Investigating the gut microbiome in farmed fish species during antibiotic treatment

BY

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A Thesis Submitted to the University of Stirling for the Degree of DOCTOR OF PHILOSOPHY

August 2020

Institute of Aquaculture

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Declaration

I hereby declare that the work and findings presented in this thesis have been composed entirely by me. In addition, the studies described in this thesis, except where specifically acknowledged, have been conducted by me. This thesis has not been submitted for any other degree or qualification

SIGNED: CJPayne
DATE: 26.08.2020

Abstract

Antibiotics can disturb the gut microbiome of numerous vertebrate animals. However, the influence of commercially licensed antibiotics on the gut microbiome in some farmed fish species remains unclear. As the gut microbiome serves vital functions to support the physiology of the fish host, any alteration of this community may have detrimental consequences on the health and production of farmed fish. To this end, the aim of this PhD was to investigate the distal gut microbiome communities of two farmed fish species, in response to the commercially licensed antibiotic compound oxytetracycline. In addition, a secondary aim was to explore the changes in gut physiology in response to oxytetracycline and changes in the gut microbiome. Before addressing these research aims, the first study sought to determine whether titrating bacterial DNA concentration could reduce the inter-individual variability observed in the fish gut microbiome. Whilst titrating bacterial DNA did not reduce the individual variation observed in the distal gut microbiome between fish, this method of library generation improved gut microbiome characterisation through increased sequencing performance and reduced introduction of microbial DNA contamination. The next study explored the longitudinal changes in the microbiome and expression of key inflammatory cytokine genes within the distal guts of rainbow trout (*Oncorhynchus mykiss*), in response to and following oxytetracycline treatment. Findings from this study demonstrated that oxytetracycline rapidly stimulated community changes in the distal gut microbiome of rainbow trout, which continued after antibiotic treatment was terminated. Furthermore, these community changes led to a more diverse distal gut microbiome in treated fish following a two-week withdrawal period. Despite considerable changes in the gut microbiome, oxytetracycline treatment did not significantly affect the expression of key inflammatory cytokines within the distal gut of rainbow trout. A further study was conducted to investigate the effect of oxytetracycline on the gut health in Nile tilapia (*Oreochromis niloticus*). To achieve this, changes in the microbiome community within the distal gut of fish before and after antibiotic treatment was profiled, along with the abundance of antimicrobial resistance genes, and the expression of host genes related to immunity, metabolism and gut function. In this study, oxytetracycline was also found disrupt the distal gut microbiome of treated fish, but these community changes led to less diverse microbiome communities by the end of a two-week withdrawal period. Several bacterial taxa did however increase in sequence abundance in response to oxytetracycline, and had strong associations with several antimicrobial resistance genes. In addition, despite oxytetracycline not having a significant effect on the expression of marker genes related to gut physiology, antibiotic-induced microbiome changes were highly correlated with the expression of several immune-related genes.

Acknowledgements

I would like to acknowledge the Fisheries Society of the British Isles who funded the work in this PhD, as part of their PhD studentship program. I would also like to thank them for providing a platform to share my research through their annual symposium.

I would also like to thank my supervisors; Dr. Margaret Crumlish and Professor Simon MacKenzie, for their continued support and guidance throughout the entire PhD program - You have not only taught me how to work like a scientist, but also how to think like one too! Thank you for also allowing me to develop my skills as an academic researcher through the countless teaching and additional research opportunities you provided during my PhD. These will become vital for me as I embark on the next stage in my career.

Thanks also go to Dr. John Taggart, Dr. Michaël Bekaert and Jacquie Ireland for their invaluable help and advice throughout the molecular and bioinformatic stages of this PhD project. Thank you to the staff at the Niall Bromage Freshwater Research Unit and the Tropical Aquarium for their technical assistance during the feeding trials, I really couldn't have conducted these experiments without your expertise and assistance. Thank you also to Professor James Turnbull for being a statistics wizard and providing guidance in the statistical analysis of my data.

A huge thank you also goes to Chelsea Broughton, Francis Legario, Ahmed Ahmed, Alex Pargana, Shankar Mandel, Savitree Ritchuay and Dr. Ben Clokie for their assistance during sampling or sample processing. Also, thanks to all the staff and students within the Emerging Aquatic Infectious Disease, and Welfare and Behaviour groups at the Institute of Aquaculture.

I am eternally grateful to my parents for their support throughout my entire education and always encouraging me to reach my full potential. I hope I can find some way of paying you back in the future! Also, to my sisters who have been an amazing support network, and when required, provided some sibling rivalry to help push me towards my goals – Thank you.

Finally, a special thank you to my partner Matthew as without you, I would not be where I am today. Thank you for supporting my decision to move to Scotland to pursue my academic career and always believing in me. You have helped in this journey in ways you will never understand.

Conferences & Presentations

Oral Presentations

American Fisheries Society **2020**

International Fisheries Section Virtual Business Meeting 2020

Titled: Exploring the impact of oxytetracycline on gut health in Nile tilapia (*Oreochromis niloticus*)

Institute of Aquaculture PhD Symposium **Stirling, UK, 2019**

Titled: Oxytetracycline induces collapse and restructuring of the gut microbiome in rainbow trout (*Oncorhynchus mykiss*, W. 1792)

Fisheries Society of the British Isles Symposium **Hull, UK, 2019**

Advances in eDNA-based Approaches to Fish Ecology and Management

Titled: Exploring the impact of oxytetracycline on gut microbiome homeostasis in rainbow trout (*Oncorhynchus mykiss*, W. 1792)

Wellcome Genome Campus **Cambridge, UK, 2019**

Animal Genetics & Diseases 2019

Titled: Exploring the impact of oxytetracycline on gut microbiome homeostasis in rainbow trout (*Oncorhynchus mykiss*, W. 1792)

Fisheries Society of the British Isles Symposium **East Anglia, UK, 2018**

The Sustainable Use and Exploitation of Fishes

Titled: Exploring the influence of bacterial DNA variation in sequence-based fish gut microbiome studies

Poster Presentations

Institute of Aquaculture PhD Symposium **Stirling, UK, 2019**

Titled: Oxytetracycline induces collapse and restructuring of the gut microbiome in rainbow trout (*Oncorhynchus mykiss*, W. 1792)

Fisheries Society of the British Isles Symposium **Hull, UK, 2019**

Advances in eDNA-based Approaches to Fish Ecology and Management

Titled: Exploring the impact of oxytetracycline on gut microbiome homeostasis in rainbow trout (*Oncorhynchus mykiss*, W. 1792)

RESEARCH COMMUNICATION

ARCH-UK Finfish/Shellfish Microbiome Workshop **Aberdeen, UK, 2018**

Titled: Don't get mad, get even: The influence of bacterial DNA variation in sequence-based fish gut microbiome studies

Fisheries Society of the British Isles Symposium **East Anglia, UK, 2018**

The Sustainable Use and Exploitation of Fishes

Titled: Don't get mad, get even: The influence of bacterial DNA variation in sequence-based fish gut microbiome studies

4th Institute of Aquaculture PhD Conference **Stirling, UK, 2018**

Titled: Don't get mad, get even: The influence of bacterial DNA variation in sequence-based fish gut microbiome studies. (2nd Prize for best student poster)

Fisheries Society of the British Isles Symposium **Exeter, UK, 2017**

50th Annual Research Symposium: Understanding Fish Populations

Titled: Exploring the role of the rainbow trout (*Oncorhynchus mykiss*) gut microbiome in supporting fish health

Publications

Perry, W.B., Lindsay, E., **Payne, C.J.,** Brodie, C. and Kazlauskaite, R. (2020) The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society B* **287**, 20200184.

Paris, J.R., Sherman, K.D., Bell, E., Boulenger, C., Delord, C., El-Mahdi, M.B.M., Fairfield, E.A., Griffiths, A.M., Gutmann Roberts, C., Hedger, R.D., Holman, L.E., Hooper, L.H., Humphries, N.E., Katsiadaki, I., King, R.A., Lemopoulos, A., **Payne, C.J.,** Peirson, G., Richter, K.K., Taylor, M.I., Trueman, C.N., Hayden, B. and Stevens, J.R. (2018) Understanding and managing fish populations: keeping the toolbox fit for purpose. *Journal of Fish Biology* **92**, 727-751.

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Abbreviation List

AMR	Antimicrobial Resistance
ANOVA	One-Way Analysis of Variance
ARG	Antimicrobial Resistance Gene
AWERB	Animal Welfare and Ethical Review Body
bDNA	Bacterial DNA
BFT	Biofloc Technology
C3	Complement Component Factor 3
cDNA	Complementary DNA
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DPW	Days Post Withdrawal
GCN	Gene Copy Number
gDNA	Genomic DNA
GF	Germ-free
GID	Gastrointestinal Digesta
Gn	Gnotobiotic
GNB	Gram Negative Bacteria
GPB	Gram Positive Bacteria
HGT	Horizontal Gene Transfer
Ig	Immunoglobulin
IM	Insect meal
IoA_MB_STD	Synthetic Microbiome Community Standard
IoA_Seq_CTL	Internal Sequencing Control
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
LEfSe	Linear Discriminant Analysis Effect Size
LPS	Lipopolysaccharides
MAMP	Microbe-associated Molecular Patterns
MAS	Motile *Aeromonas* Septicaemia
MGE	Mobile Genetic Elements
mRNA	Messenger RNA
NBFRU	Niall Bromage Freshwater Research Unit
NCIMB	National Collection of Industrial, Food and Marine Bacteria

ABBREVIATIONS

NF-κB	Nuclear factor- κB
NGS	Next Generation Sequencing
nMDS	Non-Metric Multidimensional Scaling
NSC	Negative Sequencing Control
NSC_Diet	Negative Sequencing Control (Diet Samples)
NSC_Fish	Negative Sequencing Control (Fish Digesta)
NSC_Tank	Negative Sequencing Control (Tank/ TA Biofilms)
NT-library	Non-titrated 16S rRNA Library
NTC	No Template Control
OMP	Outer Membrane Proteins
OTC	Oxytetracycline
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PE	Paired-end
PERMANOVA	Permutational Multivariate Analysis of Variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PRR	Pattern Recognition Receptors
qPCR	Quantitative-PCR
R	Pearson's Correlation Coefficient
RAS	Recirculating Aquaculture Systems
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SBM	Soybean meal
SBS	Sequencing by Synthesis
SCFA	Short Chain Fatty Acids
SE	Single-end
ssDNA	Single Stranded DNA
T-library	Titrated 16S rRNA Library
TA	Tropical Aquarium
tgDNA	Total Genomic DNA
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UK	United Kingdom
UoS	University of Stirling
USD	United States Dollar

CHAPTER 1. General introduction and literature review

Section 1.4 of this chapter was included in a co-authored review article accepted for publication in Proceedings of the Royal Society B.

1.1. Overview of the global aquaculture industry

1.1.1. Current status of global aquaculture

The practice of farming fish is embedded in human culture and can be dated back to as early as 10,000 BC (Lucas et al., 2019). However, during the last 70 years; aquaculture has witnessed vast intensification and diversification into the current production sector, which plays a vital role in the supply of aquatic animal protein (Metian et al., 2019). The importance of this industry in food security is evident in its change in global production (Figure 1.1), which rose by 8.6% annually between 1980 and 2012 (Nadarajah & Flaaten, 2017). Furthermore, this importance is also apparent when trends in annual fish consumption are taken into consideration, as this has risen twice as high as population growth since 1961 (FAO, 2018). Currently, more than 590 aquatic species are farmed in various production systems across the sector, which in 2016 recorded a global production of 80 million tonnes of food fish (FAO, 2018; Ahmed et al., 2019). Of these species, finfish comprise more than 60% (FAO, 2018). Whilst carps currently represent the most important group of farmed fish worldwide in terms of production (Dawood & Koshio, 2016); Nile tilapia (*Oreochromis niloticus*), and rainbow trout (*Oncorhynchus mykiss*) are major players within the industry contributing 8% and 2% of the global production in 2016, respectively (FAO, 2018).

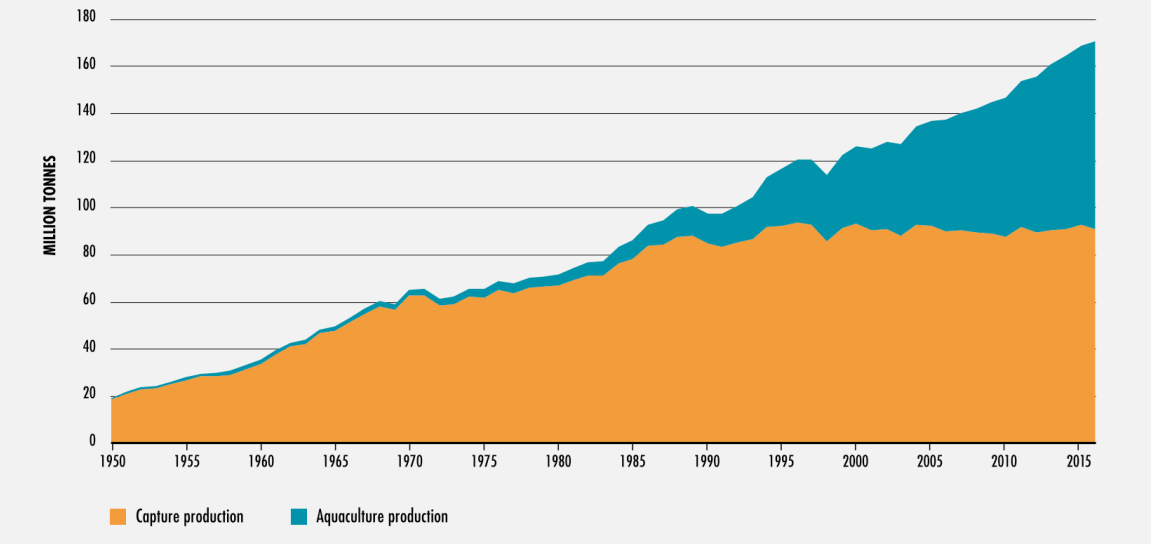

Figure 1.1. World capture fisheries and aquaculture production from 1950 – 2015. Excludes aquatic mammals, crocodiles, alligators, caimans and aquatic plants. Taken from FAO 2018.

Tilapias, also called the "aquatic chicken" are a tropical freshwater group of fish native to Africa (Eknath et al., 1998). Nile tilapia in particular, is an omnivorous species of tilapia indigenous to the waterways of the Nile, Niger and Tchad rivers, as well as in lakes of the Middle East (Lèveque, 2002). However, since its early introduction as a "farmed" fish species within irrigated ponds in 15th Century BC Egypt (Harache, 2002), production of this species has become global and now occurs in more than 100 countries (Behera et al., 2018). The widespread introduction of Nile tilapia is likely attributed to its fast growth, low production costs and well-established breeding protocols (Turker et al., 2003; Dawood et al., 2020). Of these countries however, China currently dominates the production of Nile tilapia, reporting yields of 1.2 million tonnes and more than 25% of the global supply in 2018 (FAO, 2021). Due to its extreme hardy nature, Nile tilapia can be grown in a wide array of production systems, ranging from low density ponds to highly intensive raceways and cage culture systems (Prabu et al., 2019). Despite this, earthen pond production as practiced by small to medium size tilapia farms typically dominate the production sector (Mengistu et al., 2019). Semi-intensive culture of tilapia has been favoured in the past due to its low production costs. In this approach, farmers typically use fertilisers and supplementary feeding to enrich primary production and phytoplankton biomass as a food source for the fish (Lèveque, 2002). Furthermore, many rural farmers also adopt an integrated approach by farming tilapia alongside livestock to further reduce production costs, using livestock manure as natural fertilisers (Brown et al., 2014). However, in recent years, the sector has witnessed a gradual shift to more intensive practices with a heavy reliance on formulated feeds (El-Sayed, 2013). The transition to intensive production has helped to meet the growing demand for this species through improved seed quality and yield, as well as growth promotion.

Rainbow trout, a carnivorous salmonid fish native to the North Pacific Ocean (Taylor, 1995) is now produced on every continent except Antarctica making it the most widely cultured cold, freshwater fish in the world (Liu et al., 2017; Ortega & Valladares, 2017). The global rainbow trout industry has witnessed a steep rate of intensification in the last 50 years with world production increasing from 39,671 tonnes in 1965 to 805,765 tonnes in 2014 (FAO, 2017). This rapid growth is the result of a rise in popularity of this species for human consumption, as well as for angling purposes (Fausch, 2007). This is particularly evident in the United Kingdom (UK) where this species dominates freshwater production (FAO, 2017). In 2018, Iran was the largest producer of rainbow trout globally, producing a total of 173,384 tonnes and 20% of the global supply (FAO, 2021). The market for trout can be separated into two components; notably the small, portioned sized fish, which constitute white meat, and large size fish with considerable red meat, which has been considered a good substitute for salmon (Lasner et al., 2017). Whilst the freshwater production of this species dominates the sector; production is also practised in marine environments with the anadromous ecotype of this species, named steelhead or sea trout.

Production of steelhead trout occurs in a land to sea phase approach whereby reproduction and smoltification occur in freshwater hatcheries followed by grow-out in open sea cages (Xiong et al., 2019). In freshwater production; farming of rainbow trout is conducted in an array of production systems, ranging from low intensity ponds to super-intensive structures such as open net cages, recirculation systems or raceways (Kumar, 2017; Stoyanova & Staykov, 2017). Further, in some countries; polyculture farming of rainbow trout is also practiced or being explored with other fish species such as brook trout (*Salvelinus fontinalis*), channel catfish (*Ictalurus punctatus*) and South American catfish (*Rhamdia quelen*) (Beem et al., 1988; Delihasan Sonay & Başçinar, 2017; Pereira et al., 2018).

1.1.2. Disease and antibiotic treatment in aquaculture

Global human population is estimated to reach nine billion by 2050 (FAO, 2018), however, the growth across global regions will not be distributed evenly. Whilst high-economic regions e.g. Europe are expected to remain static, "developing" regions e.g. Asia, Middle East and Africa are expected to significantly and disproportionately increase (Stentiford et al., 2012). As aquatic animal food (both captured and farmed) already contribute more than 16% of the global population's animal protein intake (Pradeepkiran, 2019); demand on farmed seafood will undoubtably increase in line with human population growth. Despite this, production in many countries is already reaching static levels as current production systems operate close to maximum biological thresholds, making further intensification challenging. This is particularly evident in the Atlantic salmon (*Salmo salar*) sector which whilst blessed with annual growths of 8% since 1995; is expected to experience diminished growths (ca. 4%) over the next few years (Marine Harvest, 2018). Currently, disease outbreaks are one of the biggest constraining factors limiting future production and economic growth within the global aquaculture industry.

As with other vertebrate animals, fish are susceptible to a range of infectious bacterial, viral, fungal and parasitic diseases, which can impose severe economic challenges for the global aquaculture industry (Rodger, 2016). For example, Streptococcosis, caused primarily by the bacteria *Streptococcus agalactiae* and *Streptococcus iniae*, is a significant disease challenge for the aquaculture industry, where these pathogens can infect a number of species including channel catfish and Nile tilapia (Chen et al., 2011; Li et al., 2014b). In 2008, global economic losses as a result of this disease was estimated to reach $250 million USD (Osman et al., 2017). Whilst the interaction of pathogens with their host and environment is extremely complex; further intensification of production systems to meet the growing demand for seafood will likely be met with an increased prevalence of disease. This will be likely as higher stocking densities allow for better transmission of pathogens and are often accompanied by water quality issues and induced stress which can impact disease resilience. Antimicrobial treatments including antibiotics,

antivirals and antifungals are a common strategy employed by farmers to control for such diseases. A number of antibiotics have been licensed for use in aquaculture across the world (Table 1.1), although many of these may not be immediately available for individual countries depending on regulations. For example, only four antibiotics are currently prescribed for the treatment of fish farmed in the UK, specifically amoxicillin, florfenicol, oxolinic acid and oxytetracycline (UK-VARSS, 2019).

Table 1.1. Antibiotic classes and examples used in the global aquaculture industry, diseases they have been used to treat and examples of resistant bacteria isolated from aquaculture environments.

