4 16S rRNA copies/μL (Rubin et al., 2014) were not processed further.

For the remaining DNA samples, the V3–4 region of the bacterial 16S rRNA gene, which has been suggested to provide superior taxonomic assignment compared with other sub-regions (Almeida et al., 2018), was amplified using the primer set 16S_F/16S_R (Table 1), which included Illumina® adapter sequences. Briefly, a total of 6.76×10^5 16S rRNA gene copies were amplified in each DNA sample through triplicate PCR reactions, which comprised of 5 μL 2× NEBNext Q5 high fidelity master mix (New England Biolabs (UK) Ltd., Herts, UK), 1 μL of each primer (1 μM final concentration), template DNA and nuclease-free water to bring the volume to 10 μL. Amplification was performed using the following conditions: 98 °C for 1 min, followed by 25 x cycles of 98 °C for ten seconds, 55 °C for 30 s and 65 °C for 45 s. All PCR reactions underwent a final extension stage at 65 °C for 5 min. Following PCR, triplicate PCR products were pooled and the libraries purified using the AxyPrep Mag PCR clean up Kit (Appleton Woods Ltd., Birmingham, UK) with a modified 1:1 volume of PCR product to Mag PCR beads. A total of 2.5 μL of each library was indexed using the Nextera XT index primers N7XX and S5XX (Illumina®, California, United States). A library was also generated for the negative sequencing control using the method described previously. Final libraries (length ~ 600 bp) were pooled in equal molar concentration (3.6 nM) and sequenced using the Illumina MiSeq® NGS system with the Illumina® MiSeq Reagent Kits v2 (2 × 250 bp; 500-cycle) at UoS.

2.5. Bioinformatic analysis

Raw Illumina reads underwent demultiplexing with Casava v. 1.8 (Illumina®) and reads representing the PhiX/internal controls or reads not matching Illumina indices were removed. The open-source program Mothur (Schloss et al., 2009) was used to process the sequence read data generated. Reads were first quality-filtered to remove those which contained ambiguous bases, homopolymers longer than 8 bp, and reads with sequences <460 bp or >500 bp. Next, the dataset was further denoised by removing chimeric and undesirable sequences assigned to "chloroplast", "mitochondria", "unknown", "archaea" and "eukaryota". Final sequences were assembled into operational taxonomic units (OTUs) according to their taxonomy (phylotype-binning) using the default phylotype command implemented in Mothur. Final OTUs were classified against the SILVA-based bacterial reference alignment [Release 132, December 2017] (Quast et al., 2013) with a minimum confidence bootstrap threshold of 80% for each assignment. Finally, OTUs which only had one sequence across the whole dataset were removed from the final dataset. The final dataset was rarefied to 8058 sequences, the lowest number of sequences per sample, prior to performing any further downstream analysis. The final microbiome sample size after rarefaction was $n = 5$ across most treatment groups and time points, except for day 0 and day 8, OTC group, which had a sample size of $n = 4$. All sequence data used in this study was deposited to the European Nucleotide Archive (ENA accession number: PRJEB34853).

2.6. Statistical analysis

Statistical analysis was performed in JMP® version 14 and RStudio

Table 1
Primer sets used in this study.

Primer	Primer Sequence (5′–3′)	Size	Application
341F	CCTACGGGNGGCWGCAG	464 bp	16S rRNA qPCR
805R	GACTACHVGGGTATCTAATCC		
16S_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[341F]	571 bp	Illumina Libraries
16S_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[805R]		

Version 1.1.41. All data was checked for distribution and homogeneity, and log10 transformed if required. Differences in the final mean length and weight of fish across treatment and time was evaluated with two-way analysis of variance (ANOVA). Two-way ANOVA was also used to test differences in alpha diversity as measured by Chao1 richness (microbial richness), Inverse Simpson (microbial diversity), and the Shannon Diversity indices (microbial evenness), with treatment and time as factors. In addition, beta diversity and the dissimilarity in microbiome community composition between groups (e.g. sample type or time and treatment groups), were analysed using the relative abundance of OTUs and the Bray-Curtis distance metric (Bray and Curtis, 1957). The Bray-Curtis distance matrix was then visualised using non-metric multidimensional scaling ordination methods and the significance of any clustering between groups tested using PERMANOVA (vegan; adonis function) (Anderson, 2001). In all tests, PERMANOVA was conducted using 10,000 permutations. The composition of the topmost abundant bacterial genera in the distal gut of individual fish was profiled using the phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2011) packages. Furthermore, linear discriminant analysis of effect size (LEfSe) (Segata et al., 2011) and Metatstats (White et al., 2009) analyses were performed to determine whether any OTUs were differentially abundant between control and treated groups, respectively. For all statistical analyses, differences between groups were considered significant if $p \leq 0.05$.

3. Results

3.1. Fish health

All fish consumed both diets readily. Final mean length and weight of fish did not significantly differ across treatment group or time ($p = 0.85$ and $p = 0.82$), as seen in Table 2. No mortalities or internal clinical signs of disease were observed in either treatment group throughout the study. Externally, some fish ($n = 19$) across both treatment groups presented superficial erosion of the dorsal fins. In addition, there was also a low incidence of superficial lesions on the pectoral ($n = 2$) and anal fins ($n = 1$), as well as the mouth ($n = 6$) of fish. On day 2, one fish from each of the diet groups presented superficial lesions on the operculum and skin below the lateral line, respectively. Bacterial swabs taken from the affected site and kidney of each fish resulted in non-significant growth (< 5 colony forming units) on general purpose agar following incubation at 4 °C for 7 days.