Drug class	Example	Disease	Resistant bacteria	Multiple resistance[a]	Origin
Aminoglycosides	Streptomycin	Gram-negative infections	*Edwardsiella ictaluri*	Yes	Diseased striped catfish (*Pangasianodon hypophthalmus*), Vietnam
Amphenicols	Florfenicol	Furunculosis	*Enterobacter* spp. and *Pseudomonas* spp.	Yes	Freshwater salmon farms, Chile
Beta-lactams	Amoxicillin	Furunculosis	*Vibrio* spp., *Aeromonas* spp. and *Edwardsiella tarda*	Yes	Different aquaculture settings, Australia
Fluoroquinolones	Enrofloxacin	Furunculosis, Enteric redmouth and Vibriosis	*Tenacibaculum maritimum*	Yes	Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*), Spain and Portugal
Macrolides	Erythromycin	Bacterial kidney disease	*Salmonella* spp.	Yes	Marketed fish, China
Nitrofurans	Furazolidone	Broad spectrum but also use in treatment of some parasitic diseases	*Vibrio anguillarum*	Yes	Diseased sea bass and sea bream, Greece
Quinolones	Oxolinic acid	Furunculosis	*Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp.	Yes	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines
Sulphonamides	Sulphadiazine	Furunculosis, Enteric redmouth and Vibriosis	*Aeromonas* spp.	Yes	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), India
Tetracyclines	Tetracycline	Mycobacteriosis	*Aeromonas hydrophila*	Yes	Water from mullet and tilapia farms, Egypt
Tetracyclines	Oxytetracycline	Furunculosis, Bacterial cold water disease and Enteric redmouth	*Aeromonas salmonicida*	Yes	Atlantic salmon & culture facilities, Canada

[a] denotes resistance to antibiotics belonging to different classes in at least one isolate.

Table modified from Defoirdt et al., (2011).

Antibiotics are usually given orally as in-feed medication although other routes of administration are applicable e.g. injection and topical application through submersion/baths (Alderman & Hastings, 1998; Rodgers & Furones, 2009). Whilst the oral route of administration is considered the most cost-effective method to treating a large population of animals, the application requires that most animals are still eating and thus should be applied at the earliest opportunity to maximise effect (Wegener, 2003). This route of administration is however promoted as a route for exacerbating or contributing to the development of antimicrobial resistance (AMR; Table 1.1), as antibiotic residues from uneaten feed or faecal matter can build up in the environment where they are available for the natural microbial flora (Cabello, 2006; Chen et al., 2015). Previous estimations suggest that at least 75% of the antibiotics administered through feed in aquatic production systems are released into the surrounding environment (Topal & Arslan Topal, 2015).

The concern surrounding the emergence of AMR has led to the development of stringent regulatory controls and a subsequent decline in the use of antibiotics to treat aquaculture diseases in many countries, particularly across Europe and North America (Defoirdt et al., 2011). For example, in the UK, sales of active ingredient for antibiotics in aquaculture reduced from 10 tonnes to 2 tonnes between 1993 and 2000, albeit with national production increasing by more than 80,000 tonnes between the same years (Rodgers & Furones, 2009). Further in 2018, when UK production of Atlantic salmon and rainbow trout reached volumes of 156,025 and 11,859 tonnes, respectively (FAO, 2021), this volume was reduced even further with sales of antibiotic active ingredients totalling just 1.16 tonnes, representing 6.5 and 13 mg kg^{-1} of antibiotics used in Scottish salmon farms and UK trout farms, respectively (UK-VARSS, 2019). These values are considerably lower than other UK meat-sectors including cattle (dairy and beef), pig and poultry (chicken, duck and turkey) production, which saw 38, 110 and 60.8 mg kg^{-1} of antibiotics used, respectively in 2018 (UK-VARSS, 2019). The decline in antibiotic use in many countries has been associated with a shift in husbandry practices and the adoption of other alternative strategies which offer the prophylactic control of diseases. These include vaccination and the use of pro- and prebiotic compounds in feed, and have been reviewed elsewhere (Song et al., 2014; Dadar et al., 2017; Sharifuzzaman & Austin, 2017). Despite the trends seen across most of the global industry, some countries still have relaxed regulatory control resulting in the overuse or misuse of antibiotics. In Chile for example, where antibiotic use has increased in contrast to other countries across the world; the salmon sector alone reported a total antibiotic use of 382.5 tonnes or 530 mg kg^{-1} in 2016 (Miranda *et al.*, 2018). These are likely driven by financial reasons, perceived barriers to alternative strategies, or poor understanding of AMR and the importance of antimicrobial management (Mo et al., 2017).

1.2. Current tools to study the fish microbiome

The term 'microbiota' was first suggested in 2001 to describe "the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space" (Lederberg & McCray, 2001). When discussing the microbiota in relation to their environment and collective genome, it is generally accepted to use the term 'microbiome', however these two terms are often used interchangeable in the literature (Iqbal & Quigley, 2016). Herein, the term 'microbiome' will be used when discussing all aspects of microbial communities within a particular habitat.

Currently five approaches are employed to investigate microbiome communities in fish (Figure 1.2). The first is based on a culture-dependant approach employing bacterial identification from intestinal homogenates and direct microscopy (Austin, 2006). Although this approach was instrumental in providing our first insights into the microbial inhabitants colonising the guts of fish, they were retrospectively also met by several challenges which limit their usefulness in microbiome research. These limitations include being time-consuming, expensive and the inability to differentiate between indigenous and transient populations e.g. from ingested feed or water (Austin, 2006; Mulle et al., 2013). In addition, studies which employ these techniques were also previously thought to be limited in their recovery of microorganisms from biological/environmental samples, otherwise known as "the great plate count anomaly". In fact, some have previously estimated that only 0.01 - 0.1% of bacteria from marine environments can be cultured *in vivo* through standard culture-dependant techniques (Connon & Giovannoni, 2002). Likewise, traditional culture-dependant methods have been estimated to only recover a minority (10-50%) of the total bacterial community within the human gut microbiome (O'Toole & Claesson, 2010). These assumptions lead to the notion that these types of studies possessed limited sensitivity and were subject to biased interpretation, often leading to distorted community profiles. As such, there has been a consensus to move away from culture-dependant approaches when studying the fish microbiome. However, these claims have recently been weakened by the advent of "culturomics" and the development of several innovative techniques including microfluidics, cultivation chips and single cell manipulation (Gutleben *et al.*, 2018), with some methods such as diffusion chambers allowing for 300 times more bacterial cells to be cultured from environmental samples compared with standard petri dish-based approaches (Lagier *et al.*, 2015). Despite the advancement in high-throughput cultivation techniques, "culturomics" are yet to be routinely applied in fish microbiome research. However, the benefits associated with these types of methods including the identification of "uncultured" taxa, allowing for further strain characterisation e.g. probiotic suitability and determining specific host-microbe interactions, show great promise for fish microbiome research in the future.

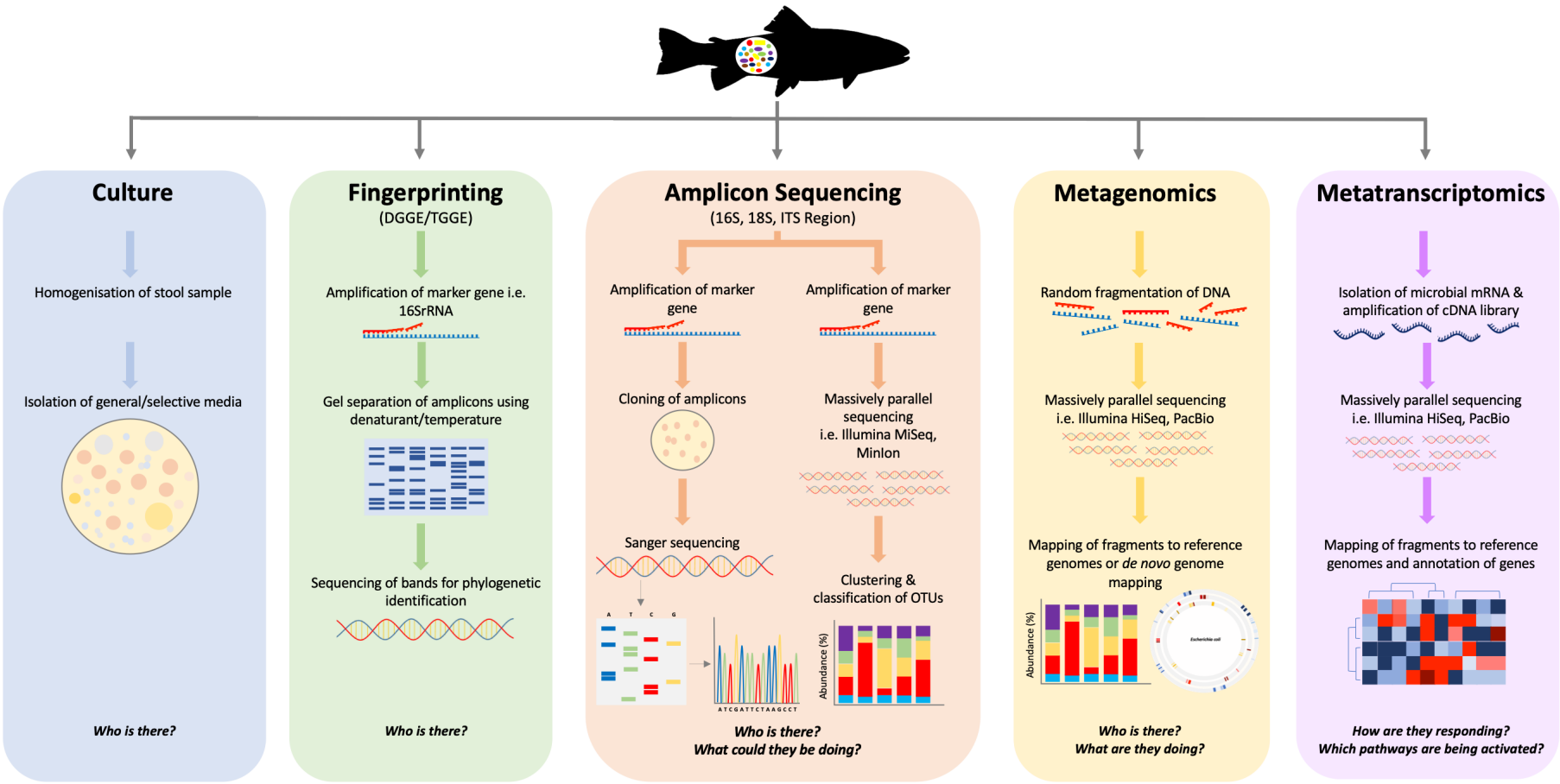

Figure 1.2. Available culture dependant and independent approaches for studying the gut microbiome and its interaction with the fish host. Adapted from National Academies of Sciences, Engineering and Medicine (2018).

In the 1980's, a new era of techniques which were more rapid, cost effective and reproducible were developed. These techniques were designed to infer the phylogenetic relationships of bacterial communities from biological and environmental samples, by comparing a stable part of their genetic code. In most cases the 16S ribosomal RNA (rRNA) is chosen for these approaches, as this particular gene is highly conserved across bacterial species and present in high abundance within the genome, making it a valuable target for phylogenetic analysis (Clarridge, 2004). Further, amongst the conserved loop regions within its structure; the 16S rRNA gene also contains hypervariable regions, which due to their differential evolutionary rates, allow phylogenetic resolution down to modern lineages such as genera (Yarza et al., 2014). Since the development of the polymerase chain reaction (PCR), nine regions (V1-V9) have been discovered on the bacterial 16S rRNA gene (Figure 1.3) (Wang & Qian, 2009). As these hypervariable regions are flanked by conserved sequences in most bacteria, amplification of target hypervariable sequences via PCR is possible using universal primer sequences (Chakravorty et al., 2007). Although no single hypervariable region is able to distinguish between all bacteria; the V2 and V4 regions are suggested to provide the best taxonomic assignment results at genus level (Armougom, 2009).

Several techniques have been developed based on targeting the bacterial 16S rRNA to characterise the commensal community within the gut microbiome of fish (Xing et al., 2013). Temperature or denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism analysis are common bacterial fingerprinting methods, which provide a snapshot of the microbial community composition within a particular environment (Inglis et al., 2012). Traditionally, these approaches employ universal or specific PCR primers that amplify a desired target region e.g. 16S rRNA gene within the DNA extracted from a given sample. The amplified DNA is then subjected to gel electrophoresis to separate the bacterial community and subsequently sequenced (Huys et al., 2008) (Figure 1.2). These approaches are also popular amongst microbiologists as they allow the identification of other microbial organisms such fungi and archaea through fungal ribosome genes and archaeal 16S rRNA genes, respectively (Schütte et al., 2008). Whilst these fingerprinting methods provide rapid, cost-effective, reproducible tools with the possibility of multiplexing; it is difficult to relate minor changes in banding patterns to species (Hamady & Knight, 2009) and the techniques do not provide the potential to translate taxonomic information.

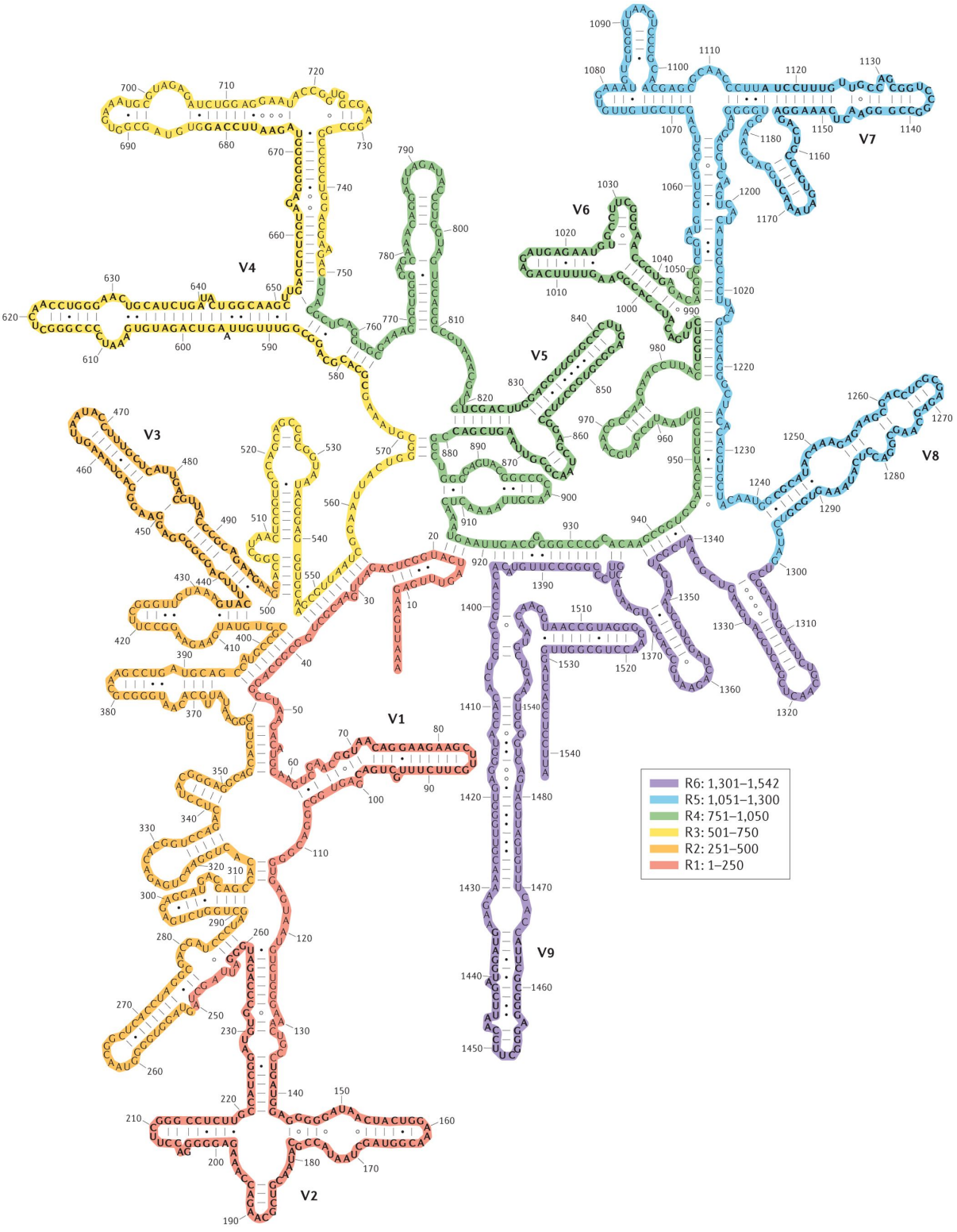

Figure 1.3. Variable regions on the 16S rRNA. Secondary formation of the *Escherichia coli* 16S rRNA gene. Gene sequences colour coded according to R fragments of ~250 nucleotides. Taken from Yarza et al., 2014.

The advances made in DNA sequencing methods over the last 40 years has provided researchers unparalleled access to characterise the diversity of microbial communities colonising different environments. Next Generation Sequencing (NGS) was first developed at the dawn of the 21st century following a goal set by the National Human Genome Research Institute to reduce the cost of human genome sequencing to $1,000 USD (van Dijk et al., 2014). Since then, it has become the "go-to" approach for characterising the microbial communities colonising fish. Whilst NGS was developed following the revolutionary advances made by first generation DNA sequencing methods e.g. Sanger sequencing, which adopt a capillary-based sequencing approach (Sanger et al., 1977; Morozova & Marra, 2008) (Figure 1.2); NGS platforms differ to these original approaches in multiple aspects. These include: (i) employing cell-free *in vitro* DNA amplification; (ii) employing solid-surface sequencing e.g. bead (Roche/454 or SOLiD pathways) or microfluidic channel (Illumina pathways); (iii) being "massively-paralleled" as the sequencing process e.g. nucleotide addition and detection, is a stepwise reaction series, among others (van Dijk et al., 2014; Gyllensten et al., 2016; Levy & Myers, 2016). Whilst three main technologies went on to dominate the NGS market over its first decade including Illumina/Solexa, T Roche/454, and the Applied Biosystems SOLiD; the latter two were unable to overcome certain limitations and became uncompetitive in the rapidly evolving NGS market. As a result, in 2016 both Roche and Thermofisher shutdown their platforms, catapulting other NGS technologies into the front line of fish microbiome research (van Dijk et al., 2014; Ghanbari et al., 2015; Levy & Myers, 2016).

Since its introduction onto the NGS market in 2006 (Liu et al., 2014), the Illumina technology has been massively embraced by researchers and their machines now dominate more than 70% of the DNA sequencing market (Sasaki et al., 2016). This is likely associated with the lower costs per base and improved volumes of data output, which in 2014 was the highest compared with all other NGS platforms on the market (van Dijk et al., 2014; Ghanbari et al., 2015). The Illumina platform takes a similar approach to sequencing as the Sanger method but sequences with reversible dye terminators (Mardis, 2013). The amplification and sequencing of the DNA fragment library by an Illumina NGS platform is outlined in Figure 1.4. Illumina has been extremely successful in developing its platforms over the last 15 years to improve its sequencing capabilities and cement itself as a front runner in the NGS market. Specifically, the technology employed by Illumina platforms allows for either single-end (SE) or paired-end (PE) reads to be generated (Degnan & Ochman, 2012). The development of PE sequencing overcame a significant limitation in the short ca. 100 bp read length originally only possible with Illumina, as now PE reads can be merged, generating reads up to 600 bp in length (Ghanbari et al., 2015). This higher read-length makes it possible to perform *de novo* genome assembly and metagenomics using the Illumina technology, a role once taken by the Roche/ 454 sequencing platform (van Dijk et al.,

2014). In addition, PE reads also improve the alignment of sequence reads during data analysis as they provide a higher overall certainty of base placement compared with SE reads (Mardis, 2013). Illumina now also offer the possibility of capturing high quality reads at an unprecedented scale with the introduction of the NovaSeq 6000, which can generate 20 billion reads per run (Illumina, 2020). This considerably reduces the overall sequencing costs, which can be far below that initially set by the National Human Genome Research Institute. Finally, the flow cell technology and chemistry employed by Illumina platforms also allows for multiplex sample sequencing with the addition of unique index sequences. Currently, Illumina offer the potential of multiplexing up to 384 libraries with their Nextera XT index kits. However, the development of phasing amplicon sequencing (Wu et al., 2015a), among other methods, has vastly increased the possible number of libraries which can be pooled and sequenced. Although Illumina have dominated the present NGS market, current global sequencing capacity is near to satiation thus as competing technologies are being introduced onto the market, the growth of Illumina in the future NGS market may not be as impressive as previous years.

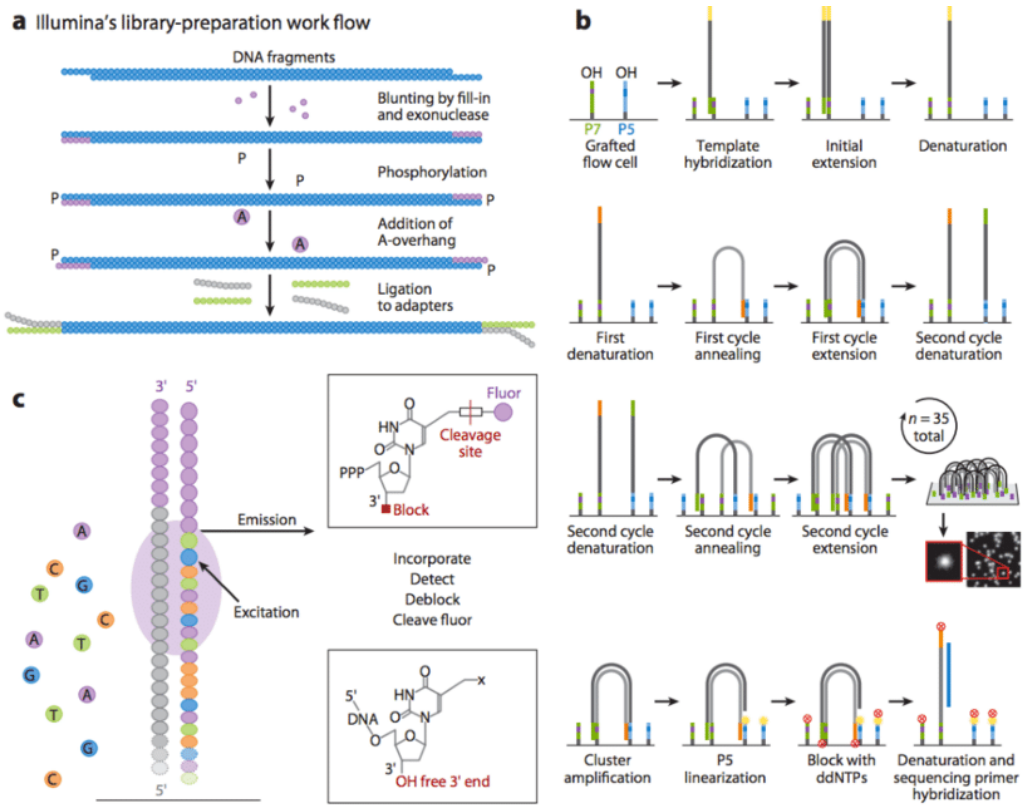

Figure 1.4. Illumina Sequencing Process. (a) Illumina® library-construction process. An extra A nucleotide is added to the 3' end of each strand of fragmented DNA (100-300 bp) to increase efficiency during the ligation process. Adapter sequences, which have a T overhang to increase efficiency are ligated to each end of the DNA fragment. Each fragment will then undergo size selection on an agarose gel to select for fragments within the desired ~200-400 bp range include in the final library. The DNA is PCR amplified using primer constructs required for the binding and clustering of the DNA fragment onto the flowcell. This adds a total of 53 bp to the fragment between the two ends (28 bp to the P5 end and 25 bp to the P7 end) resulting in a total adapter length of 119 bp between the two ends. (b) Illumina® cluster generation by bridge amplification. The single stranded DNA (ssDNA) is attached to the flow cell via covalently decorated complimentary sequences on the surface. During the annealing process, the ssDNA strand bends over and attaches to the second complimentary oligonucleotide sequence forming a bridge. DNA polymerase then synthesises the reverse strand, the two strands then release from one of the complimentary strands and straighten. Each strand then forms a new bridge and the process is repeated. After the final PCR round, DNA templates not attached to the flow cell are washed away leaving the synthesised strands attached to the P5 & P7 oligonucleotide sequences, each strand dissociates from the P5 complimentary oligonucleotide sequence and straightens allowing the release of the P5 ends in preparation of sequencing. (c) Sequencing by synthesis (SBS) with reversible dye terminators. The SBS technology uses four fluorescently labelled nucleotide bases to sequence millions of clusters on the flow cell surface. During each sequencing cycle, a single labelled deoxynucleoside triphosphate (dNTP) is incorporated into the synthesised chain. The removable blocking group attached to the 3' OH end of the ribose sugar on the nucleotide base acts as a terminator for polymerase. Unincorporated nucleotides are washed away and then lasers are passed over the flow cell to activate the fluorescent label on the nucleotide base, the resulting fluorescent dye is then imaged to identify the base using an optical scanner of charged couple device. The 3' OH group is chemically de-blocked and then the fluorescent groups are chemically cleaved to allow for the next incorporation event. The sequencing cycle is repeated many times (<300 nucleotide addition reactions depending on Illumina platform) to determine the sequence of each fragment, one base at a time. This information is the aligned and compared to a reference to produce sequence reads. Taken from Mardis et al. (2013).

Currently, NGS methods are the lead choice when characterising the community composition and functional potential of microbiome communities using amplicon sequencing, metagenomic or metatranscriptomic approaches, respectively. Whilst both amplicon sequencing and metagenomic approaches allow researchers to profile the taxonomic diversity of microbial communities, metatranscriptomics are applied in microbiome studies for gene expression profiling of the entire microbial population within a given sample (Figure 1.2). In 16S rRNA amplicon-sequencing; generic primers are used to amplify a desired hypervariable region on the 16S rRNA genes present within DNA samples. Following this, DNA libraries containing the 16S rRNA amplicons of interest can then be pooled (e.g. Illumina) and sequenced on a given NGS platform either by sequencing-by-synthesis or sequencing-by-ligation methods (Ghanbari et al., 2015). Generated sequence reads are then clustered into operational taxonomic units (OTUs) and subsequently classified against a curated database e.g. SILVA (Quast et al., 2013) to identify the bacterial taxonomy from which these reads originated from in the sample. Currently, two main bioinformatics software are used in fish microbiome research to process reads generated using NGS methods; mothur (Schloss et al., 2009) and QIIME (Caporaso et al., 2010).

16S rRNA amplicon-sequencing has frequently been applied in fish microbiome research to study the intestinal microbial community in different fish species, as well as how they respond to certain treatments/disturbances (Lyons et al., 2017a; Gupta et al., 2019; Kuebutornye et al., 2020). However, the short read length associated with 16S rRNA sequencing has been suggested to limit the taxonomic resolution of such studies (Claesson et al., 2010). As a result, identification of bacterial taxa within microbiome communities are often only reported down to class or family level. Further, the functional potential of given microbiome communities can only be predicted through computational analysis e.g. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013). Finally, profiled microbiome communities from 16S rRNA NGS studies also appear to be influenced by experimental design parameters related to DNA extraction method, sample collection and storage, sequencing platform and primer choice (Gorzelak et al., 2015; Castelino et al., 2017; Rintala et al., 2017; Wang et al., 2018b; Witte et al., 2018). To date, DNA extraction method, sample type and sample storage have been demonstrated to influence fish microbiome communities profiled using 16S rRNA NGS methods (Carda-Diéguez et al., 2014; Lyons et al., 2017a; Han et al., 2018). These experimental biases, among others, may partly explain the inter-individual variability in microbiome communities, which has been previously reported in several fish species including Atlantic cod (*Gadus morhua*) (Fjellheim et al., 2012), Atlantic salmon (Webster et al., 2018) and rainbow trout (Mansfield et al., 2010). However, further analytical work is required to refine 16S rRNA NGS methods to improve the accuracy and reproducibility of fish microbiome data.

Metagenomic approaches have recently been applied in fish microbiome research to sequence the taxonomic composition and functional potential of the intestinal microbiome in Atlantic cod and freshwater carp (*Labeo rohita*), amongst others (Riiser et al., 2019; Tyagi et al., 2019). In this approach; the entire collection of genomic material in a given sample is sequenced, irrespective of origin e.g. host and microbe. More specifically, genomic material (e.g. DNA) extracted from samples is used as a template and sequenced directly, without any previous amplification of marker genes e.g. 16S rRNA (Figure 1.2). The vast amount of sequence data generated is then classified against certain databases such as SILVA (Quast et al., 2013) to characterise the taxonomic composition within the sample. As entire microbial genomes can be captured and organised, metagenomic studies can characterise microbial communities down to species/strain levels (Mas-Lloret *et al.*, 2020), offering a considerable advantage over 16S rRNA amplicon sequencing approaches. In addition, the functional potential within given microbiome communities can also be profiled, as the entire collection of genes available within the community can be captured and subsequently queried using programs such as MEGAN (Mitra et al., 2011) or HUMAnN (Abubucker et al., 2012). This approach is not without barriers however, particularly with regards to cost, which can be considerably higher compared with 16S rRNA sequencing approaches as a higher sequencing depth is required to capture the entire collection of genomes present. Another challenge exhibited by metagenomics is that when sequencing DNA, this approach may also capture DNA from dead microbial populations (Tringe & Rubin, 2005), making it difficult to identify live microbial populations and the true functional potential of the microbiome community. A number of approaches have been developed to overcome this hurdle including the use of promidium monoazide (Erkus et al., 2016), which binds to free DNA (e.g. from dead cells) within a sample and prevents downstream sequencing. Another approach may be to sequence the entire RNA material within a sample instead of DNA, otherwise known as metatranscriptomics.

As described previously, metatranscriptomics allows the global transcriptome e.g. host and microbiome, within a sample to be profiled through massively paralleled sequencing of RNA. The approach follows similar steps to that discussed for metagenomics except RNA template material is isolated, converted to complementary DNA (cDNA) and subsequently sequenced (Figure 1.2). As a result, this approach can be extremely beneficial for capturing the diversity and composition of the viable microbiome community. Likewise, as this approach also utilises RNA rather than DNA material, it also profiles the entire gene expression within a given sample at a specific timepoint, under a specific condition. Thus, metatranscriptomics allows researchers to elucidate how the microbiome responds functionally to external stresses and how the host responds to changes in the microbiome community, respectively. This information is crucial to better understand host resilience and host-microbiome interactions. Metatranscriptomics also has

its challenges, particularly with regards to microbial messenger RNA (mRNA) structure. Unlike eukaryotes; prokaryotic mRNA lacks a poly-A tail, which prevents conventional cDNA synthesis through oligod(T) primers (Bashiardes et al., 2016). Likewise, microbial mRNA only represents a small fraction of the global RNA material within a sample which is often dominated by rRNA of microbial and eukaryotic origin. However, several strategies have been developed to enrich prokaryotic mRNA material for use in metatranscriptomics studies to overcome this problem (Tveit et al., 2014). Whilst this approach offers huge potential to better understand the diversity and functional potential of microbial communities, it has not yet been widely used in fish microbiome research with the exception of a small number of studies (Wu et al., 2015b).

1.3. The fish gut microbiome

1.3.1. Community composition

The gut environment of fish is a complex ecosystem estimated to harbour more than 10^{11} bacteria g^{-1} and include both autochthonous (resident, mucosa-associated) and allochthonous (transient, digesta-associated) bacterial populations, which play distinctive roles in host physiology (Nava & Stappenbeck, 2011; Navarrete et al., 2012). To date more than 500 different bacterial species have been detected in the intestinal tract of fish, primarily dominated by aerobic or facultative aerobes (Talwar et al., 2018). Despite this huge diversity of bacteria observed across the teleost lineage; evidence from numerous studies collectively suggest that the fish gut has relatively low phylogenetic diversity with up to 90% of the gut microbiome being represented by three bacterial phyla including Proteobacteria, Firmicutes and Fusobacteria (Talwar et al., 2018). The phyla Actinobacteria, Bacteroidetes are also present to a lesser extent followed by Tenericutes and Verrucomicrobia (Xing et al., 2013; Ghanbari et al., 2015; Lowrey et al., 2015; Llewellyn et al., 2016). There are a number of recent studies with exceptions to this, particularly with salmonid fish as both Atlantic salmon and rainbow trout have been shown to be dominated by Tenericutes (Lyons et al., 2017b; Fogarty et al., 2019).

Despite the presence of a core gut microbiome community in fish, a number of external factors can induce selective pressures on the microbiome community leading to differential abundance of individual taxa between fish species. For example, diet is suggested to modulate gut microbiome diversity in fish, increasing from carnivorous to herbivorous fish species, respectively (Liu et al., 2016). Likewise, differences are also observed in the actual composition of phyla in the gut microbiome community between fish with different feeding preferences. Whilst carnivorous fish species often display a dominance of Proteobacteria members e.g. Desulfovibrionales and Aeromonadales within their gut microbiomes, Firmicutes members e.g.

Clostridiales are observed more frequently in herbivorous fish species (Sullam et al., 2012). Further, there also appears to be a relationship between the microbiome community and external salinity conditions. In general representatives from *Aeromonas*, *Plesiomonas*, *Pseudomonas* and the families Enterobacteriaceae and Fusobacteriaceae appear to be dominant in the gut microbiome of freshwater fish, whereas *Vibrio* species as well as *Alteromonas*, *Micrococcus* and *Moraxella* display a more prominent position in the guts of marine species (Nayak, 2010; Talwar et al., 2018). Finally, environmental temperature appears to influence the nature of the gut microbiome in fish as some fish species have been observed to fluctuate both their community composition, and microbial load according to particular seasons (Naviner et al., 2006). For example, the gut microbiome communities of certain salmonid species e.g. Atlantic salmon and rainbow trout, appear to have increased representation of Gram-negative organisms e.g. Vibrionaceae in the warmer summer months whilst Gram-positive members e.g. Firmicutes, show greater abundance when temperatures fall during the colder months (Naviner et al., 2006; Zarkasi et al., 2014).

Endogenous selection pressures can also shape the gut microbiome community in fish. This was first proposed more than 60 years ago by Liston (1957) who suggested that the fish gut applies host-specific pressures to select the structure of the microbiome community. In the study, *Vibrio* representatives were found to dominate the microbial community colonising the guts of skate (*Raja* species) and lemon sole (*Pleuronectes microcephalus*), despite these species consuming different diets. It was argued that the microbiome cannot simply arise from mechanical transfer from prey alone, but that the gut conditions themselves exert selective pressures on the bacteria ingested. Since then, numerous studies have provided evidence for this theory which collectively suggests that host genetics play a crucial role in community assembly (Bledsoe et al., 2018). This can likely be attributed to the role of genetics in modulating gut histology and physiology which all interact to influence gut microbiome composition (Li et al., 2014a). In fact, genetic-based selection was recently reported to influence the gut microbiome communities in the freshwater fish species silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Aristichthys nobilis*) and blunt snout bream (*Megalobrama amblycephala*) by Li et al., (2012). In their study; microbiome communities were observed to cluster according to fish species, despite all being reared in the same production environment and on similar diets. Similarly, both wild and reared Atlantic salmon have also been shown to possess a gut microbiome structure that is greater influenced by developmental stage rather than geography (Llewellyn et al., 2016; Dehler et al., 2017). However, host genetic-based selection does not appear to have a critical role in microbiome community retention, as endogenous processes have been reported to be more pronounced in larvae and juvenile stages compared with adult stages. In fact, deterministic or genetic selection of bacterial communities was previously demonstrated

to account for up to 60% of the influence on community structure in larval individuals of grass carp, Chinese perch (*Siniperca chuatsi*) and *Silurus meridionalis* (Yan et al., 2016). However, these same influential processes were reduced to account for up to 33% in adult individuals of the same species. Similarly, in gibel carp (*Carassius auratus gibelio*), the gut microbiome has been shown to form distinct communities at different stages of development, with host filtering playing a larger role in this establishment at larval stages and weakening during development (Li et al., 2017). Both studies provide important data which suggest that whilst genetic-based selection may lead to the assembly of certain bacterial taxa in the gut of larval individuals; deterministic processes become less distinct as fish develop when drift or stochastic processes become the dominating influential factor.

1.3.2. Functional potential

Due to the importance of fish in food security and recreation, their microbiome communities have received considerably more attention than other non-mammalian vertebrates. However, to date, most research has been conducted to characterise the taxonomic landscape of the bacterial community in different fish species through 16S rRNA amplicon sequencing. As a result, our understanding surrounding the importance of the microbiome in host physiology and health primarily descends from predictions of functional potential through computation analysis e.g. PICRUSt (Talwar et al., 2018). Despite this, early microbiome studies employing germ-free (GF; those that are born and raised in the absence of microbes) or gnotobiotic (Gn; those colonised with selected/specific microbes) animals were instrumental in our initial understanding of the microbiome's role in fish (Cheesman & Guillemin, 2007). Likewise, there is now a growing trend in fish microbiome research for studies to utilise metagenomic or metatranscriptomic approaches to understand host-microbe interactions and microbiome functions. To date, the gut microbiome community has been associated to serve a variety of functions in the fish host which are important in brain signalling, behaviour and growth (Li et al., 2013; Borrelli et al., 2016), although a primary focus for a number of studies has been on the role of the gut microbiome in digestion/metabolism and disease resilience.

Acquisition, digestion and metabolism of dietary nutrients is vital for fish growth and survival. A number of studies have recently implicated the gut microbiome in serving important dietary functions within the fish host, particularly through microbial fermentation and nutrient synthesis. This appears critical for herbivorous fish species which feed on high plant diets containing complex carbohydrates, and corresponds with the higher bacterial diversity observed in these fish species. Whilst fish endogenously produce a number of enzymes for the hydrolysis and acquisition of simple and some complex carbohydrates (Krogdahl et al., 2005), they are unable to produce cellulase which is needed to breakdown cellulose, a primary structural component in

plant cell walls (Saha et al., 2006). However, cellulose fermenting bacteria such as *Clostridium*, *Citrobacter* and *Leptotrichia* have been reported in the guts of a number of herbivorous species including blunt snout bream and grass carp (Liu et al., 2016), suggesting a possible host-acquired function through the microbiome. Likewise, a high dominance of cellulase-producing *Aeromonas*, *Gordonia* and *Mycobacterium* were also found in the guts of wood-eating catfish (*Panaque nigrolineatus*) (McDonald et al., 2019). Desai et al., (2012) previously reported higher proportions of Firmicutes compared with Proteobacteria in the gut microbiome community of adult rainbow trout reared on a diet composed of plant-material, further cementing the role of the gut microbiome in supporting digestion and metabolism. Higher proportions of Firmicutes in fish fed plant-based diets has been associated with the accompanying increase in digestibility of plant materials (Salze et al., 2008). In fact, previous studies in herbivorous species such as carp have attributed high growth rates with a dominance of Firmicutes and Bacteroidetes, likely owing to improved digestion and metabolism of nutrients (Li et al., 2013). The prominent role of the microbiome community in nutrient synthesis is further evident in the detection of a number of phytase-producing bacteria including *Bacillus atrophaeus* and *Bacillus subtilis* in the guts of herbivorous and omnivorous fish species, which help metabolise the antinutritional factor phytate found in plants (Khan & Ghosh, 2012). In contrast to herbivorous fish, Proteobacteria members with lipase and protease activity e.g. Aeromonadales, Enterobacteriales and *Halomonas* have been reported in high abundance in the gut microbiomes of carnivorous species such as Atlantic salmon, brown trout (*Salmo trutta*), mandarin fish (*Siniperca chuatsi*), rainbow trout and topmouth culter (*Culter alburnus*) (Sullam et al., 2012; Liu et al., 2016). However, carnivorous fish gut microbiomes can also be populated by cellulolytic or phytase members similar to herbivorous fish through the mechanical transfer of microbes from their prey (Khan & Ghosh, 2012).

Members of the microbiome community also play vital roles in vitamin and energy synthesis, as well as mineral uptake. Fusobacteria and in particular *Cetobacterium* have been frequently observed to dominate the gut microbiomes of freshwater fish such as Amazonian piracucu (*Arapaima gigas*), common (*Cyprinus carpio*) and gibel carp, and Nile tilapia (She et al., 2017; Ramírez et al., 2018; Chang et al., 2019; Suphoronski et al., 2019), where they are thought to play a role in vitamin B-12 production (Ramírez et al., 2018). Similarly, Firmicutes members such as lactic acid bacteria (LAB) provide a source of short chain fatty acids (SCFA) to the fish host including acetic, propionic and n-butyric acids from plant-derived carbohydrates (Kihara, 2008; Komatsu & Sakata, 2016). Short chain fatty acid production by gut microbiome members has been demonstrated in several teleost species including common carp and Nile tilapia (Kihara & Sakata, 1997, 2002). In addition to serving in disease resilience as described later, SCFAs are also thought to serve in digestion and metabolism through (i) provision of energy for epithelial cells;

(ii) enhanced epithelial cell proliferation; (iii) increased surface area for absorption of minerals; (iv) modification of mineral transport mechanisms; and (v) increased absorption of trace elements e.g. phosphorous (Allameh et al., 2017; Hoseinifar et al., 2017).