3.2. In vitro antimicrobial testing

Zones of inhibition in bacterial growth was observed only in the *Y. ruckeri* bacterial lawns exposed to the OTC-coated pellets. These were measured at a mean (± SD) diameter of 23.33 ± 4.93 mm. Control pellets which lacked OTC, produced no inhibition zones after 48 h incubation.

Table 2
Final mean (+SD) length and weight measurements for control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment.

Treatment group	Day	Length (cm)		Weight (g)	
		Mean	SD	Mean	SD
Baseline	0	23.06	1.90	134.50	34.31
Control	2	23.62	1.59	146.83	29.89
OTC	2	24.00	2.26	152.17	41.51
Control	8	22.93	2.15	138.33	29.18
OTC	8	24.38	1.52	149.83	30.21
Control	10	23.07	1.90	126.50	31.06
OTC	10	22.87	0.99	124.33	12.13
Control	15	23.77	2.00	144.33	39.98
OTC	15	23.62	1.06	138.17	25.66
Control	22	23.73	1.28	140.33	25.51
OTC	22	23.45	1.70	138.00	30.55

3.3. Gut microbiome dynamics

3.3.1. Microbiome diversity

Oxytetracycline treatment led to significantly higher microbial richness on average in OTC-treated fish compared with controls ($F = 4.2893$, $p = 0.0436$) (Fig. 1). In addition, microbial richness significantly increased over time in OTC-treated fish ($F = 7.9191$, $p = 0.0070$). No significant difference was observed between treatment groups for Inverse Simpson ($F = 0.3243$, $p = 0.5717$) and Shannon Diversity ($F = 0.5950$, $p = 0.4442$) indices. The gut microbiome of fish showed significant difference in alpha diversity (richness, diversity and evenness) over time irrespective of treatment group ($p < 0.05$). In general, microbial communities were found to have higher alpha diversity in fish sampled between days 8 and 22 compared with those sampled on day 0 (Fig. 1). Non-metric multidimensional scaling of Bray-Curtis dissimilarities found microbiome communities to differ significantly in their composition according to sample type (e.g., diet, fish & tank water) (Fig. 2A), which was confirmed through PERMANOVA ($p < 0.001$). Time and treatment were significant factors in the composition of distal gut microbiome communities in fish, as samples clustered according to sampling day (PERMANOVA; $p = 0.026$) and exposure to OTC (PERMANOVA; $p = 0.041$) (Fig. 2B). In addition, average Bray-Curtis dissimilarities revealed that the gut microbiomes of fish varied more between sampling days than treatment group (Fig. 2C). However, average distances from the baseline group were higher for OTC-treated fish compared with the control group on days 8, 15 and 22. Inter-individual sample diversity was found to increase over time from day 0 to 22, irrespective of OTC treatment.

3.3.2. Microbiome community

A total of 899 OTUs were detected across all microbiome samples. Of these OTUs, 442 were observed in samples originating from fish gut digesta, whereas 344, 596 and 105 OTUs were detected in the diet, tank water or the negative sequencing control samples, respectively. The OTUs detected in the distal gut of fish were assigned to 411 bacterial genera. The top 25 genera present in the distal gut microbiome communities of fish across time or treatment group is presented in Fig. 3. At day 0, the most abundant genera in the pre-treated fish microbiomes were *Mycoplasma* followed by *Deefgea*, *Brevinema* and *Bacillus*.

The distal gut microbiome of treated fish was found to shift throughout the 7-day OTC treatment period (Fig. 3). This shift was largely driven by OTUs assigned to *Bacillus*, *Clostridium_sensu_stricto_1* and *Mycoplasma*, which decreased in sequence abundance within the guts of treated fish during antibiotic treatment between days 0 and 8. This was accompanied by an increase in the prevalence in *Aeromonas, Deefgea* and *Pseudomonas* within the OTC-treated group. Further analysis with LEfSe and metastats found several OTUs including OTU0031, assigned to *Clostridium_sensu_stricto_1*, to be significantly lower in the distal guts of treated fish compared with control fish on day 8 (Table 3). The enrichment of *Aeromonas* in treated fish persisted after OTC treatment had stopped, with an *Aeromonas* OTU (OTU0017) being detected at significantly higher levels compared with the control group by day 10. There was a notable increase in the prevalence of *Pseudomonas*, *Shewanella* and *Yersinia* following a 1-week withdrawal from OTC treatment. *Clostridium_sensu_stricto_1* was also detected in higher abundance in treated fish by day 15, with OTU0031, which was assigned to this genus, present at a significantly higher abundance within the distal guts of treated fish. By day 22, the distal gut microbiome of treated fish looked markedly different to the control group. *Bacillus*, which had continued to decline in abundance within treated fish from day 10, was notably depleted in the distal guts of treated fish by day 22. Further, a *Bacillus* OTU (OTU0002) was found to have significantly lower abundance in treated fish compared with the control group by the end of the trial. This was accompanied by lower abundances in other taxa

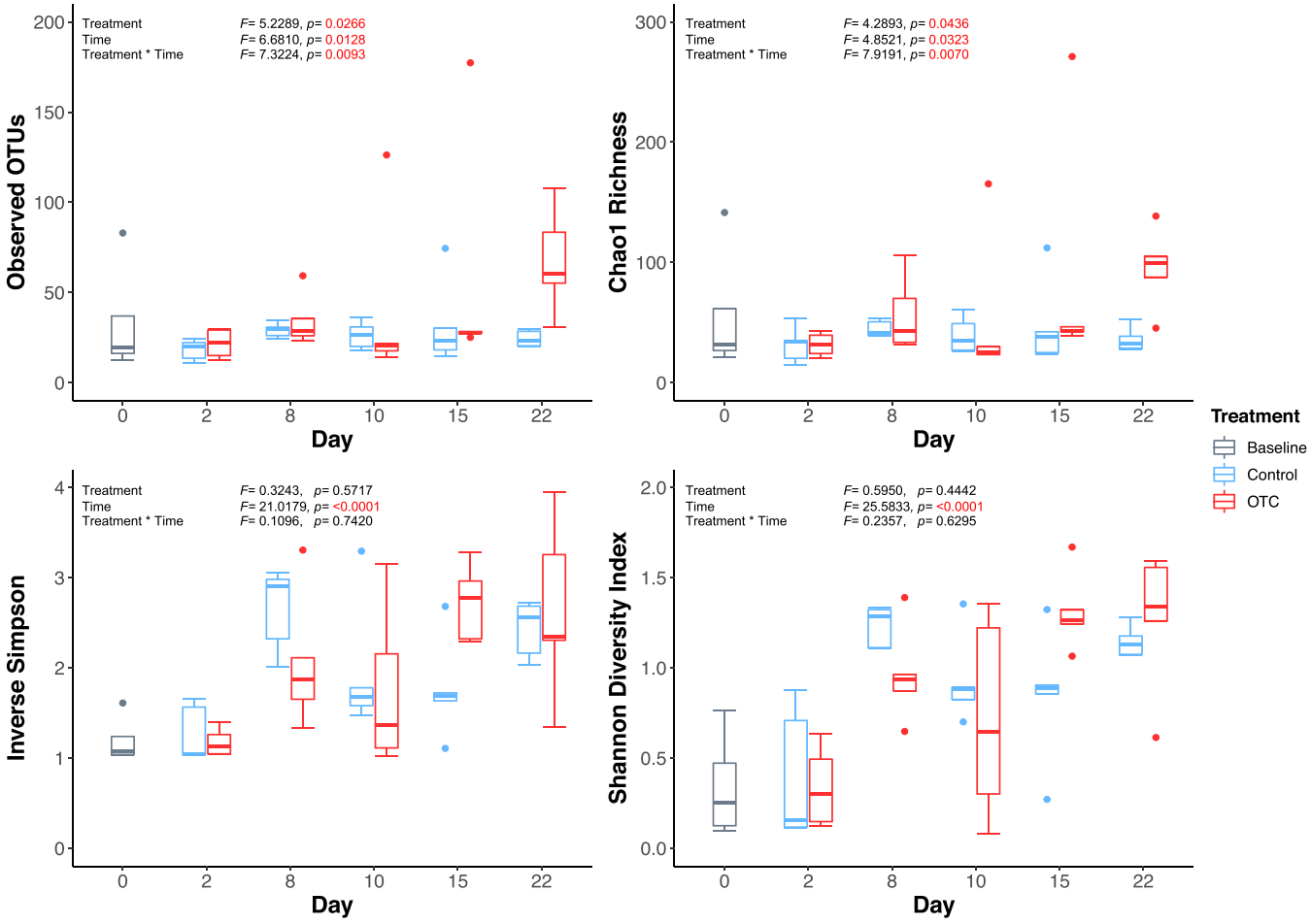

Fig. 1. Alpha diversity measures of microbiome communities in the distal gut of control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

including *Mycoplasma* and *Clostridium_sensu_stricto_1* compared with control fish. Despite this, an increased number of taxa was detected within the distal guts of treated fish by day 22, following the results from alpha diversity analysis (Fig. 1). Further, OTUs assigned to *Legionella* (OTU0046), *Polynucleobacter* (OTU0020), *Pseudomonas* (OTU0011) and *Undibacterium* (OTU0153) all became significantly elevated in the distal guts of treated fish compared with the control group at this time point. This was also accompanied by other taxa which showed elevated abundances including *Aeromonas*, *Brevinema*, *Deefgea* and *Pantoea* in treated fish by day 22. The genus *Chloroplast_ge*, dominated by a single OTU (OTU0005), was also found in higher abundance within the distal guts of treated fish compared with the control group on day 22. On closer inspection, this genus had become enriched in treated fish immediately following OTC treatment at day 8, further increasing in prevalence through days 10–22.