The gut microbiome in vertebrates is also suggested to provide a first line of defence against communicable disease. This is thought to occur through colonisation resistance pathways which can either be "direct" through microbiome-pathogen interactions, or "indirect" through interactions with the host's innate and adaptive immune system. In the first pathway; the gut microbiome can inhibit colonisation of pathogenic bacteria through passive microbial-mediated inhibition or competitive exclusion, as well as active mechanisms (Kamada et al., 2013). Passive mechanisms employed by the commensal microbiome include reducing niche availability and occupying available binding sites within the intestinal environment (Buffie & Pamer, 2013). This has previously been demonstrated to exist in fish by Rendueles et al., (2012). In their study, authors demonstrated that the protective effective of two probiotic *Escherichia coli* strains against *Edwardsiella ictaluri* infection in Gn zebrafish (*Danio rerio*) was not correlated to enhanced inflammatory potential or production of bioactive antimicrobials, but instead to the presence of adhesion factors such F pili on the probiotic strains. Competitive exclusion can also be achieved through passively mediated nutrient limitation (Buffie & Pamer, 2013). It is thought that throughout host-microbiome evolution, commensal members have become better competitors in the complex microbial food-web that exists within the vertebrate intestinal environment. Therefore, the commensal community can effectively starve competing pathogens of available nutrients, making them less able to colonise the surface of the gut (Stecher & Hardt, 2008). Whilst this mechanism has already been demonstrated in higher vertebrate animals (Stecher & Hardt, 2011), further studies are required to explore whether similar mechanisms could also be employed by members of the fish gut microbiome. The microbiome can also mediate colonisation resistance actively through the production of toxic secondary metabolites such as antimicrobials e.g. bacteriocins, hydrogen peroxide and carbon dioxide (Gill, 2003; Stecher & Hardt, 2008). More recently it has emerged that SCFAs produced by the commensal microbiome also facilitate in the active colonisation resistance of pathogen establishment (Shapiro et al., 2014). A link between intestinal SCFA production and disease resistance has long been thought to exist in fish and has been demonstrated in Javanese carp (*Puntius gonionotus*), as fish reared on diets containing the LAB species *Enterococcus faecalis* displayed significantly elevated levels of intestinal SCFAs and reduced susceptibility to *Aeromonas hydrophila* (Allameh et al., 2017). These compounds are thought to reduce the pH within the immediate intestinal environment (Hoseinifar et al., 2017), which induces an inhibitory effect on the growth of certain bacteria. However, as indicated in the study by Allameh et al., (2017), this may not solely arise through intestinal pH modulation but also through interactions with the host's immune response.

To date, current knowledge on host-microbe interactions in fish mostly derive from work employing GF or Gn fish models. Collectively, evidence from these studies suggest that the microbiome community aids in "indirect" colonisation resistance through the development and maturation of gut-associated lymphoid tissue, and modulation of host immune functions. These effects are thought to arise through interactions with the *MyD88* signalling pathway (Cheesman et al., 2011; Galindo-Villegas et al., 2012), which is a key regulator in host-microbiome interactions. This pathway begins following detection of key molecular signatures termed microbe-associated molecular patterns (MAMP) e.g. microbial nucleic acid, β-glucan, peptidoglycan and lipopolysaccharides (LPS) (Magnadóttir, 2006; Ito, 2014), by intracellular or surface-bound pattern recognition receptors (PRR) e.g. toll-like receptors or nucleotide-binding oligo-merization domain- like receptors. Both commensals and pathogens can display MAMPs, which when detected by PRRs can exert stimulatory or suppressive effects on immune cells (Kelly & Salinas, 2017).

When compared with conventional animals (those with an established microbiome); Gn fish which lack a commensal microbiome show poor gut development and differentiation, altered gut physiology and delayed intestinal epithelial proliferation/turnover (Rawls et al., 2004; Galindo-Villegas et al., 2012). Gnotobiotic fish also display poor mucosal tolerance as they lack intestinal alkaline phosphatase at the brush border, an important enzyme responsible for LPS detoxification (Bates et al., 2007; Malo et al., 2010), suggesting a pivotal role played by the microbiome in immune homeostasis. The role of the gut microbiome in immune modulation and education has also been demonstrated using Gn fish, as individuals can display weakened or delayed expression of immune-related genes along with depleted populations of gut-associated immune cells (Cheesman & Guillemin, 2007; Galindo-Villegas et al., 2012; Tan et al., 2019). In Gn larvae, these characteristics have been accompanied by an increased susceptibility to diseases caused by spring viremia of carp virus and *Vibrio anguillarum* in zebrafish and European sea bass (*Dicentrarchus labrax*), respectively (Galindo-Villegas et al., 2012; Reyes-López et al., 2018). Single-species colonisation in Gn fish have highlighted potential roles played by the microbiome in modulating specific immune responses in the host. These include induction of serum amyloid a, which is an important acute phase protein (Rawls et al., 2004; Wei et al., 2013) and in higher vertebrates, has been associated with chemotaxis and priming of immune cells, and opsonisation of Gram-negative bacteria (Lee et al., 2017). Likewise Gn fish studies have also revealed microbiome-derived transcriptional responses in myeloperoxidase and complement component factor 3 (C3), which are important for neutrophil-derived antimicrobial compounds and immunoregulatory functions, respectively (Holland & Lambris, 2002; Castro et al., 2008). However, whilst some host functions can be served by different bacterial taxa colonising the gut, there is increasing evidence that specific bacterial taxa may be responsible for certain host

responses. For example; *A. hydrophila* and not *Pseudomonas aeruginosa*, was previously demonstrated to induce expression of C3 mRNA in Gn zebrafish to similar levels as seen in conventional individuals (Rawls et al., 2004). Likewise, in Gn zebrafish, upregulation of innate-related genes were induced only by conventional zebrafish-associated microbiome transplants and not microbiome communities transplanted from mice (Rawls et al., 2006). Furthermore, activation of the transcription factor Nuclear factor- κB (NF-κB) by *P. aeruginosa* resulted in increased expression of specific innate immune genes leading to enhanced neutrophil activity in zebrafish (Kanther et al., 2011). These specific host-microbiome interactions may arise through the production of certain SCFAs by different bacteria, as SCFAs were recently demonstrated to be important regulators of the vertebrate immune system (Shapiro et al., 2014), however further Gn work is required to better understand individual host-microbe interactions in fish.

1.4. Manipulating the fish gut microbiome community – a consequence for microbiome function?

1.4.1. Manipulating the environmental microbiome

Recirculating aquaculture systems (RAS) and Biofloc technology (BFT) are revolutionary forms of aquaculture, which utilise the environmental microbial community to minimise the exchange of rearing water, associated excess nutrients and pathogens with the surrounding environment. As such, these approaches are thought to provide environmentally sustainable alternatives to traditional aquaculture. In these systems, microbial reconditioning of the rearing water is vital as fish are stocked at high densities resulting in elevated concentrations of organic material in the form of fish waste, which can promote microbial growth (Aruety et al., 2016). Here, selection of K-strategist bacteria (competitive, slow growing) shifts the community from an autotrophic to heterotrophic state. This transition allows for a more stable microbial community to establish, which maintains water quality through nutrient recycling and inhibits the growth of r-strategists and opportunistic bacterial pathogens (Skjermo et al., 1997; Ahmad.H et al., 2016). In RAS, selection of K microbial communities is achieved by passing rearing water through heterotrophic biofilters (Vadstein et al., 2018). However, in BFT; a high carbon to nitrogen ratio within rearing water is conditioned by the addition of carbohydrate sources, manipulating the microbial community towards heterotrophic (K-strategist) communities (Liu et al., 2019). In addition, the high carbon conditions also promote nitrogen uptake into microbial biomass within the controlled microbial communities, which form bacterial “flocs” and provide a protein rich food source to fish, reducing artificial feed inputs (Pérez-Fuentes et al., 2016).

The microbial communities associated with live feed cultures are often comprised of opportunistic pathogens. As such, alteration of the associated microbial communities is critical to the

production of fish larvae and traditionally involve non-selective, temporary methods e.g. physical or chemical disinfection (Skjermo & Vadstein, 1999). More recently however, efforts have shifted towards targeted manipulation through probiotics and bacteriophages which have been associated with a decrease in opportunistic pathogens within associated microbial communities (Qi et al., 2009; Kalatzis et al., 2016). Live feed also appears to play a critical role in the delivery and establishment of the initial gut microbiome in fish larvae upon first feeding (Reid et al., 2009). As such, supplementation of live feed cultures with beneficial microbes e.g. *Lactobacillus* species or *Pediococcus acidilactici* has become common practice in hatcheries with beneficial effects observed in growth, mucosal immunity and stress tolerance of fish larvae (Carnevali et al., 2004; Rollo et al., 2006; Azimirad et al., 2016).

1.4.2. Genetics

Within aquaculture, genomic selection has been routinely applied to increase production through better growth and disease resilience, as well as selecting for desirable characteristics relating to flesh colour and body shape (Yáñez et al., 2015; Zenger et al., 2019). Recent evidence suggests genetic components may play a fundamental role in determining the gut microbiome community in fish (Li et al., 2018). This likely occurs as a result of the role played by host genes in modulating gut morphology and physiology, which can influence attachment sites and environmental conditions for the commensal community. The interaction between host genes and the gut microbiome supports the "hologenome" concept, which proposes that the host organism along with their commensal microbial community form one unit of selection (Zilber-Rosenberg & Rosenberg, 2008). Therefore, in line with this concept, it's possible that traits selected during genomic improvement programs may not solely arise through selection of host genetic components, but also through changes in microbial communities and their interactions with the host. Motivated by this theory, a number of pyrosequencing studies have recently investigated the interaction between genetic traits and the gut microbiome in fish. Findings from these studies have supported the hologenome theory, as significant differences were identified in the composition and diversity of the gut microbiome in *Flavobacterium psychrophilum*-resistant and susceptible lines of rainbow trout (Brown et al., 2019). Likewise, differences in microbiome structure and diversity have also been reported between cold-resistant and cold-sensitive blue tilapia (*Oreochromis aureus*) (Kokou et al., 2018). Whilst these studies have provided evidence to support a link between host genetic selection and the microbiome community structure; future work involving metagenomic and transcriptomic approaches is necessary to provide a better understanding of how these microbial changes contribute to the selected traits in the animal.

1.4.3. Diet

Developing sustainable feed has become a top priority for the aquaculture sector. As such, there has been a considerable rise in the number of studies investigating the influence of alternative protein sources on the gut microbiome of fish and host-microbiome interactions. Soybean meal (SBM) is one such alternative protein source used in commercial aquafeeds. However, increasing evidence suggests that the antinutritional factors and antigens present can disturb the gut microbiome of fish towards an undesirable community which has consequences for their immune response. This was recently demonstrated using 16S rRNA amplicon sequencing in northern snakehead (*Channa argus*) by Miao et al., (2018). In their study, fish fed increasing levels of SBM displayed a depletion in Firmicutes abundances within their gut microbiome, compared with control fish. This was accompanied by significantly upregulated expression of pro-inflammatory cytokines, indicating enteritis or mucosal inflammation within their intestinal tissue. Likewise, disturbed microbiome communities as a result of SBM or suboptimal dietary formulations e.g. high unsaturated fatty acid deficiency, have also been associated with reduced stress-resilience (Batista et al., 2016; Gatesoupe et al., 2016) as well as changes in gut functions related to metabolism (Gatesoupe et al., 2018). These changes could be microbial mediated, as fish fed plant-protein based diets exhibit alterations in their intestinal morphology including tissue damage and disruption to the lamina propria and mucosal folds (Wang et al., 2017). These changes alter the attachment sites on mucosal surfaces for commensal bacteria (Ringø & Gatesoupe, 1998), and therefore may impact on gut microbiome diversity or alter the community composition. Insect meal (IM) is becoming a common component in aquafeed as an alternative protein source with a high nutritional value (Magalhães et al., 2017). Whilst several studies have shown the potential for their use in manipulating the gut microbiome community in fish e.g. increased ratio of Firmicutes:Proteobacteria in rainbow trout (Bruni et al., 2018; Huyben et al., 2019), the influence of IM substitution on microbial-mediated functions in the fish host remains underexplored. However, as insects are rich in chitin, this component has been suggested to be targeted by beneficial commensal bacteria e.g. LAB, which in turn can improve the performance and health of the fish host (Bruni et al., 2018), thus this avenue offers a huge platform for future research.

1.4.4. Dietary supplements - probiotics & prebiotics

In view of the challenges associated with antimicrobial compounds; the use of alternative prophylactic measures to improve the health and resilience of farmed fish has received particular attention in recent decades. The ability of probiotic bacteria to induce changes in host physiology and function through the microbiome has been well documented in fish (Ridha & Azad, 2016; Ramos et al., 2017; Ghori et al., 2018). In particular, several studies have reported beneficial effects on fish growth following targeted microbiome manipulation e.g. increased Firmicutes

abundance, using probiotic *Bacillus* species, *Lactobacillus rhamnosus* or yeast species. Improved growth in fish is thought to occur directly through probiotic-induced, microbial-mediated regulation of the genetic components or endocrine signalling pathways involved in growth and appetite, respectively (Falcinelli et al., 2016; Giorgia et al., 2018; Jurado et al., 2018). Probiotic-induced increases in growth can also occur indirectly through microbial-mediated alterations in intestinal morphology (Elsabagh et al., 2018), which can lead to improved digestion and metabolism (Falcinelli et al., 2015; Liu et al., 2018). Gut microbiome alteration through probiotic bacteria can also provide an alternative approach to controlling for disease outbreaks in production systems by improving disease resilience in fish (Safari et al., 2016; Schmidt et al., 2017). This is thought to occur through their positive effects on immune homeostasis. When supplemented in the diet, bacteriocin-producing probiotic bacteria such *Bacillus* species and *Lactobacillus* species, have been reported to enhance immune pathways in fish through the stimulation of innate defences (Chen et al., 2019). Likewise, these groups of bacteria have also been demonstrated to induce adaptive defences through the promotion of mucin-releasing goblet cells (Topic Popovic et al., 2017) and regulation of pro-inflammatory cytokines (Ringø et al., 2018). As described previously, production of SCFAs by some probiotic bacteria also facilitate in the active colonisation resistance of bacterial pathogens by regulating intestinal pH levels. However, whilst SCFA production by probiotic bacteria has been demonstrated in fish (Asaduzzaman et al., 2018), the effect of SCFA on environmental conditions and pathogen establishment within the gut of fish remains to be explored *in vivo*.

Modulation of the gut microbiome through non-digestible fibres and other prebiotic compounds has received increased attention in recent years, since they have been reported to exert beneficial effects on numerous physiological parameters and functions within fish. Such effects include changes in intestinal morphology (Cerezuela et al., 2013), which has been associated with improved mucosal barrier function (Yang et al., 2018). 16S rRNA amplicon-sequencing has recently confirmed this effect in gilthead sea bream (*Sparus aurata*) fed sodium butyrate (Piazzon et al., 2017). In this study, increased microbial diversity and Firmicutes abundance in treated fish was associated with enhanced mucosal barrier function, leading to a decrease in intestinal parasitic load and improved disease resilience following experimental challenge with *Photobacterium damselae* subsp. *piscicida*. Enhanced disease resilience through prebiotic manipulation of the mucosal barrier has also been linked with enhanced immune pathways in certain fish species. In fact, both arabinoxylo-oligosaccharide and raffinose have been shown to improve immune parameters relating to phagocytosis, as well as respiratory burst and myeloperoxidase activities through enrichment of Firmicutes within the gut microbiome of fish (Geraylou et al., 2013; Xu et al., 2018).

1.4.5. Chemotherapeutants

Intensification of the global aquaculture industry to meet the growing demand for animal protein has been associated with increased disease outbreaks and a reliance on chemotherapeutants such as antibiotics for treating infections in production systems. However, as most antibiotics given in aquaculture display broad-spectrum activity (Armstrong et al., 2005), they may also have unintentional effects on non-target populations within the gut microbiome. This has been demonstrated using NGS in the gut microbiome communities of several fish species in response to antimicrobial compounds found within the aquatic environment (Narrowe et al., 2015; Gaulke et al., 2016). Likewise, NGS and 16S rRNA fingerprinting methods have also demonstrated microbiome disturbances in fish following treatment with a number of licensed antibiotics currently being used in aquaculture. For example, the gut microbiome of Atlantic salmon has been found to increase in diversity, albeit with different bacterial taxa e.g. Proteobacteria and Tenericutes, following antibiotic treatment with florfenicol and oxolinic acid, respectively (Gupta et al., 2019). Similar findings have also been reported for oxytetracycline, as low levels of this antibiotic compound has been reported to increase the diversity of gut microbiome communities in Nile tilapia (Limbu et al., 2018). Likewise, oxytetracycline has also been associated with detectable increases in the prevalence of Proteobacteria members within the gut microbiome of Senegalese sole (*Solea senegalensis*) (Tapia-Paniagua et al., 2015). However, this same antibiotic was found to reduce gut microbiome diversity and richness in Atlantic salmon and zebrafish, respectively (Navarrete et al., 2008; Zhou et al., 2018b). In addition, in the same study by Zhou et al., (2018b), sulfamethoxazole was not found to significantly alter the gut microbiome community richness or diversity in zebrafish. Collectively, evidence from these studies suggest that antibiotic compounds do not have universal effects on the gut microbiome communities of different fish species. Furthermore, gut microbiome responses are not universal for different antibiotic compounds, even within the same fish species. Thus, further research is required to better understand the relationship between antibiotics and the gut microbiome community in fish. This is particularly important for the global aquaculture industry due to the large diversity of fish species farmed, and the wide variety of antibiotic compounds used during treatment.

Fish are thought to possess a complex network of interactions with certain members within the gut microbiome to provide important functions such as colonisation resistance and immune regulation (Van der Waaij et al., 1971; Kelly & Salinas, 2017). This raises concerns for antibiotic treatments within aquaculture, as alterations in gut microbiome diversity and composition could have unintentional side effects on the health and physiology of the fish host. In aquaculture, this could have consequences for production. In fact, reduced microbial diversity in the gut has previously been associated with pathogen establishment in farmed ayu (*Plecoglossus altivelis*) during an experimental challenge with *V. anguillarum* (Nie et al., 2017). In their study, authors

attributed the establishment of *V. anguillarum* in challenged fish to the observed reduction in co-operative networks within the microbiome community. However, the reduced diversity seen in infected fish may have also increased the availability of vacant attachment sites, which could allow for the opportunistic pathogen to colonise and become established. Whilst antibiotic treatment was not investigated in this study; antibiotic-induced disruptions in gut microbiome community networks or diversity as described previously, may increase the likelihood of disease outbreaks in farmed fish.

Recent evidence from several fish species have also demonstrated that immune regulation and activity may also be disrupted by antibacterial agents through the associated disturbances in the gut microbiome. In a recent experimental study by López Nadal et al., (2018), zebrafish larvae treated with oxytetracycline were found to have shifts in gut microbiome community composition, accompanied by a reduction in the expression of IL-1β, an important pro-inflammatory cytokine. Similar immunosuppressive effects were also reported for zebrafish in response to the antibiotic olaquindox, as treated fish were found to have significantly lower relative expression levels of pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokines (He et al., 2017). In this study, disruptions in immune status were associated with a significant reduction in gut microbiome community diversity and shifts in the community composition in favour of Proteobacteria. Together, despite short-term pathogen control; the changes in gut microbiome homeostasis and immune potential may lead to long-term disruption in disease resilience of farmed fish, increasing the risk of future disease outbreaks. This particular consequence seems likely following the recent study by Zhou et al., (2018a) who reported increased mortality rates in zebrafish following treatment with oxytetracycline and subsequent challenge with *A. hydrophila*. In their study, an increase in mortality following *A. hydrophila* challenge was observed in antibiotic treated fish alongside significant changes in their gut microbiome. More specifically, treated fish were found to have reductions in gut microbiome community richness, associated with a decline in the abundance of Proteobacteria and Bacteroidetes compared with the control group. Similar findings were also reported by He et al., (2017) in zebrafish when treated with olaquindox, as treated fish displayed lower gut microbiome diversity but higher mortality rates following experimental challenge with *A. hydrophila*. Therefore, antibiotic treatment could undoubtably increase the risk of future disease outbreaks if the gut microbiome communities of treated fish do not recover. However, further research is warranted to determine in more detail, how changes in both the gut microbiome community and host-microbiome interactions can compromise the disease resilience of the fish host.

To date, most studies have investigated the immediate effects of antibiotic treatment on the gut microbiome community in fish. As a result, the recovery of the fish gut microbiome following

antibiotic treatment has been relatively underexplored. A recent study by Narrowe et al., (2015), did however reveal that the gut microbiome community of fathead minnows (*Pimephales promelas*) was able to re-stabilise following exposure to an antimicrobial compound. In their study, authors found that the differences in gut microbiome community structure seen immediately following exposure to triclosan, diminished after two weeks of withdrawal from the antimicrobial compound. In higher vertebrate animals, gut microbiome recovery has been found to vary considerably. For example, the gut microbiome in chickens (*Gallus gallus*), has been reported to recover following a 12-day withdrawal period (Videnska et al., 2013), whereas in humans, considerable differences in microbiome composition can remain for up to four years following antibiotic treatment (Jakobsson et al., 2010). Fish represent a diverse group of vertebrate animals encompassing ca. 30,000 species (Ravi & Venkatesh, 2018), with varied morphology, physiology and life histories. As a result, it is likely that the gut microbiome communities of different fish species may also vary in their recovery following antibiotic treatment. Further investigation is therefore required to better understand gut microbiome resilience and recovery across fish species. This is particularly pertinent for farmed fish species as antibiotic treatment is common practice within the industry.

1.5. Aims and objectives

The aim of this PhD study was to determine the effect of antibiotic treatment, a common aquaculture husbandry practice, on the distal gut microbiome community and function in farmed fish species. This thesis will test the hypothesis that oxytetracycline can alter the composition of the distal gut microbiome community, which disrupts host-microbiome interactions and gut health in fish. Two model fish species were studied as part of this investigation; Nile tilapia and rainbow trout, which are major contributors to either global or UK aquaculture production, respectively, and thus provide important representatives for the aquaculture industry. Results from this study will enhance knowledge on the inter-individual variability that exists within the distal gut microbiome communities of fish and the importance of sample processing in NGS-based gut microbiome studies (Chapter 2). Findings from this study will also improve our understanding of the longitudinal impacts of oxytetracycline, a licensed antibiotic for aquaculture, on the distal gut microbiome community in different farmed fish species (Chapters 3 & 4). Furthermore, this work will report on the short-term consequences of this same antibiotic on fish health, through changes in the relationship between the gut microbiome community and gut physiology (Chapter 4).

The following objectives were set out:

1. To optimise 16S rRNA amplicon-sequencing methods for profiling the gut microbiome community in fish (Chapter 2).
2. To describe the microbiome community established within the distal gut of Nile tilapia and rainbow trout (Chapters 2, 3 & 4).
3. To map the longitudinal changes in distal gut microbiome diversity and composition following antibiotic treatment in Nile tilapia and rainbow trout (Chapters 3 & 4).
4. To profile the dynamics of gut health status in Nile tilapia in response to antibiotic-induced microbiome community changes (Chapters 4).

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CHAPTER 2. Titrating bacterial DNA concentration improves characterisation of the fish gut microbiome but does not reduce inter-individual variation

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2.1. Abstract

Next generation sequencing (NGS) has become a popular tool to investigate the bacterial microbiome colonising fish. Despite the significant advances made in NGS, inter-individual variability in microbial community diversity and composition can limit the data analysis and clarity of findings from fish gut microbiome studies. The aim of the following study was to determine whether standardising bacterial DNA (bDNA) concentration in 16S rRNA libraries, could reduce individual variability in the distal gut microbiome community of rainbow trout (*Oncorhynchus mykiss*) characterised using the Illumina MiSeq platform. In this study, authors optimised a library preparation protocol targeting the V4 region of the bacterial 16S rRNA gene. In addition to traditional steps, this new protocol also included quantification and titration of bDNA in libraries to a standard 16S rRNA concentration. Titrating bDNA was found to improve the sequencing performance of titrated libraries and was associated with increased microbial diversity in most fish compared with non-titrated libraries. However, titrating bDNA was not found to reduce the inter-individual variability in the distal gut microbiome community profiles between fish, as this approach resulted in sample-specific changes in the alpha and beta diversity of gut microbiome communities. Despite this, titrating bDNA was associated with an increase in the detection and abundance of rare bacterial taxa (0.1% - 1% abundance). This enrichment in bacterial taxa was associated with a reduction in OTUs assigned to *Corynebacterium_1, Mycoplasma* and *Ralstonia*, which had a higher representation in the non-titrated libraries and dominated a negative sequencing control. Together, the results of the present study demonstrate the potential of titrating bDNA in 16S rRNA libraries. This approach can serve as an additional measure in microbiome studies to improve sequencing yield and the detection of bacterial taxa, whilst mitigating against the influence of foreign microbial DNA contamination. However, further work is required to better understand the sample-specific responses observed in this study, which will help to evaluate the use of bDNA titration as a technical approach to reduce the inter-individual variation in fish gut microbiome studies.

Keywords: rainbow trout, gut microbiome, amplicon-sequencing, bacteria, 16S rRNA, standardisation

2.2. Introduction

The mucosal surfaces of fish support a diverse assemblage of microorganisms, which along with their collective genomes and surrounding environment, constitute a microbiome community (Marchesi & Ravel, 2015). The largest of these mucosal-associated communities colonise the gastrointestinal tract (Navarrete et al., 2012), where it is thought to be shaped by both endogenous and exogenous factors including host genetics, environmental conditions and feeding types (Wang et al., 2018). Within this community, particular members contribute to the health and physiology of the fish host where they are involved in behavior, disease resilience, growth and nutrition (Salze et al., 2008; Li et al., 2013; Ingerslev et al., 2014; Borrelli et al., 2016; Kelly & Salinas, 2017). As the aquaculture sector intensifies to meet increasing demands for animal protein, there has been a growing interest to identify how aquaculture practices impact on the intricate relationship between the fish host and it's microbiome. This has ultimately ignited an explosion of next generation sequencing (NGS) studies profiling the fish gut microbiome.

The development of NGS has enabled researchers to profile the complexity of environmental and animal-associated microbiomes, at a greater resolution than ever before. This approach builds on the same principles as the early culture-independent methods e.g. denaturing gradient gel electrophoresis and Sanger sequencing, by targeting specific taxonomic markers such as the bacterial 16S rRNA gene to infer the genetic fingerprint of the microbial community within a sample (Wang et al., 2018). In this approach, also called 16S rRNA amplicon-sequencing, the diverse nucleotide sequence spanning one or several hypervariable regions (V1 – V9) of the bacterial 16S rRNA gene is targeted and sequenced. The generated sequence profiles are then assigned to individual bacterial taxa using curated taxonomic databases (Chaudhary et al., 2015). Recent advances in NGS has enabled hundreds of thousands of 16S rRNA sequences to be analysed from a single sample (Jesmok et al., 2016), thus providing the opportunity to detect both the dominant and rare members within the microbial communities investigated. This improved power coupled with reduced running costs and high-throughput capabilities (Clooney et al., 2016) has enabled NGS to become a standard approach when studying the fish gut microbiome.

As public awareness surrounding animal research intensifies, it is important that microbiome studies are designed with the minimal number of animals required to provide robust and reproducible data. This is in line with the 3Rs framework, which encourages efforts to reduce, refine and replace the use of animals in research (Bara & Joffe, 2014). However, previous studies in farmed Atlantic cod (*Gadus morhua*) (Fjellheim et al., 2012), wild Atlantic salmon (*Salmo salar*) (Webster et al., 2018) and farmed rainbow trout (*Oncorhynchus mykiss*) (Mansfield et al., 2010), have revealed that microbiome research in fish may be hampered by the variability that exists in the microbiome community diversity and composition observed between individuals.

This inter-individual variation in community dynamics can be problematic when it comes to data analysis, as it can mask changes between different treatment groups, impair the reproducibility of data, and ultimately impact on cross-experiment comparisons. To address this limitation, larger sample sizes may be required to improve the clarity of observations from such studies. However, this does not support the 3R's framework, which is increasingly being promoted in animal research within the United Kingdom by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (Burden et al., 2015). Whilst the individual differences in microbiome community likely arise through host factors (Wang et al., 2016); technical factors within the experiment e.g. sample processing, may exacerbate the problem of inter-individual variability within NGS-based fish microbiome studies. For example, both sample type and storage methods have been reported to alter the microbial communities characterised from fish gut samples (Carda-Diéguez et al., 2014; Lyons et al., 2017). Therefore, to reduce the use of animals in fish microbiome research, any attempt to minimise the degree of variability through technical approaches should be encouraged.

Most animal gut microbiome studies conducted to date have focused on standardising the amount of genomic DNA (gDNA) material, which is used as a template for 16S rRNA amplification prior to sequencing. However, as samples used in these studies are often derived from animal tissue and/or bodily fluids, the gDNA recovered will comprise of varied amounts of both host and microbial DNA (Feehery et al., 2013). Thus, generating DNA libraries using this approach could result in libraries with varied bacterial DNA (bDNA) template material e.g. 16S rRNA gene, for amplification and sequencing. This may subsequently interfere with the downstream analysis and increase the level of inter-individual variation observed in the diversity and structure of the microbial communities profiled. Despite this, the importance of standardising template bDNA material in 16S rRNA amplicon-sequencing studies remains to be explored for any vertebrate animal. Therefore, the primary objective of the following study was to determine the effect of standardising bDNA on the characterisation of the distal gut microbiome in rainbow trout. We hypothesised that standardising bDNA template between 16S rRNA libraries, would reduce the inter-individual variability of the final microbiome communities profiled, as each library would be given equal sequencing opportunity to reflect the actual microbial composition present. To test this hypothesis, real-time quantitative-PCR (qPCR) methods were applied to measure the bDNA yield in gDNA samples recovered from rainbow trout distal gut digesta material. Then, high-throughput sequencing was used to compare two 16S rRNA library generation protocols: (i) non-titrated (standard) and (ii) titrated according to 16S rRNA gene concentration (Figure 2.1). The level of variability between 16S rRNA libraries from each protocol was evaluated based on sequencing performance, as well as the alpha and beta diversity, and taxonomic profiles of the gut microbiome communities characterised.

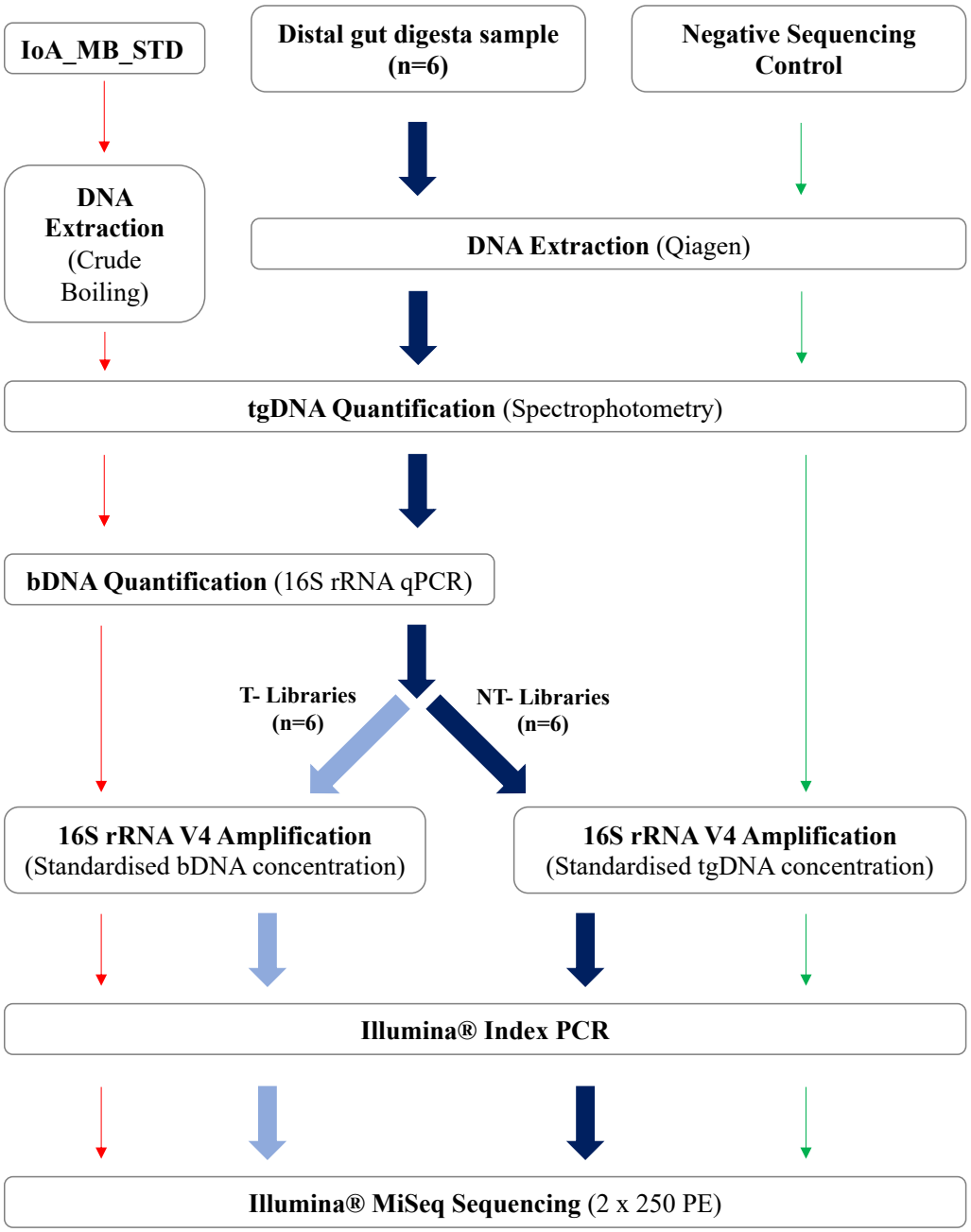

Figure 2.1. Experimental Design. This figure summarises the steps taken during 16S rRNA library generation. Distal gut digesta material from rainbow trout was used to generate two sets of 16S rRNA libraries, differing in the titration of genomic material. Titrated (T-) libraries were generated by titrating bacterial DNA (bDNA) template according to 16S rRNA concentration. Non-titrated (NT-) were generated using a standardised concentration of total genomic DNA (tgDNA) which comprise varying amounts of host and microbial genomic material. Both 16S rRNA library sets were indexed and sequenced to characterise the distal gut microbiome community. The IoA_MB_STD was included as a positive sequencing control and comprised DNA from five bacterial isolates. Nuclease-free water was used to generate a negative sequencing control to identify all possible sources of contamination within 16S rRNA libraries.

2.3. Methods

2.3.1. Fish collection and DNA extraction

Rainbow trout were obtained from a stock held onsite at the Niall Bromage Freshwater Research Unit (NBFRU) aquaria, University of Stirling (Stirling, UK). Prior to sampling, fish were reared in 300 L tanks, under 12:12 light:dark photoperiod and ambient water temperature (2.88 ± 1.55°C) for six days. Fish were reared on a commercial diet using T-Elite FR 4 mm complete trout feed (20% oil content, 39% protein content) (Skretting, France) at a feeding rate of 0.7% bodyweight day^{-1}. Feeding was terminated 16 hours prior to sampling. Fish had originally been sourced from a local trout farm. Following transport to NBFRU, all fish were subjected to a saltwater (Instant Ocean; Aquarium Systems®, France) treatment at a concentration of 2 g L^{-1} for one hour, followed by two separate Halamid (Tosylchloramide Sodium) (Axcentive SARL®, France) treatments, both at a rate of 5 ppm for one hour. This was recommended by the onsite veterinarian to treat a low protozoan parasite infection observed during the pre-transfer health check. A period of six days took place between the saltwater/ Halamid treatments and sampling. Gastrointestinal digesta (GID) material was collected from the distal gut of six adult rainbow trout, weighing approximately 145.3 ± 36.1 g. As there were no published guidelines for the minimal sample size required for microbiome studies at the time of conducting this study, this sample size was chosen to follow international recommendations for RNA-seq experiments (Schurch et al., 2016), which uses similar molecular methods.

Following death by a lethal dose of the tricaine methanesulfonate (Tricaine 1000 mg g^{-1} powder; Pharmaq®, UK), fish were sampled for GID material using a protocol described by Lyons et al., (2017): (i) fish were swabbed with 100% ethanol to remove skin-associated microflora which could potentially contaminate the GID samples; (ii) an incision was made into the abdomen from just below the gills towards the vent; (iii) the internal viscera were then exposed and checked for any gross abnormalities; (iv) after aseptic removal of fat deposits and pyloric caeca surrounding the gut tissue, the distal portion of the gut was located (Figure 2.2.) and aseptically removed using sterile scissors; (v) a total of 150 mg GID material was collected from each fish by squeezing the distal gut tissue with sterile tweezers into a sterile 2 mL microcentrifuge tube (Alpha Labs®, Hampshire, UK), containing 1 mL ASL lysis buffer (Qiagen®, Hilden, Germany). No intact feed pellets were observed within the GID material of any fish sampled. GID samples were transported on dry ice (ca. 30 minutes) to the Institute of Aquaculture, University of Stirling where they were stored at -70°C until DNA extraction.

Figure 2.2. Dissection of rainbow trout. Arrow indicates location of distal gut region sampled for gut digesta material.

Total genomic DNA from both host and microbial cells hereby referred to as tgDNA, was extracted from the GID samples using the QIAamp DNA Stool Mini Kit (Qiagen®, Hilden, Germany). Extraction of DNA was performed following the manufacturer's instructions, but with minor modifications as described by Lyons et al., (2017). First, GID components were disrupted using a Mini bead-beater 16 (Biospec®, Bartlesville, OK, USA) at maximum speed (3.5 x 1000 rpm) for four separate cycles of 30 seconds each. To improve the lysis and recovery of DNA from Gram-positive bacteria, the resulting suspension was then vortexed for 15 seconds and heated at 95°C for ten minutes in a static heat block. The amount of inhibitEX® tablets to bind PCR inhibitors in GID samples was reduced to 0.5 x tablets, which was added to the 700 µL supernatant from each sample. Lastly, the final tgDNA samples were eluted in 50 µL elution buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0), supplied in the Qiagen kit. After extraction, the concentration and purity of eluted tgDNA samples were analysed by spectrophotometry using the Nanodrop® 2000c (Thermo Scientific®, Glasgow, UK). Ten microlitre aliquots were prepared and stored at -20°C until required.

A microbial community standard called IoA_MB_STD was produced using the genomic DNA from five bacterial isolates (Table 2.1). The IoA_MB_STD standard was generated for use as a positive control during the quantification of bDNA step, and to calculate sequencing error following 16S rRNA amplicon-sequencing. The bacterial species used in the IoA_MB_STD were chosen as they had previously been detected in fish (Austin & Austin, 2012; Traoré et al., 2015), and where possible the reference strains were used as their full genome was publicly available. With the exception of the *Salmonella enterica* subsp. *enterica* isolate, the bacterial isolates were purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB) culture collection. The *S. enterica* subsp. *Enterica* isolate was obtained from the collection held onsite at the Institute of Aquaculture. All bacterial isolates had previously been stored at -70°C using the

Protect Microorganism Preservation system (Scientific Laboratory Supplies Limited, UK). Bacteria were recovered from bead stocks on general purpose agar and incubated at respective temperatures for 48 hours prior to performing purification and identification checks. Most bacteria were inoculated onto tryptone soya agar (TSA; Oxoid, UK) and incubated at 28°C, except for *Vibrio anguillarum* NCIMB 6 which was inoculated on TSA supplemented with 2% (w/w) sodium chloride and incubated at 22°C. Following purity checks where one type of colony forming unit was observed on agar plates, all recovered bacterial isolates were identified by primary biochemical tests. These tests included Gram stain, cytochrome-oxidase activity using oxidase strips (Oxoid, UK), motility by hanging drop method and the oxidation/fermentation test (Hugh & Leifson, 1953; Frerichs, G.N and Millar, 1993). All recovered isolates conformed to the expected biochemical profiles for respective species (Table 2.1) (Buller, 2004; VetBact, 2009). Following identification, bacterial isolates were inoculated into tryptone soya broth (Oxoid, UK), with or without supplementation with sodium chloride, and incubated for 18 hours at the respective temperatures described previously. Genomic DNA from each individual isolate was then extracted from bacterial suspensions in exponential growth phase following the crude boiling method similar to that described by Queipo-Ortuńo et al., (2008), except the final DNA was eluted in nuclease-free water. The concentration and purity of extracted DNA was analysed using the Nanodrop® 2000c. Finally, the IoA_MB_STD sample was prepared so that total genomic material from each bacterial isolate comprised 20% of the total abundance. The final IoA_MB_STD sample was serially diluted ten-fold to give a total concentration of 5 ng μL^{-1} using nuclease-free water and stored at -20°C until required.