Analysis of microbial communities associated with the diets found similar communities across treatment group and time (Fig. 4A), supporting results from beta diversity analysis (Fig. 2A). Further, diet samples were dominated by a single OTU (OTU0005), assigned to *Chloroplast_ge*. Overall, distinct microbiome communities were detected between the diet and tank water samples (Fig. 4), following findings from beta diversity analysis. Analysis of OTUs detected in the tank water revealed similar membership across treatment groups and time, although abundances between genera varied (Fig. 4B). On closer inspection however, the microbial communities within the tank water samples did display similar composition to the NSC used in this study, and as such were not analysed further.

4. Discussion

In this study, low-level OTC treatment was sufficient to induce a decline in members of the Firmicutes (*Bacillus* and *Clostridium_sensu_stricto_1*) and Tenericutes (*Mycoplasma*) phyla within the distal guts of treated fish between days 2 and 8. Whilst a decline in these bacterial phyla within OTC-treated fish was in contrast to a similar study in rainbow trout (Roy Choudhury et al., 2021), this trend does follow findings from studies in other vertebrate animals treated with OTC (Ghanbari et al., 2019; Mu et al., 2017). As Gram-positive bacteria, members of the Firmicutes phylum lack an outer lipopolysaccharide membrane layer (Miller and Salama, 2018). Furthermore, the Tenericutes phylum lack a cell wall all together (Brown, 2018). The lack of outer cellular protection in these taxa therefore may be correlated with a reduced resilience against antibiotic compounds that target intracellular mechanisms of the bacterium, such as OTC (Leal et al., 2019). Indeed, *Mycoplasma* in particular falls within the spectrum of activity for OTC, as tetracyclines are often used when treating *Mycoplasma* bacterial diseases in farmed poultry (Puvača et al., 2020).

Oxytetracycline was also found to disrupt members belonging to the Gram-negative Proteobacteria phylum, demonstrating that even at low levels, this antibiotic compound can affect a diverse range of organisms within the gut microbiome of rainbow trout. However, unlike other taxa, OTC treatment in this study led to higher abundances of the Proteobacteria members *Aeromonas, Deefgea* and *Pseudomonas* in treated fish between days 2 and 8, compared with animals in the control group. These findings would indicate some level of resistance in members of

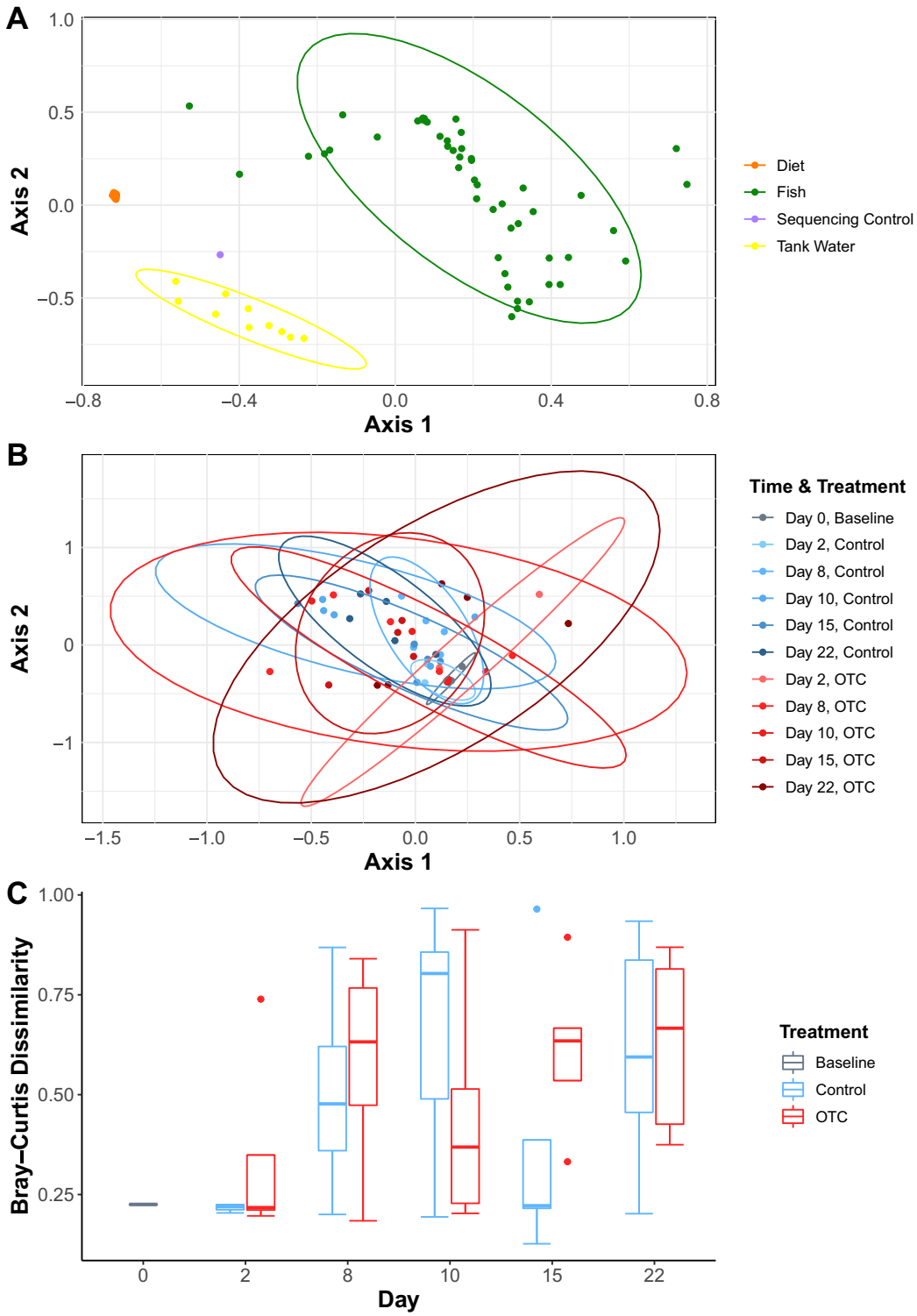

Fig. 2. Comparison of gut microbiome communities in rainbow trout within and between sampling days and treatment groups. Non-multidimensional scaling ordination of microbiome community composition based on Bray-Curtis distances using the relative abundance of OTUs (A & B). Distances were generated for the complete dataset including diet, tank water and sequencing control samples (A), and within the distal gut of rainbow trout alone (B). Boxplot showing inter-individual sample diversity based on Bray-Curtis dissimilarities (C). Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

this phylum to the OTC compound and follows reports in other fish species, such as Atlantic salmon and Senegalese sole (*Solea senegalensis*), exposed to the same antibiotic compound (Navarrete et al., 2008; Tapia-Paniagua et al., 2015). Resistance to OTC in Proteobacteria could arise intrinsically due to the outer lipopolysaccharide membrane layer found in all Gram-negative organisms (Miller and Salama, 2018), as this outer membrane barrier makes the cell highly impermeable to harmful toxic compounds like antibiotics (Ebbensgaard et al., 2018). Likewise, the resilience of Proteobacteria to OTC observed in this study, could also be correlated to acquired resistance through the expression of antibiotic

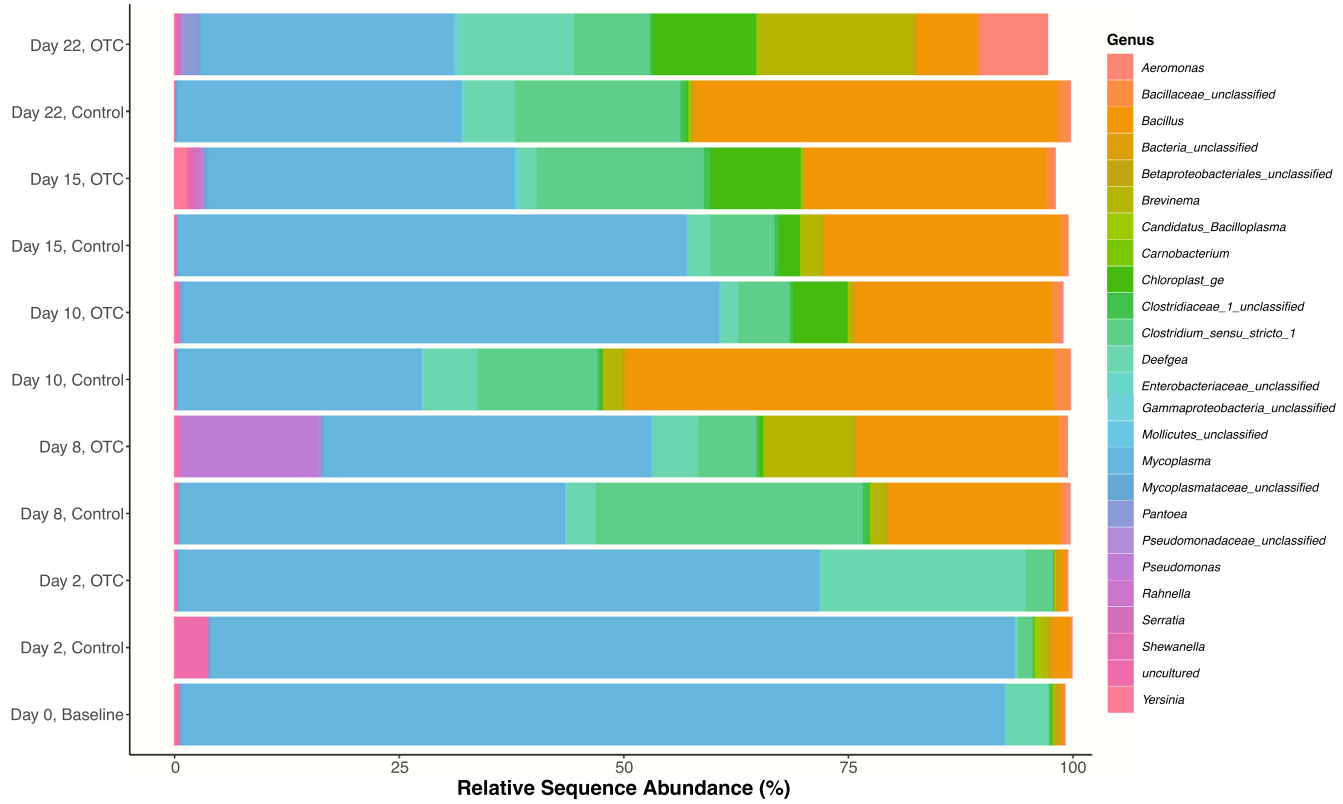

Fig. 3. Mean relative sequence abundance (%) of the top 25 bacterial genera in the distal gut of control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment.