Table 2.1. Composition and biochemical characteristics of bacterial isolates used in the synthetic microbiome community standard.

Bacterial isolate	Gram morphology	Motility	Oxidase	Oxidation/ Fermentation
Aeromonas hydrophila NCIMB 9240	Negative, short straight rods	Motile	Positive	Fermentative
Edwardsiella ictaluri NCIMB 13272	Negative, short straight rods	Non-motile	Negative	Fermentative
Salmonella enterica subsp. *Enterica*	Negative, straight rods	Motile	Negative	Fermentative
Vibrio anguillarum NCIMB 6	Negative, curved rods	Motile	Positive	Fermentative
Yersinia ruckeri NCIMB 2194	Negative, straight thin rods	Non-motile	Negative	Fermentative

Total genomic DNA was also extracted for a negative sequencing control (NSC) with the QIAamp DNA Stool Mini Kit, using nuclease-free water as a starting material and stored in AE buffer at -20°C until required. This sample extraction was prepared in an attempt to obtain DNA sequences from all potential sources of contamination in the DNA extraction and library preparation protocol.

2.3.2. 16S rRNA gene cloning and plasmid preparation

Plasmid DNA standards for the V4 16S rRNA hypervariable region were prepared and used in real-time quantitative-PCR (qPCR) standard curves. Bacterial DNA from *Aeromonas hydrophila* NCIMB 9240 was extracted using crude boiling methods as described above and the V4 hypervariable region of the 16S rRNA gene (length 292 bp) was amplified in a one-step PCR reaction using the primer set described by Lyons et al., (2017): 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Eurofins Genomics UK, Wolverhampton, UK). All PCR reactions were prepared to a total of volume of 25 µL and comprised of 12.5 µL 2X HS MytaQ mastermix (Bioline®, London, UK), 8.5 µL ultrapure water, 1 µL of each primer at 10 pM concentration and 2 µL (200 ng) of gDNA. The V4 16S rRNA hypervariable region was amplified in PCR reactions using the following parameters; 95°C for two minutes, followed by 30 x cycles of 95°C for 15 seconds, 53°C for 20 seconds and 72°C for one minute. A final extension cycle was performed at 72°C for two minutes. Amplified PCR products were then purified using the NucleoSpin® Gel & PCR clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and 30 ng of the purified PCR product was ligated into the pGEM-T Easy Vector system (Promega Corporation, Madison, USA) following the manufacturer's instructions. The ligation reaction containing V4 16S rRNA PCR products was then transformed into *Escherichia coli* strain DH5α (fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR1) (ThermoFisher Scientific, Basingstoke, UK) using a high efficiency transformation protocol described by New England Biolabs® with minor modifications. First, transformed cells were inoculated into 250 µL of Luria-Bertani (LB) broth and incubated at 37°C for three hours at low shaking speed (50 rpm). Following incubation, 200 µL of the transformed *E. coli* DH5α suspension was used to create a bacterial lawn on LB agar supplemented with 0.001% (v/v) ampicillin at a concentration of 50 mg mL^{-1}. Inoculated LB + ampicillin agar plates were incubated for 18 hours at 37°C.

After the 18-hour incubation; three recombinant colonies were processed for plasmid DNA extraction with the NucleoSpin® Plasmid QuickPure kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Briefly, individual colonies were isolated and inoculated into sterile 1 mL LB broth + 0.001% (v/v) ampicillin at a concentration of 50 mg mL^{-1} and incubated at 37°C for

six hours at 150 rpm. After incubation, bacterial suspensions were centrifuged at 11,000 x g for 30 seconds (Sigma 4K15c; Sigma Laborzentrifugen GmbH, Germany) before plasmid DNA was extracted following the manufacturer's instructions. The final plasmid DNA stock was quantified by spectrophotometry using the Nanodrop® 2000c. After extraction, 50 ng of plasmid DNA was screened for the presence of the 16S rRNA V4 gene insert by one-step PCR using the primers 515F/806R and conditions described previously. Following PCR amplification, PCR products were visualized on a 1.5% agarose gel under UV illumination. A plasmid DNA sample was confirmed as having the 16S rRNA V4 gene insert by the presence of a single band with a molecular weight of 292 bp. Finally, the plasmid DNA sample was serially diluted ten-fold in nuclease free water to concentrations containing $8.0 \times 10^8 – 8.0 \times 10^4$ V4 16S rRNA copies μL^{-1}. All plasmid DNA stocks were stored at -20°C until required.

2.3.3. Quantification of 16S rRNA gene copy number by real-time qPCR

Real-time qPCR was applied to quantify the absolute abundance of 16S rRNA genes in tgDNA samples as a measure of bDNA yield from GID samples. Prior to quantification, tgDNA eluted in AE buffer was re-dissolved in nuclease-free water using an ethanol-precipitation method. Briefly, the tgDNA sample was added at a volume of 1:2 into a suspension of 100% (v/v) ethanol and 0.3 M sodium acetate. The tgDNA:ethanol suspensions were stored overnight at -20°C to precipitate the tgDNA before being washed twice in ice-cold 70% (v/v) ethanol and centrifuged at 17,000 x g for 15-20 minutes to remove the sodium acetate. After allowing washed tgDNA pellets to air-dry, tgDNA was then resuspended in 30 µL nuclease-free water. Final tgDNA stocks were quantified by spectrophotometry using the Nanodrop® 2000c and stored at -20°C until required.

Real-time qPCR was performed on a Stratagene Mx3005P QPCR System (Agilent Technologies LDS UK Ltd, Cheshire, UK) using 96-well plates and the primer pair 515F/806R described previously. All qPCR reactions were prepared in triplicate for each tgDNA sample to a total volume of 20 µL and contained 10 µL 2X Luminaris Color HiGreen qPCR Master Mix (ThermoFisher Scientific, Basingstoke, UK), 2 µL (20 ng) template tgDNA, 0.5 µL of each primer at 10 pM concentration and 7 µL nuclease free water. The 16S rRNA copy number was also quantified from the IoA_MB_STD sample which was included in respective qPCR reactions as a positive control and at a concentration of 5 ng μL^{-1}. To confirm reagents and subsequent 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 three minutes, absolute quantification was conducted over 40 x cycles at 95°C for 15 seconds, 53°C for 30 seconds and 72°C for 20 seconds. 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

seconds. This analysis was conducted to identify any primer dimer formation and the specificity of amplified PCR products. Cycle threshold (Ct) values were generated using the fluorescence data for each qPCR reaction collected at the end of each extension cycle. The 16S rRNA gene copy number per 10 ng of tgDNA was calculated from the final Ct values in each qPCR reaction using the standard curve generated with serially diluted plasmid standards described above. The final real-time qPCR achieved correlation coefficients of 0.99 and efficiencies of 1.694. Total 16S rRNA copy number was then calculated per gram of GID sample.

2.3.4. Amplification of the 16S rRNA V4 hypervariable region and Illumina sequencing

Two Illumina-compatible libraries were generated using tgDNA from each GID sample and the 16S Metagenomic Sequencing Library Preparation protocol provided by Illumina® with minor modifications. Non-titrated (NT-) libraries were prepared from a total of 50 ng of template tgDNA using the primer pair 515F/806R with additional Illumina-associated adapters attached (Eurofins Genomics UK, Wolverhampton, UK). The Illumina adapter sequences for each respective primer were as follows; 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[515F sequence]-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[806R sequence]-3'. PCR reactions were prepared to a total volume of 30 µL and included 15 µL 2X NEBNext Q5 high fidelity master mix (New England Biolabs (UK) Ltd, Herts, UK), 3 µL of each primer (1 µM final concentration), 7 µL nuclease-free water and 2 µL (50 ng) of template tgDNA. The 16S rRNA V4 region (length 292 bp) was amplified in each tgDNA sample using the following one-step PCR conditions; 98°C for one minute, followed by 25 x cycles of 98°C for ten seconds, 53°C for 30 seconds and 65°C for 45 seconds. All PCR reactions underwent a final extension stage at 65°C for five minutes. All samples were amplified in triplicate 10 µL reactions and 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. Following the first round of amplification; 2.5 µL of purified PCR products from the first PCR was used as the template material for the second round of PCR. The second round of PCR amplification served to attach the dual index sequences and Illumina® sequencing adapters included in the Nextera XT index kit (Illumina®, California, United States) to amplified PCR products. The index PCR was performed in 25 µL reactions containing 2.5 µL purified PCR product, 12.5 µL 2X NEBNext Q5 high fidelity master mix, 2.5 µL of each Nextera XT index primer N7XX and S5XX, and 5 µL nuclease-free water. Dual indexing was conducted using similar PCR conditions to that described for the first round of PCR except the cycle number was reduced to eight. Barcoded PCR products were purified using the AxyPrep Mag PCR clean up Kit at the same volume used previously. Titrated (T-) libraries were prepared from each tgDNA sample as described above except in the first round of PCR; template bDNA was titrated and added at a concentration of 3.12×10^6 16S rRNA copies. Illumina libraries were also prepared for the IoA_MB_STD and NSC samples. The IoA_MB_STD 16S rRNA

library was prepared using the same concentration of 16S rRNA copies used in T-libraries, whereas the NSC library was generated using 2µL (3 ng) template tgDNA.

Final Illumina libraries (length 428 bp) which included dual indices, and Illumina® sequencing adapters were quantified fluorometrically with Qubit™, using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Basingstoke, UK). The 16S rRNA libraries were pooled in equal concentration (7.4 nM) and the final pooled library was quantified again with Qubit™, using the same high sensitivity dsDNA kit. The final pooled library was denatured in fresh 0.2 M NaOH and diluted to a final concentration of 20 pM in pre-chilled HT1 buffer (Illumina®). To increase the diversity within the final library and provide quality control data, the diluted library was combined with a PhiX control (Illumina®) and an internal sequencing control (IoA_Seq_CTL) containing genomic DNA from multiple sources, both at 4% of final library concentration. The final pooled library at 4 pM concentration was sequenced using the Illumina MiSeq® NGS system with the Illumina MiSeq® Reagent Kits v2 (500-cycle) at the Institute of Aquaculture.

2.3.5. Bioinformatics

Reads were demultiplexed with Casava v. 1.8 (Illumina®) and reads representing the PhiX/ IoA_Seq_CTL or reads not matching indices were removed. The open-source program Mothur (Schloss et al., 2009) was used to process the sequence read data generated. Demultiplexed 16S rRNA reads were merged using the make.contigs command. Resulting contig reads were quality filtered to remove those which contained ambiguous bases, homopolymers > 8 bp, and reads with sequence lengths > 315 bp. Remaining sequences were aligned to the SILVA SSU Ref NR [Release 132, December 2017] database (Quast et al., 2013) after customising the reference alignment to the specific V4 region amplified by the primers used in this study (length=292 bp). Sequences were aligned using the align.seqs command and was implemented using the default needleman alignment method. Sequence reads within the dataset were further denoised using the pre.cluster command allowing for up to three differences between duplicate sequences. To ensure accurate diversity estimates, further quality filtering was performed to remove reads with chimeric sequences as well as those classified to non-target taxa. Chimera removal was completed with the UCHIME (Edgar et al., 2011) algorithm in Mothur. Removal of undesirable sequences was completed using the remove.lineage command and was set to remove sequences classified as either “chloroplast”, “mitochondria”, “unknown”, “archaea” or “eukaryota”. Sequence error rate was calculated using the seq.error command in Mothur and utilised the sequence reads associated with the IoA_MB_STD sample. Sequence reads belonging to IoA_MB_STD sample were removed from the final dataset after calculating sequencing error. Taxonomic annotation was completed using the cluster.split and classify.otu commands. Final sequences were assembled into operational taxonomic units (OTUs) at a distance of 0.03 using the default opticlust algorithm

implemented in Mothur. Final OTUs were aligned to the SILVA-based bacterial reference alignment [Release 132, December 2017] with a minimum confidence bootstrap threshold of 80% for each assignment. The final dataset was rarefied to the lowest number of sequences per sample (e.g. 736,417) prior to performing any further downstream analysis. Sequence information from the NSC sample (10,197 sequence reads) was used as a reference of potential contaminating OTUs within GID samples and thus was kept separate from the final dataset.

2.3.6. Data analysis

Sequencing depth was assessed for each library using rarefaction curve analysis of Chao1 richness in Mothur. Rarefaction curves were visualised using the ggplot2 (Wickham, 2011) and reshape2 (Wickham, 2007) packages within RStudio Version 1.1.419. Alpha diversity metrics were also calculated for each library and included Chao1 richness, Inverse Simpson diversity index and Shannon diversity, as measures of OTU richness, diversity and evenness, respectively. Distal gut microbiome community structure in the NT-libraries and T-libraries was evaluated by creating a distance matrix of beta diversity using the thetaYC coefficient (Yue & Clayton, 2005) and Bray-Curtis dissimilarity (Bray & Curtis, 1957) calculators in Mothur. Distance matrices were visualised and compared between NT-libraries and T-libraries using non-metric multidimensional scaling (nMDS) with the Vegan package in RStudio Version 1.1.419. For all distance measures; PERMANOVA (vegan; adonis function) (Anderson, 2001) at 10,000 permutations, was used to test the influence of fish and 16S rRNA library method on the variation in distal gut microbiome community composition. Visualisation of top bacterial genera characterised using NT-libraries and T-library methods was performed using the ggplot2 package. Finally, metatstats (White et al., 2009), linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) and Indicator (McCune et al., 2002) analyses were performed within Mothur, to detect any discriminatory OTUs between NT-libraries and T-libraries, respectively.

2.4. Results

2.4.1. DNA recovery & sequencing performance

The tgDNA yield recovered from the six GID samples ranged from 42.1 and 466.2 ng μL^{-1} as seen in Table 2.2. DNA purity as determined by 260:280 and 260:230 ratios were acceptable for all tgDNA samples (Table 2.2). Bacterial DNA yield recovered from the GID samples ranged from 4.68 x 10^7 to 6.03 x 10^9 16S rRNA gene copies g GID^{-1} (Table 2.2). On closer inspection, the highest recovery of bDNA was observed from the GID samples of Fish 4, followed by Fish 3, which were also observed to recover the least tgDNA compared with all other fish. However, no clear trend was observed between tgDNA and bDNA recovery yield as Fish 2 which recovered the highest tgDNA was not found to have the lowest bDNA yield.

Table 2.2. DNA yield and purity, total and filtered sequencing reads, and operational taxonomic units (OTUs) detected in non-titrated (NT-) libraries and titrated (T-) 16S rRNA libraries.

Fish	tgDNA Yield[a]	DNA Purity		bDNA Yield[b]	Total Reads		Filtered reads		Observed OTUs	
		$_A$260:280	$_A$260:230		NT-	T-	NT-	T-	NT-	T-
1	243.9	2	2.31	8.76E+08	1.39E+06	1.19E+06	1.28E+06	1.08E+06	209	434
2	466.2	1.96	2.25	2.45E+08	9.00E+05	1.22E+06	7.36E+05	9.91E+05	147	377
3	92.2	1.89	2.15	1.63E+09	1.01E+06	1.28E+06	8.85E+05	1.14E+06	195	134
4	42.1	1.98	1.73	6.03E+09	1.36E+06	1.14E+06	1.28E+06	1.06E+06	240	134
5	302.9	2.03	1.9	1.80E+08	1.08E+06	1.15E+06	9.16E+05	8.78E+05	151	395
6	184.6	1.96	2.19	4.68E+07	1.14E+06	1.33E+06	9.50E+05	1.01E+06	252	670

[a] ng μL^{-1}

[b] Bacterial DNA (bDNA) measured as 16S rRNA gene copies g GID^{-1}

A total of 15,554,654 reads were obtained from the Illumina MiSeq® system with a minimum and maximum of 900,115 and 1,386,750 sequence reads per sample, respectively. The sequencing error rate for this experiment was calculated at 0.4%. Titrating 16S rRNA libraries to a standard bDNA concentration increased the number of reads generated in the 16S rRNA libraries and reduced the variability in the number of reads generated between samples (Table 2.2). More specifically, the average ± SD number of reads generated on the Illumina MiSeq platform in NT-libraries and T-libraries was 1,146,427 ± 194,006 and 1,216,426 ± 74,063 sequence reads, respectively. Further, following quality control, the average ± SD number of filtered reads were 1,008,845 ± 224,479 compared with 1,028,027 ± 91,442 for NT-libraries and T-libraries, respectively.

The number of OTUs detected from the NT-libraries was found to vary and range between 147 and 252 OTUs in Fish 2 and Fish 6, respectively (Table 2.2). Overall, T-libraries detected a higher

number of OTUs compared with the NT-libraries, although titrating bDNA further increased the variability of observed OTUs between fish as seen in Table 2.2. The average ± SD OTUs recorded in NT-libraries and T- libraries were 199 ± 44 and 358 ± 203, respectively. On further analysis, titrating bDNA was observed to have contrasting effects on the number of OTUs detected in 16S rRNA libraries depending on the starting concentration of bDNA material. Whilst the highest number of OTUs were found in the T-library of Fish 6, which initially recovered the least bDNA, the lowest number OTUs were found in T-libraries from Fish 3 and Fish 4, which had the highest bDNA yield following DNA extraction (Table 2.2). All present microbial phylotypes were captured using both library methods as all 16S rRNA libraries reached similar goods coverage values of > 99%. Moreover, both library methods shared 393 OTUs which represented 99.94% and 99.78% of the total abundance in NT-libraries and T-libraries, respectively. However, both library methods also detected a unique set of rare OTUs which collectively represented less than 1% of the total sequence abundance in each group. The total number of unique OTUs was increased when bDNA was titrated with 415 and 1,104 unique OTUs detected in NT-libraries and T-libraries, respectively.

2.4.2. Alpha diversity

Rarefaction analysis of Chao 1 richness demonstrated a greater level of microbial diversity in the T-libraries of four out of six fish compared with their NT-libraries, respectively (Figure 2.3). However, titrating bDNA in 16S rRNA libraries had the opposite effect for Fish 3 & Fish 4 as both T-libraries displayed lower slopes than their NT-library counterparts. All curves reached saturation phase indicating similar sequencing depth between the NT- library and T-library groups (Figure 2.3). Similar to the findings observed for detected OTUs, there was a low level of variation found in the alpha diversity of the NT-libraries from the six fish as seen in Figure 2.4. Overall, titrating bDNA had a positive effect on alpha diversity as T-libraries from Fish 1, 2, 5 & 6 were found to have increased community richness, diversity and evenness compared with their NT-libraries, respectively (Figure 2.4). However, again titrating bDNA was found to increase the variability observed in alpha diversity measures between fish, following findings in observed OTUs described previously. This was likely associated with the non-linear change in alpha diversity found between individual pairs of NT-libraries and T-libraries (Figure 2.4). Whilst alpha diversity patterns remained similar between fish, the increase in alpha diversity within T-libraries was highest when the alpha diversity was already high in the NT-library. This can be seen in Fish 6 for all alpha diversity measures (Figure 2.4). Furthermore, titrating bDNA was also found to have a negative effect on alpha diversity for Fish 3 and to some extent Fish 4, whereby T-libraries displayed lower community richness and diversity than their NT-library counterparts (Figure 2.4).