Table 3
Operational taxonomic units (OTU) identified as discriminatory according to oxytetracycline (OTC) exposure by LEfSe and Metastats algorithms in Mothur.

Day	Phylotype		Mean abundance (%)		*p* value	
	OTU ID	Genus	Control group	OTC group	LEfSe	Metastats
2	OTU0019	*Bacillales_unclassified*	0.02	0.00	–	0.013
8	OTU0007	*Clostridiaceae_1_unclassified*	0.74	0.21	–	0.006
	OTU0021	*Clostridium_sensu_stricto_4*	0.07	0.01	–	0.009
	OTU0031	*Clostridium_sensu_stricto_1*	29.89	6.67	–	0.006
10	OTU0013	*Gammaproteobacteria_unclassified*	0.00	0.02	–	0.007
	OTU0017	*Aeromonas*	0.00	0.27	–	0.012
	OTU0080	*Reyranella*	0.01	0.00	–	0.044
15	OTU0008	*Brevinema*	2.36	0.11	–	0.035
	OTU0031	*Clostridium_sensu_stricto_1*	7.29	18.81	–	0.032
22	OTU0002	*Bacillus*	40.63	6.82	–	0.002
	OTU0003	*Bacillaceae_unclassified*	1.16	0.20	–	0.002
	OTU0007	*Clostridiaceae_1_unclassified*	0.67	0.16	–	0.025
	OTU0009	*Mycoplasmataceae_unclassified*	0.11	0.03	–	0.006
	OTU0010	*Burkholderiaceae_unclassified*	0.00	0.06	–	0.002
	OTU0011	*Pseudomonas*	0.01	0.19	–	0.016
	OTU0019	*Bacillales_unclassified*	0.07	0.02	–	0.037
	OTU0020	*Polynucleobacter*	0.00	0.08	–	0.020
	OTU0033	*Sporichthyaceae_unclassified*	0.00	0.00	0.044	–
	OTU0046	*Legionella*	0.00	0.03	–	0.021
	OTU0051	*Bacilli_unclassified*	0.02	0.00	–	0.003
	OTU0089	*NS11-12_marine_group_ge*	0.00	0.01	0.002	–
	OTU0097	*Microbacteriaceae_unclassified*	0.00	0.01	–	0.017
	OTU0153	*Undibacterium*	0.00	0.03	–	0.015

resistance genes, as several *tet* genes, which mediate resistance to tetracyclines, have been detected in Proteobacteria (Dang et al., 2008; Roberts, 2005).

In this study, the distal gut microbiome of treated fish experienced a series of successional events following antibiotic treatment. Bacterial succession in the gut microbiome has been reported in other vertebrate animals following disturbance events, such as antibiotic treatment in golden Syrian hamsters (*Mesocricetus auratus*) (Peterfreund et al., 2012) or *Vibrio cholerae* infection in humans (David et al., 2015). The series of community level changes in the gut microbiome observed in this study, may be correlated with the dynamic changes in bacterial competition during and following antibiotic treatment, as proposed by Peterfreund et al. (2012). Indeed, it is possible that the decline in antibiotic-sensitive members in treated fish in response to OTC treatment, allowed those