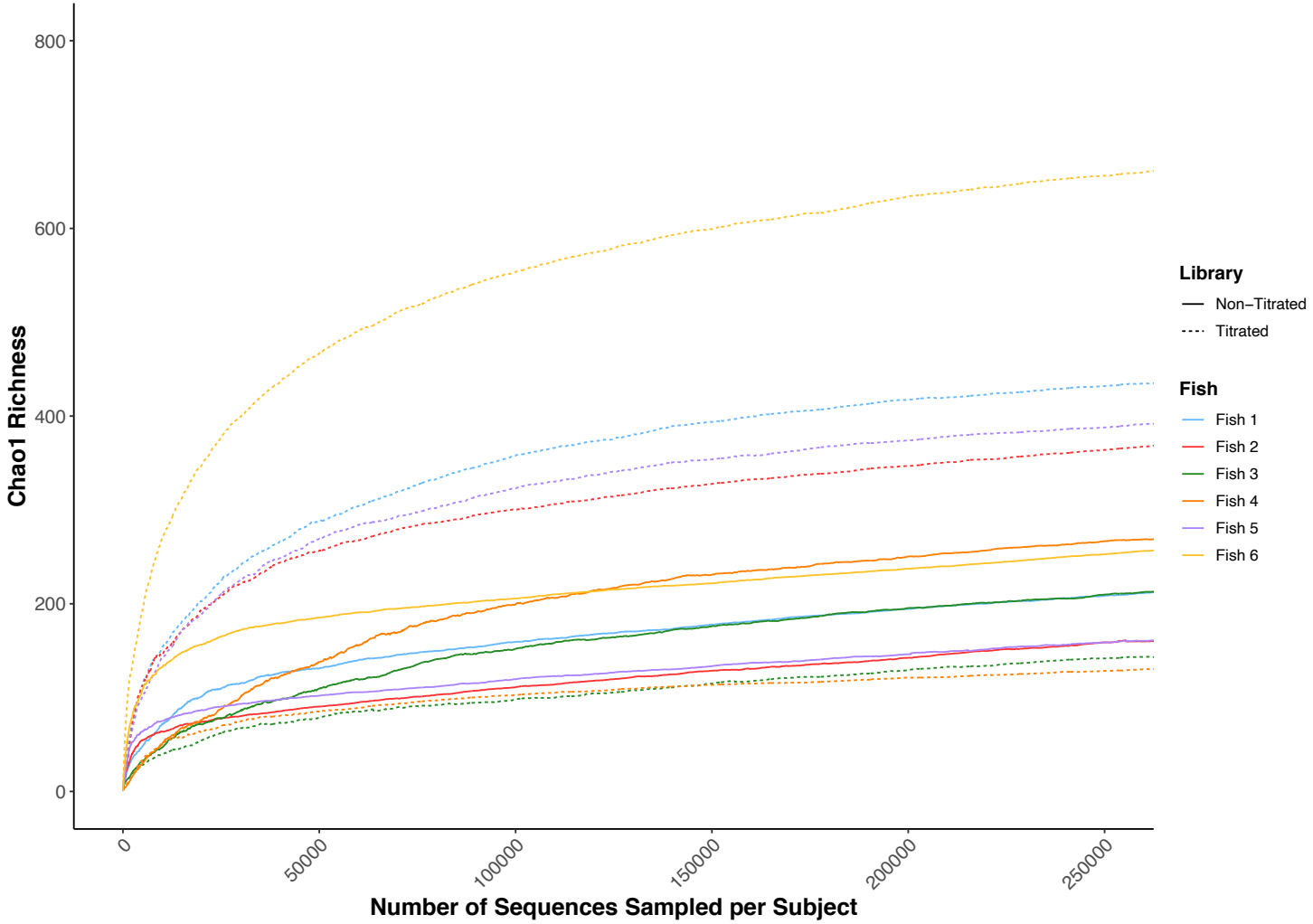

Figure 2.3. Rarefactions curves for individual fish sampled, and characterised from either non-titrated or titrated 16S rRNA libraries. Curves represent Chao1 richness per sample as a function of the sequencing effort. OTUs are clustered according to a 97% sequence similarity cut-off value.

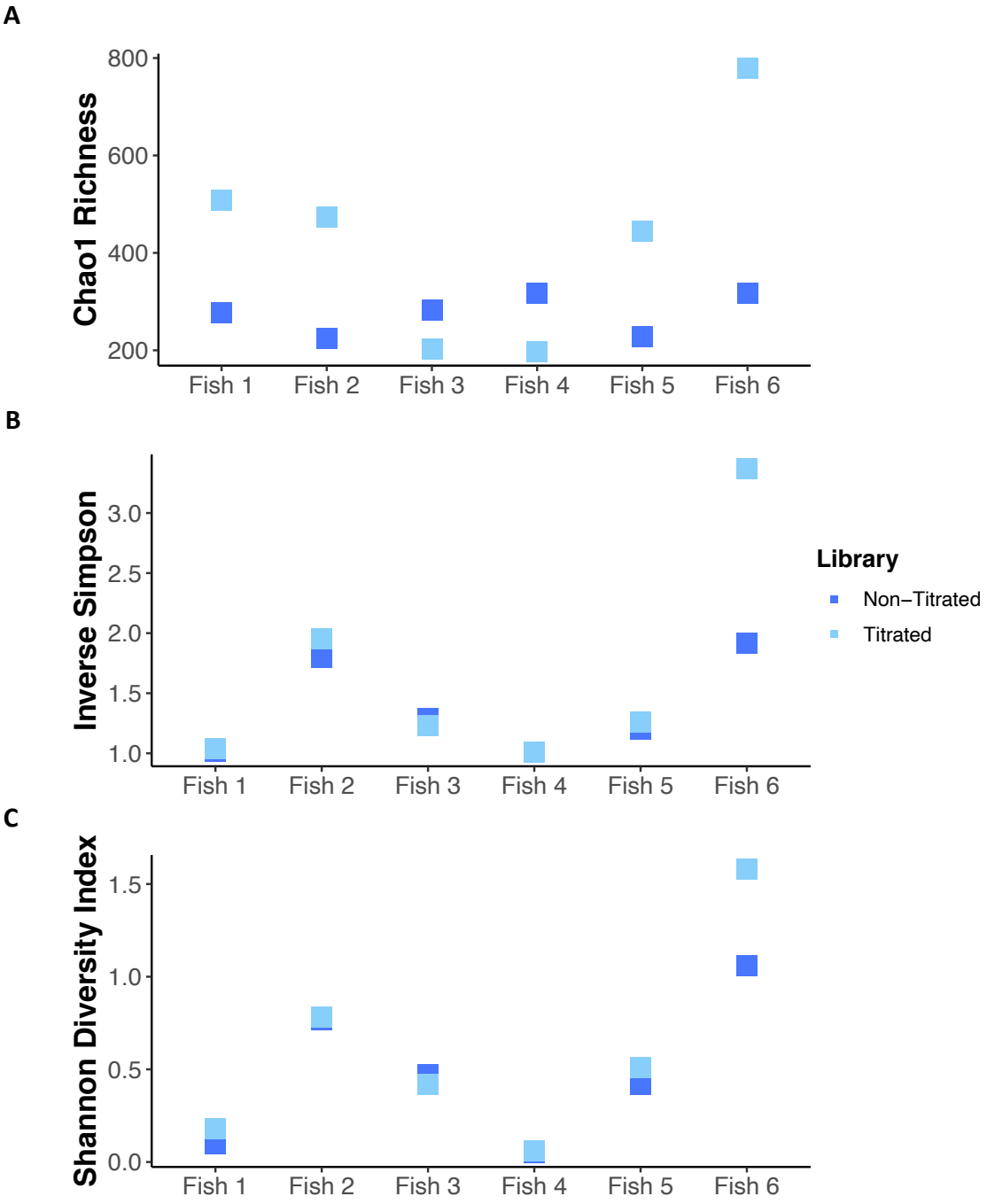

Figure 2.4. Alpha diversity measures of the distal gut microbiome in rainbow trout characterised from either non-titrated or titrated 16S rRNA libraries. Chao1 richness estimations (A), Inverse Simpsons diversity measures (B) and Shannon evenness (C) are shown for each fish. Colour indicates 16S rRNA library type.

2.4.3. Beta diversity

The relationship between individual sample microbiome community structure, and the effect of titrating bDNA was examined using the nMDS of ThetaYC and Bray-Curtis distance matrices (Figure 2.5). A significant amount of inter-individual variability was observed in both microbial community membership (ThetaYC) (Figure 2.5 A) and composition (Bray-Curtis) (Figure 2.5 B) between fish (PERMANOVA; ThetaYC $p = 0.007$; Bray-Curtis $p = 0.0003$). On closer inspection, whilst distal gut microbiome communities from Fish 1, 3, 4 and 5 clustered close together, Fish 2 & Fish 6 were found to form separate clusters away from other fish, both in community membership (Figure 2.5 A) and composition (Figure 2.5 B). When 16S rRNA library type was taken into consideration, titrating bDNA was not found to have an effect on beta diversity of the microbiome communities profiled (PERMANOVA; ThetaYC $p = 0.67$; Bray-Curtis $p = 0.3$) and did not reduce the inter-individual variability observed in beta diversity between fish (Figure 2.5 A and B). Despite this, titrating bDNA was observed to visually shift the microbiome community membership and composition of Fish 2 & Fish 6, indicating sample-specific responses in beta-diversity to bDNA titration.

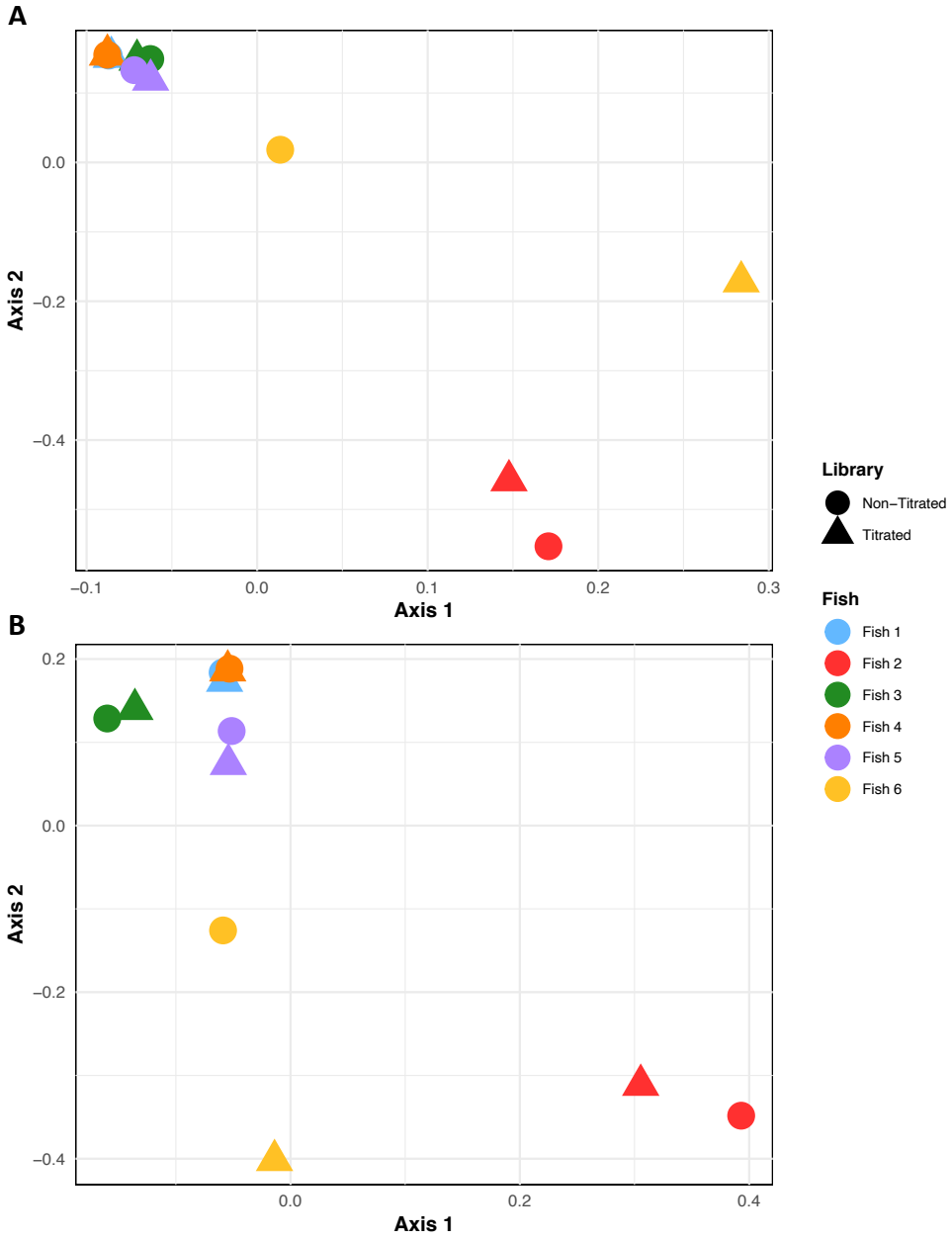

Figure 2.5. Non-metric multidimensional scaling on ThetaYC (A) and Bray-Curtis (B) distances of the distal gut microbiome in rainbow trout characterised from either non-titrated or titrated 16S rRNA libraries. Each point represents a single sample. Colours and shapes represent the origin of each sample.

2.4.4. Microbial community

The microbiome community of rainbow trout in this study was dominated by four bacterial genera, which in order of abundance were *Mycoplasma*, *Brevinema*, *Clostridiaceae_1_unclassified* and *Deefgea* (Figure 2.6). All four genera were identified in both sets of 16S rRNA libraries where they represented a mean sequence abundance of 97.68% and 97.27% in NT-libraries and T-libraries, respectively (Table 2.3 (S1); Table 2.4 (S2)). Variability was noted amongst NT-libraries in the sequence abundance of the four dominant genera (Table 2.3 (S1)). This was particularly seen in Fish 2 & Fish 6 which were found to have considerably higher and lower abundances of *Brevinema* and *Mycoplasma* compared with other fish, respectively. Titrating bDNA in T-libraries was found to reduce the variability observed between fish in the sequence abundance of *Brevinema* and *Deefgea* (Table 2.3 (S1); Table 2.4 (S2)). However, T-libraries were observed to have greater variability in the sequence abundances of *Clostridicaeae_1_unclassified* and *Mycoplasma*, compared with NT-libraries, respectively (Table 2.3 (S1); Table 2.4 (S2)).

Collectively, a higher number of sequences were assigned to *Brevinema* and *Clostridicaeae_1_unclassified* in T-libraries compared with NT-libraries (Figure 2.6). Titrating bDNA also increased the sequence abundance and detection of rare bacterial taxa within the gut microbiome community of rainbow trout, as the total number of genera recorded at a sequence abundance of 0.1-1% increased from 40 to 48 in NT-libraries and T-libraries, respectively (Figure 2.6). Again, variability was observed in the presence and abundance of rare genera detected within NT-libraries (Table 2.3 (S1)), and this was not found to be reduced in the T-libraries due to differential responses across bacterial genera (Table 2.4 (S2)). However, the overall increase in bacterial diversity found in T-libraries was associated with a reduction in the sequence abundance of three bacterial genera. These included *Mycoplasma*, *Ralstonia* and *Corynebacterium_1*, which decreased in sequence abundance from 79.22% to 75.94%, 0.84% to 0.39% and 0.09% to < 0.01%, in NT-libraries and T-libraries, respectively (Table 2.3 (S1); Table 2.4 (S2)). On closer inspection, all three genera were found to dominate the microbial community within the NSC sample where they represented 4.13%, 60.59% and 10.47% of the sequence abundance, respectively (Figure 2.7; Table 2.5 (S3)).

Figure 2.6. The mean relative abundance of bacterial genera observed in non-titrated (A) and titrated (B) 16S rRNA libraries generated from the gut digesta of rainbow trout (n=6). Pie plot represents dominant bacterial genera (> 1%). Bar plot represents minor bacterial genera (0.01 to 0.1%).

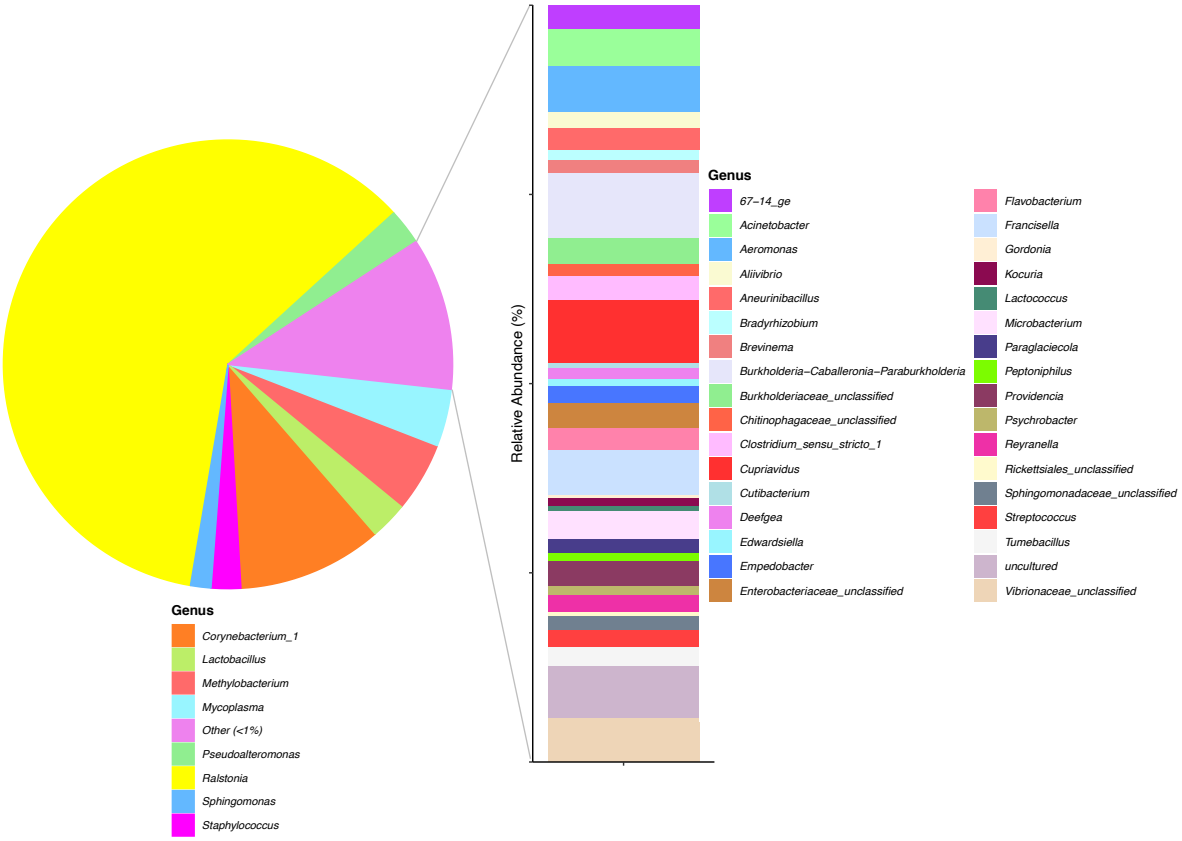

Figure 2.7. The relative abundance of bacterial genera observed in the negative sequencing control. Pie plot represents dominant bacterial genera (> 1%). Bar plot represents minor bacterial genera (0.01 to 0.1%).

Differential abundance testing was used to identify OTUs that significantly differed between the NT-libraries and T-libraries in this study ($p < 0.05$; Table 2.6 (S4)). Overall, T-libraries had a higher number of OTUs that were found to have a significantly elevated sequence abundance compared with NT-libraries (Figure 2.8), indicating greater detection sensitivity when bDNA was titrated. In contrast, only four OTUs were found to have significantly depleted sequences within T-libraries (Figure 2.8). A comparison between NT-libraries and T-libraries revealed OTU11, assigned to *Corynebacterium_1*, to be discriminatory between library preparation methods. Sequences assigned OTU11 became significantly depleted in T-libraries, displaying a log2 fold abundance of -6.95 (Figure 2.8). On further inspection, this OTU was found to dominate the NSC, where it represented > 99% of the reads assigned to *Corynebacterium_1* and 10.46% of the total microbiome community (Figure 2.7). The OTUs; OTU1 and OTU5, which contributed the majority of reads assigned to *Mycoplasma* and *Ralstonia*, respectively, were also investigated due to their observed higher abundance in the NT-libraries and NSC compared with T-libraries. However, despite sequences assigned to these OTUs becoming depleted when bDNA was titrated (Figure 2.8); OTU1 and OTU5 were not found to be discriminatory according to Metastats, LEfSe or Indicator analyses (Table 2.6 (S4); $p > 0.05$).