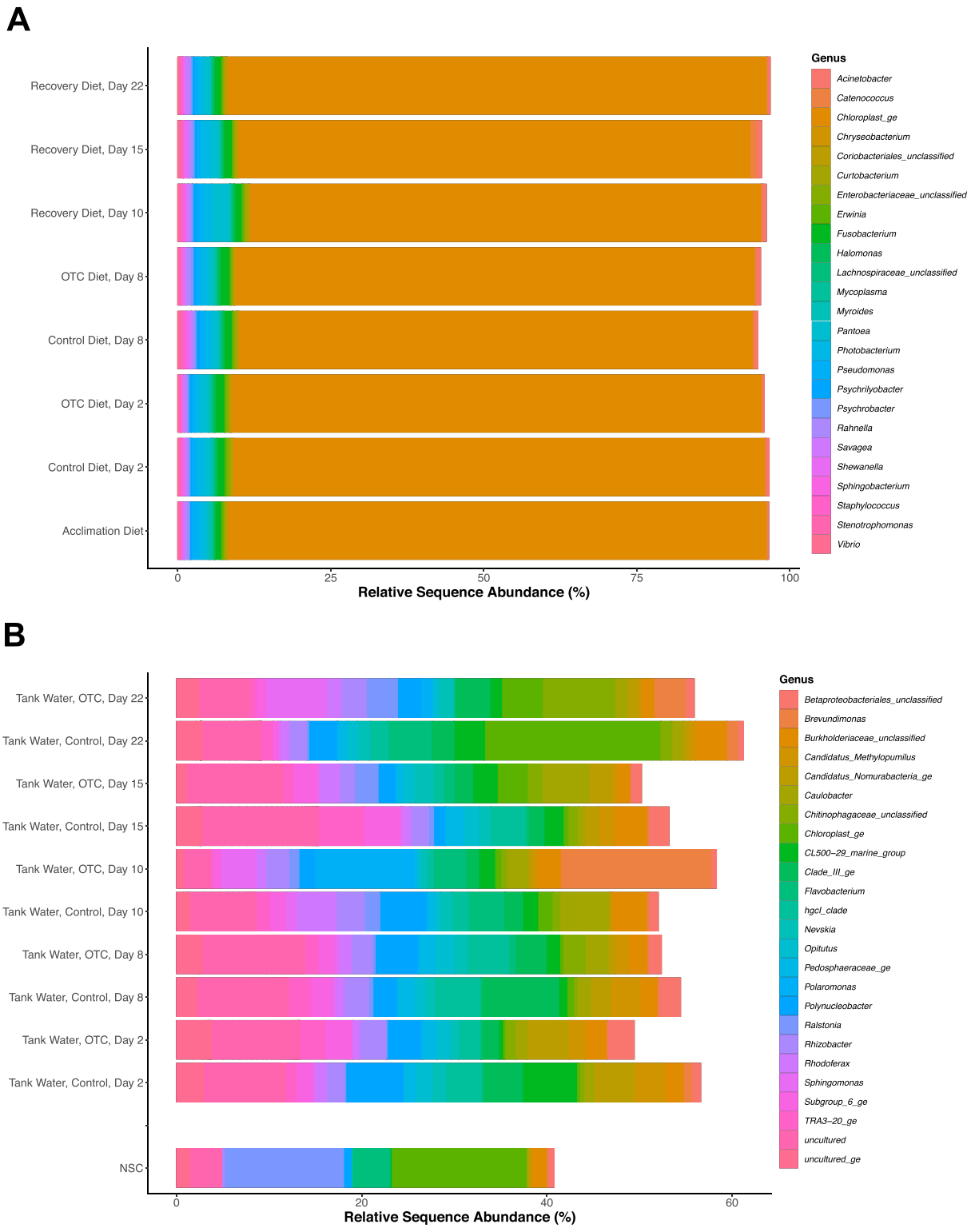

Fig. 4. Relative sequence abundance (%) of the top 25 bacterial genera in feed pellets (A), sequencing control (B) and tank water (B) across time and treatment.

more resilient and able to thrive in the new conditions to proliferate, as suggested in the present study by *Bacillus* giving way to *Aeromonas, Deefgea* and *Pseudomonas* in OTC-treated fish between days 2 and 8. However, when the antibiotic pressure was removed, the more competitive bacteria were able to outcompete early colonisers. This can be seen in the present study with the gradual increase in abundance of *Clostridium_sensu_stricto_1* in OTC-treated fish from days 8 to 15, to the detriment of *Deefgea*. The life-histories described for these bacterial taxa would support this hypothesis, as Firmicutes (*Bacillus* and *Clostridium_sensu_stricto_1*) and Proteobacteria (*Aeromonas, Deefgea* and *Pseudomonas*) are often regarded as having K- and r- life strategies, respectively (Brzeszcz et al., 2016; Ringelberg et al., 2008). In microbial ecosystems, r-strategist bacteria are usually the first to colonise newly exposed surfaces, such as those created following microbiome

disruption, however often become outcompeted by slow-growing competitive bacteria displaying K- life strategies during later successional stages (Rui et al., 2009). On the other hand, as the lamina propria and mucosal folds within the distal gut of fish function as attachment sites for commensal bacteria (Ringø and Gatesoupe, 1998), modification of these features during and after OTC treatment may have indirectly stimulated changes in the microbiome community in this study. Given that OTC has been shown to alter the intestinal villi width and muscularis thickness in Nile tilapia (Limbu et al., 2018), similar morphological changes could take place in rainbow trout.

A decrease in gut microbiome diversity was expected in this study, as OTC displays broad-spectrum activity (Leal et al., 2019). However, minimal changes in microbiome diversity were observed between treatment groups during antibiotic treatment (days 2–8) in this study. It is possible that the minimal effects of OTC observed on microbiome diversity during antibiotic treatment were related to tank conditions. Although the entire feed ration was consumed in each tank over the treatment period, the low water temperature in tanks may have resulted in a lower consumption or metabolism of the antibiotic in some fish. Therefore, further investigation in rainbow trout is warranted at optimal rearing conditions to confirm OTC effects on the microbiome. Despite this, the present study did however show that fish treated with low levels of OTC had significantly more diverse microbiome communities compared with the control group by day 22. This would agree with a recent study in Nile tilapia also exposed to low levels of the same compound (Limbu et al., 2018). Syntrophic network interactions and niche occupation are important components of colonisation resistance, a function of the stable gut microbiome in vertebrate animals whereby the commensal community limit the invasion and expansion of exogenous microorganisms and pathobionts (Buffie and Pamer, 2013; Shah et al., 2021). Therefore, findings from this study and that of Limbu et al. (2018), would suggest that low-level OTC treatment may disrupt colonisation resistance within the gut microbiomes of these fish species, allowing for resident microbiome members to expand or potential invaders to colonise. This is highly probable, as antibiotic treatment has already been demonstrated to deteriorate ecological networks within the gut microbiomes of invertebrate animals (Yang et al., 2017), thus similar responses may also occur in fish. Indeed, a recent study in European seabass (*Dicentrarchus labrax*), in which treated fish were given a low-level treatment with a cocktail of antibiotic compounds, found significantly increased bacterial counts within the guts of treated fish, accompanied by disruptions in co-operative interactions within microbiome community networks (Kokou et al., 2020).

As part of the succession process following antibiotic treatment, the Cyanobacteria genus *Chloroplast_ge*, became established within the distal guts of OTC-treated fish between days 8 and 22. Moreover, the same OTU assigned to *Chloroplast_ge* also dominated the microbial communities associated with feed pellets given during the trial. These findings would therefore indicate that antibiotic-induced disruptions in the microbiome community, allowed diet-associated microbiota members to colonise and proliferate within the distal gut of OTC-treated fish in this study. This would agree with the findings from Schmidt et al. (2017), who also demonstrated successful colonisation of diet-associated bacteria in the recovered gut microbiome of black molly (*Poecilia sphenops*), following treatment with streptomycin sulfate. Cyanobacteria has also been detected in the guts of channel catfish (*Ictalurus punctatus*), freshwater carp (*Labeo rohita*), gizzard shad (*Dorosoma cepedianum*) and silver carp (*Hypophthalmichthys molitrix*) (Burgos et al., 2018; Tyagi et al., 2019; Ye et al., 2014), thus it is possible the diet-associated *Chloroplast_ge* microbiota were able to survive the harsh conditions of intestinal tract and colonise the guts of treated fish in this study. As fish are farmed in an environment where they are in constant contact with a diverse community of microorganisms, the colonisation and expansion of environmental microbiota could have detrimental consequences for fish in aquaculture. In the present study, *Aeromonas*, *Pseudomonas* and *Yersinia* were all found to become enriched within the guts of treated fish at selected time points throughout the trial. All three bacterial genera contain pathogenic species associated with disease in rainbow trout (Austin and Austin, 2012). Moreover, members from these genera can also be found naturally in the aquatic environment as well as aquaculture facilities (Batrich et al., 2019; Chenia and Duma, 2017; Coquet et al., 2002; Gołaś et al., 2019). Therefore, results from this study suggest that OTC treatment, particularly at low levels, may increase the risk of disease in these animals. This is supported by recent evidence demonstrating the increased susceptibility of black molly and zebrafish to natural or experimental challenges with various bacterial pathogens following antibiotic treatment (Schmidt et al., 2017; Zhou et al., 2018b), and highlights the importance of good antibiotic stewardship in the aquaculture industry.

In the present study, alpha diversity and community membership in the gut microbiome of OTC-treated fish had still not stabilised after 2 weeks withdrawal of OTC. Future studies are therefore warranted to determine the time required for the gut microbiome in rainbow trout to recover following OTC treatment. In other vertebrate animals such as broilers and humans, recovery of post-antibiotic communities ranges from 12 days to more than 4 years following antibiotic withdrawal, respectively (Jakobsson et al., 2010; Videnska et al., 2013). The length of time taken for the gut microbiome to recover could have implications for farmed fish, as under a stable microbiome, members of this community provide a number of microbial-mediated functions to the fish host. In this study, OTC-treated fish were found to have lower abundances of Firmicutes members including *Bacillus* and *Clostridium_sensu_stricto_1* compared with control fish, both during antibiotic treatment and also after 2-weeks withdrawal of OTC. Both genera comprise important health-promoting bacterial species. Members of the *Bacillus* genus are often used as probiotics in aquaculture, where they have been documented to aid in digestion and antioxidant enzyme activity in fish, as well as enhance disease resistance to bacterial pathogens (Kuebutornye et al., 2019). Likewise, *Clostridium butyricum*, a member of the *Clostridium_sensu_stricto_1* genus, has been recently shown to display health-promoting qualities in common carp (*Cyprinus carpio*), such as stimulating host immunity and short-chain fatty acid content, which collectively enhances gut barrier protection against invading pathogens (Meng et al., 2021). Therefore, if during microbiome recovery, particular communities are slow to re-establish or be replaced by functionally similar bacteria, certain functions may not be provided to the fish host during this process. Furthermore, if the recovery process occurs over a long timeframe, the lack of certain functions could have long-term detrimental effects on fish health and welfare, which would limit production and thus be a concern for the aquaculture industry.

5. Conclusions

In this study, we showed that low-level OTC treatment can disrupt the gut microbiome community of rainbow trout, leading to a significant shift in community taxonomic profiles and higher microbial richness. Overall, findings showed that 1-week exposure to a low-level OTC treatment, resulted in a decline of beneficial bacterial communities accompanied by the expansion of potential opportunistic groups. Moreover, environmental microbiota associated with feed pellets colonised the distal guts of fish following antibiotic treatment. Further longitudinal studies are however required to fully characterise the recovery of the gut microbiome in rainbow trout, and evaluate the long-term impacts of OTC treatment and post-antibiotic microbiome communities on fish health and welfare.

CRediT authorship contribution statement

Christopher J. Payne: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing. **James F. Turnbull:** Formal analysis, Writing – review & editing.

Simon MacKenzie: Formal analysis, Funding acquisition, Resources, Supervision, Writing – review & editing. **Margaret Crumlish:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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