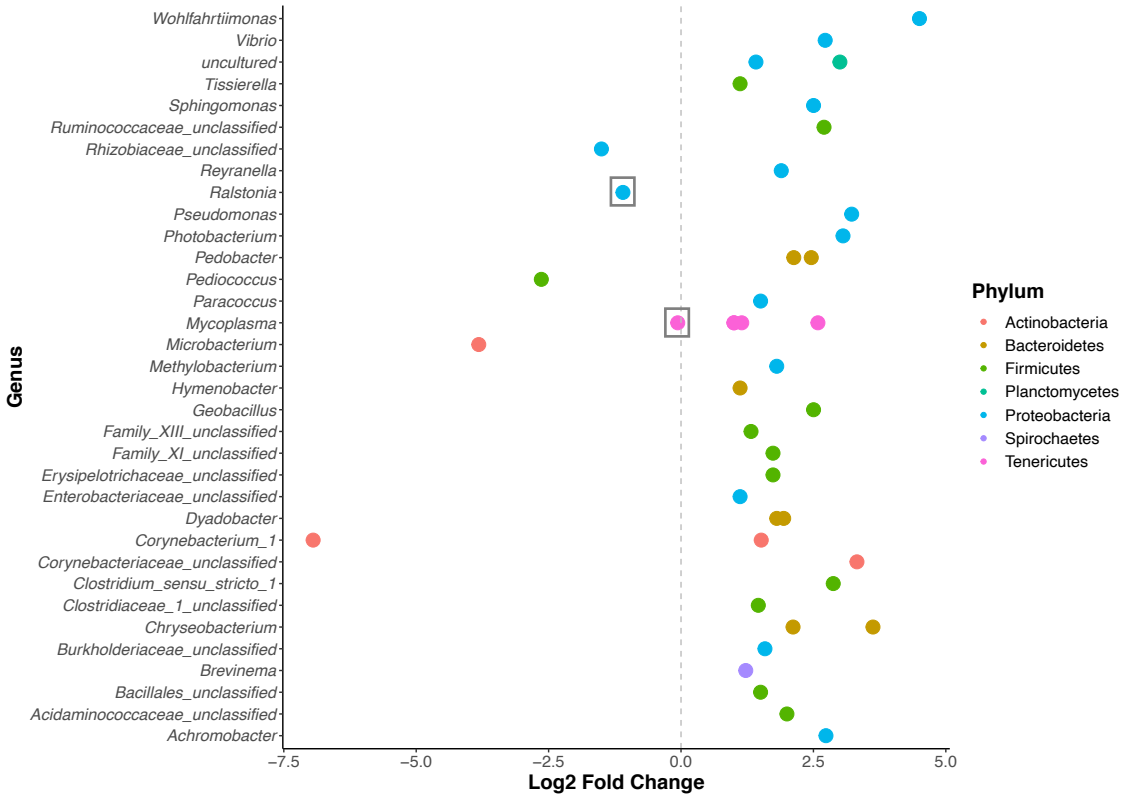

Figure 2.8. Plot of operational taxonomic units (OTU) that were significantly differentially abundant ($p < 0.05$) between library method. Effect size is represented as the log2 fold-change of each OTU observed in titrated 16S rRNA libraries compared with non-titrated 16S rRNA libraries. Each circle represents a single OTU and is coloured according to the phylum to which the OTU originates. A surrounding square indicates the OTUs which did not have significantly different sequence abundance between non-titrated and titrated 16S rRNA libraries.

2.5. Discussion

Findings from this study demonstrate that bDNA yield can differ between the GID material from the distal gut of individual rainbow trout. This was likely associated with DNA extraction bias, and has previously been suggested to arise from a number of factors including the complex physiochemical nature of the GID, which can contain inhibitory endogenous compounds e.g. aromatic acids (McOrist et al., 2002; Thomas et al., 2015). Co-extraction of inhibitory components can reduce the quality of DNA extracted and negatively affect downstream processes within these studies (Claassen et al., 2013). In addition, the complex configuration of the microbiome itself can also influence the recovery of bDNA from GID material. This is thought to occur as a result of the uneven distribution of bacterial members within the gut microbiome, as well as the variability in their cell wall structure and integrity (Wesolowska-Andersen et al., 2014). The variation in bacterial cell wall properties in particular can result in DNA extraction bias due to differences in cell lysis efficiency (Yuan et al., 2012), ultimately impacting on the recovery of bDNA within GID samples. No clear trend was observed in this study to demonstrate a relationship between the tgDNA and yield of bDNA recovered from GID material. However, the two samples with the lowest recovery of tgDNA were found to contain the highest concentration of recovered bDNA. Whilst the amount of starting material used for DNA extraction was standardised, it's possible that the samples with a higher recovery of tgDNA were more contaminated by the co-extraction of host DNA. This could occur potentially through the presence of gut mucus within the GID sample as this secreted layer covers the intestinal epithelium, and thus could have also been collected during the squeezing of intestinal tissue to retrieve the GID material. Furthermore, as the mucous layer can contain live/dead cells as well as a range of biologically active components of host origin (Gómez & Balcázar, 2008), this layer could have generated a large amount of host genomic material during the DNA extraction process. Whilst the level of host DNA was not measured in this study, the evidence from this study highlights the need for better sampling techniques or microbial DNA recovery methods for microbiome studies, which reduce host DNA contamination.

Titrating bDNA in 16S rRNA libraries was not found to reduce the inter-individual variation observed in gut microbiome diversity in this study. Furthermore, in the case of alpha diversity, titrating bDNA was even found to increase the variation observed between individuals. In general, the nature of the titration protocol used in this study, could explain the increased variability observed in OTU number and Chao1 richness estimates. Previous investigation using whole genome sequencing has revealed a huge diversity in the 16S rRNA gene copy number (GCN) of bacteria, which can range from one copy per genome to over ten in some taxa (Louca et al., 2018). In addition, variations in GCN can even exist between strains of a single species (Jung et al.,

2017). Therefore, titrating bDNA in T-libraries according to 16S rRNA concentration, could have artificially selected for bacterial taxa with a certain threshold of 16S rRNA GCNs in their genome. For example, taxa with higher GCNs of 16S rRNA may be given disproportionately greater opportunity for detection, either during the amplification or sequencing stages of microbiome characterisation. As individual fish can already vary in their gut microbiome community membership, the inter-individual variability in gut microbiome diversity can therefore be further increased during the titration of bDNA due to the variation in 16S rRNA GCN found between the different bacterial taxa. The titration protocol could also explain the reduction observed in community richness between NT-libraries and T-libraries of Fish 3 and Fish 4 in this study. As these samples contained the highest bDNA concentration, a much larger dilution was required for these samples to achieve the required concentration of 16S rRNA genes to be added into the PCR reactions. It is therefore possible that sample dilution had an effect on these particular T-libraries, leading to the observed changes in gut microbiome diversity. Diluting samples prior to PCR amplification has previously been suggested to be problematic in NGS-based microbiome studies, as it can dilute the DNA molecules from less abundant taxa (Castle et al., 2018). This further reduces the opportunity of these organisms to be amplified and sequenced. In the present study, if certain organisms (or OTUs) were already detected at low abundances in the NT-libraries of Fish 3 and Fish 4, the dilution of tgDNA samples could have ultimately led to these organisms not being detected in the final microbiome community profiled from the respective T-libraries. This seems likely as sample dilution has previously been demonstrated to induce moderate bias on the microbiome characterised from human fecal samples (Velásquez-Mejía et al., 2018). However, further work is required to explore the relationship between sample dilution and patterns of microbial biomass during fish microbiome characterisation.

Findings from this study also showed that fish host had a much stronger influence on the distal gut microbiome community structure compared with variation in bDNA template concentration. Variations in beta diversity can arise through differences in environmental conditions such as salinity or diet which can shape the microbiome community of fish (Sullam et al., 2012). However, as fish in this study were reared in the same production system; endogenous factors likely exerted a stronger influence on the individual variability in microbiome communities in this study. For example, host genetic variation, which has previously been reported to result in distinct gut microbiome communities between fish species (Li et al., 2012), may also be responsible for the variation observed between individuals of the same fish species. This is highly likely given the role played by the host's genetic components in modulating gut histology and physiology, which interact to shape the microbiome community (Li et al., 2014). As fish were sourced from a local trout farm which at the time had multiple stocks from different sources, it is possible that there was some degree of genetic variation between individuals as fish could have

come from different genetic populations. This genetic variation could have resulted in differences in gut morphology between individual fish in this study. Furthermore, as particular morphological features such as the lamina propria and mucosal folds serve as important attachment sites for the commensal community (Ringø & Gatesoupe, 1998), changes in these features could lead to different microbial loads and diversity within the distal gut microbiome of individual fish. These morphological differences may have attributed to the distinct microbiome community structures observed for Fish 2 and Fish 6 in this study, as they formed separate clusters away from other fish despite all being reared in the same production system. If this is true, then these differences in microbiome community could be exacerbated when bDNA is titrated, as observed in this study, through processes described previously.

Finally, another endogenous factor to take into consideration is the host's immune status, as under normal conditions, this system is involved in regulating the gut microbiome in fish through exclusion and neutralisation mechanisms (Llewellyn et al., 2014; Kelly et al., 2017). High inter-individual variation in innate immunity has been reported in several fish species including zebrafish (*Danio rerio*), turbot (*Scophthalmus maximus*) and gilthead sea bream (*Sparus aurata*) (Sitjà-Bobadilla et al., 2006, 2008; Rojo et al., 2007). Likewise, as inter-individual variability in the innate and adaptive immune responses of rainbow trout has previously been demonstrated (Thuvander & Caristein, 1991; Chilmonczyk & Monge, 1999), it is possible that fish in this study may have exhibited variability in their immune status. Moreover, these variations in immune activity could have led to the differences observed in the microbiome community between individuals. This theory seems likely given that a link between inter-individual variability in inflammatory potential and the gut microbiome has already been reported in humans (Schirmer et al., 2016). However, as these endogenous components were not measured in the current study, they warrant further investigation so as to determine how researchers can best reduce the individual variability in fish gut microbiome studies through biological approaches.

In the present study, it was unable to be shown whether titrating bDNA could reduce the individual variability in the distal gut microbiome community composition of rainbow trout. This was due to the fact that different bacterial genera responded differently to bDNA titration in T-libraries. The differential response of bacterial taxa was likely associated with the potential variation observed in 16S rRNA GCNs, as described previously. Together with the diversity data, these findings demonstrate that titrating bDNA may not be the best approach when attempting to reduce the inter-individual variability during gut microbiome characterisation in fish. However, as this study utilised a small number of samples, increasing the sample size would improve the clarity of the observed trends in this study, and provide greater statistical power to detect small but important changes between NT-libraries and T-libraries, respectively (Casals-Pascual et al.,

2020). For example, due to the disparity in alpha diversity between the T-libraries of Fish 3 and Fish 4, and the remaining fish samples, increasing the sample size would give a better understanding of which responses, if not both, were a real biological effect of titrating bDNA in 16S rRNA libraries.

Nonetheless, results presented in this study did demonstrate that titrating bDNA in 16S rRNA libraries, may serve a number of potential benefits for the microbiome research community. For example, titrating bDNA lead to a greater resolution in the microbiome communities profiled as a number of dominant genera increased in sequence abundance in T-libraries. Furthermore, some of the rare bacterial taxa (0.1% - 1% abundance) could only be detected when bDNA was titrated. The improved resolution was likely related to standardising 16S rRNA concentration, which in some samples meant increasing the amount of bDNA added to the PCR reactions. The increase in template material for amplification would mean that these samples had a greater sequencing opportunity to reflect the true microbial community present. Indeed, this was reflected in the improved sequencing performance of T-libraries, as these libraries were found to generate higher overall sequencing read yields compared with NT-libraries. Sequencing output is an important factor in NGS projects (Di Bella et al., 2013) as the number of reads assigned, and thus the number of bacterial taxa detected in microbiome samples has been reported to increase with increased sequencing depth (Jovel et al., 2016; Zaheer et al., 2018). In this study, the T-libraries displayed greater sequencing depth with the average sequence read yield increasing by almost 20,000 reads compared with NT-libraries. The authors acknowledge that this study contained a small number of samples and thus read depth would not be as high if sequencing at full capacity e.g. 384 libraries on the Illumina MiSeq® platform. However, the increase in sequence read yield in the T-library group could account for the observed increase in OTU assignment and subsequent alpha diversity obtained in most fish. This theory seems likely as similar patterns have been observed previously in human stool samples, among others (Jovel et al., 2016). The increased detection of OTUs and microbial taxa ultimately allowed for more detailed profiles of the distal gut microbiome community in rainbow trout to be generated. This would suggest that bDNA titration could be of benefit when profiling the microbial communities of fish species, whose microbiomes have yet to be explored, and where sensitivity is important.

Higher sequencing read yields was also accompanied with a reduced variability in sequencing depth between T-libraries. It is likely that this improvement was associated with the quantification and subsequent adjustment of bDNA template material prior to sequencing. The standardisation of bDNA template allowed for each 16S rRNA library to have an equal opportunity for sequencing coverage. This additional step could therefore be of value for studies with samples that vary significantly in bDNA template, as it allows for low or high biomass samples to be

identified and the amount of template DNA within these samples to be adjusted in 16S rRNA libraries, prior to sequencing. This is extremely important for sequencing performance as if bDNA is not titrated; samples with high target DNA concentration may utilise more of the sequencing capacity and reduce the sequencing coverage of other lower biomass samples. Likewise, samples that fall below a minimum threshold of target DNA concentration of 10,000 copies per microlitre (Rubin et al., 2014), may not be sequenced successfully.

The increased detection of rare bacterial taxa observed when bDNA was titrated, was also associated with a decline in the representation of a number of bacterial genera. More specifically, OTUs assigned to *Corynebacterium_1*, *Mycoplasma* and *Ralstonia* were found to have depleted sequence abundance within T-libraries compared with NT-libraries, respectively. Despite adding no DNA material to the NSC sample at the DNA extraction step, these OTUs were also observed to dominate this sample. Therefore, findings from this study suggest that the sequence abundance of these OTUs in the distal gut microbiome of rainbow trout may be inaccurate, as they could have originated from contaminant DNA material introduced into 16S rRNA libraries. The introduction of microbial DNA contaminants in microbiome studies is a considerable challenge recently highlighted by a number of studies (Salter et al., 2014; Velásquez-Mejía et al., 2018). Sources of potential foreign microbial DNA include molecular grade water, PCR components, DNA extraction reagents as well as the general laboratory environment (Kulakov et al., 2002; Evans et al., 2003; Shen et al., 2006; Salter et al., 2014). In this study, new DNA extraction kits and PCR components were used to minimise the chances of potential contamination, thus these findings were not expected. However, the presence of these genera in the NSC in this study does support similar findings described previously. Both *Corynebacterium* and *Ralstonia* have been detected within negative 'blank' controls (Salter et al., 2014), where they likely originate from contaminated PCR reagents or commercial DNA extraction kits, respectively (Laurence et al., 2014; Glassing et al., 2016). These results therefore highlight the importance of running a negative 'blank' control with every experiment. The inclusion of microbial DNA contaminants during sample preparation can present challenges for microbiome studies, as they are reported to generate ambiguous and inaccurate results of the communities profiled. However, samples with low microbial biomass are thought to be particularly susceptible to the effects of contaminant DNA (Weiss et al., 2014). This is thought to occur as they can provide less endogenous starting material and so become outcompeted by predominating DNA from foreign sources in the sequencing reactions (Lauder et al., 2016). Taking this into consideration, data from this study therefore also demonstrates the potential benefit of the additional quantification and bDNA titration steps in library preparation, as they can minimise the introduction of contaminant microbial DNA by adjusting the endogenous starting material in reactions prior to sequencing. These steps could be extremely valuable to particular microbiome studies working with low

biomass sample types associated with fish such as the mucus, pyloric caeca, skin and stomach tissue (Austin, 2006; Minniti et al., 2017; Egerton et al., 2018).

2.6. Conclusions

Findings from this study have shown that titrating bDNA can increase the sequence read yield, and reduce the introduction of foreign microbial DNA contamination in 16S rRNA libraries. Collectively, these advantages can improve the characterisation of the gut microbiome, through increased detection of microbial diversity and rare bacterial taxa within the GID samples of fish. However, whilst the proposed method of generating 16S rRNA libraries was intended to reduce inter-individual variability, titrating bDNA resulted in non-linear and sample-specific changes in the bacterial diversity of distal gut GID from fish. Furthermore, the bDNA titration approach did not reduce inter-individual variability in distal gut microbiome community profiles. These results therefore warrant further investigation with larger sample sizes, to better discern the global pattern observed in 16S rRNA libraries when titrating bDNA. However until then, titrating bDNA should be considered in microbiome studies, given the benefits to microbiome characterisation associated with sequencing performance and the reduced inclusion of contamination. For these reasons, the bDNA quantification and titration approach, with minor modifications, was used in chapters 3 & 4 when profiling the distal gut microbiome communities from GID material in rainbow trout and Nile tilapia, respectively.

2.7. Acknowledgements

This study was funded by the Fisheries Society of the British Isles as part of their PhD Studentship program. The authors would like to thank the staff at the NBFRU aquaria, University of Stirling for their care in the husbandry of fish used in this study. In addition, we are grateful for the advice and assistance of Dr. J. Taggart and Dr. M. Bekaert, as well as Mrs J. Ireland during the amplicon-sequencing and bioinformatic stages of the study. We would also like to thank Prof. J. Turnbull for his help during data analysis.

2.8. Author Contributions

CJP and SM developed the study concept and design. CJP collected stool samples from fish and performed all laboratory work. CJP analysed all data with input from MC and SM.

2.9. Conflicts of Interest

The authors declare that they have no competing interests.

2.10. Ethical Approval

All work carried out was approved by the Animal Welfare and Ethical Review Body (AWERB) at University of Stirling (AWERB (17 18) 006 New ASPA (B)) and followed guidelines set out by the UK Home Office Animals (Scientific Procedures) Act 1986.

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2.12. Supplementary Information

Table 2.3 (S1). Sequence abundance of top bacterial genera in the distal gut digesta of rainbow trout characterised using non-titrated 16S rRNA libraries.

Genus	Abundance							
	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Mean	SD
Acinetobacter	0.07%	0.07%	0.00%	0.00%	0.07%	0.08%	0.05%	0.03%
Actinobacteria_unclassified	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%
Aeromonas	0.00%	0.01%	0.00%	0.00%	0.01%	0.04%	0.01%	0.01%
Afipia	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%
Bacillales_unclassified	0.01%	0.00%	0.61%	0.01%	0.00%	0.13%	0.13%	0.22%
Betaproteobacteriales_unclassified	0.00%	0.01%	0.17%	0.00%	0.00%	0.00%	0.03%	0.06%
Bradyrhizobium	0.00%	0.02%	0.00%	0.00%	0.04%	0.02%	0.01%	0.02%
Brevinema	0.27%	68.69%	0.56%	0.04%	4.84%	18.98%	15.57%	24.66%
Burkholderia-Caballeronia-Paraburkholderia	0.00%	0.02%	0.00%	0.00%	0.03%	0.03%	0.01%	0.01%
Burkholderiaceae_unclassified	0.00%	0.02%	0.00%	0.00%	0.02%	0.00%	0.01%	0.01%
Candidatus_Bacilloplasma	0.00%	0.00%	0.00%	0.00%	0.00%	0.40%	0.07%	0.15%
Cloacibacterium	0.05%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%
Clostridiaceae_1_unclassified	0.00%	0.00%	0.03%	0.00%	0.00%	6.71%	1.12%	2.50%
Clostridium_sensu_stricto_1	0.00%	0.00%	0.21%	0.00%	0.00%	0.12%	0.06%	0.08%
Corynebacterium	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%
Corynebacterium_1	0.05%	0.24%	0.01%	0.01%	0.01%	0.26%	0.09%	0.11%
Cupriavidus	0.00%	0.01%	0.00%	0.00%	0.06%	0.02%	0.02%	0.02%
Deefgea	0.00%	0.00%	10.58%	0.00%	0.00%	0.02%	1.77%	3.94%
Enterobacteriaceae_unclassified	0.01%	0.19%	0.01%	0.01%	0.03%	1.31%	0.26%	0.48%
Fusobacterium	0.01%	0.00%	0.00%	0.00%	0.01%	0.05%	0.01%	0.02%
Halomonas	0.00%	0.00%	0.00%	0.00%	0.03%	0.01%	0.01%	0.01%
Kocuria	0.00%	0.02%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%
Lachnospiraceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%
Lactobacillus	0.01%	0.03%	0.00%	0.00%	0.02%	0.06%	0.02%	0.02%
Lactococcus	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.01%	0.01%
Methylobacterium	0.01%	0.05%	0.00%	0.00%	0.00%	0.11%	0.03%	0.04%
Microbacteriaceae_unclassified	0.03%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%
Mycoplasma	98.88%	28.87%	87.59%	99.44%	91.31%	69.26%	79.22%	24.65%
Pediococcus	0.00%	0.01%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%
Peptostreptococcaceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.01%	0.01%
Photobacterium	0.01%	0.00%	0.00%	0.00%	0.03%	0.11%	0.02%	0.04%
Providencia	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.01%
Pseudoalteromonas	0.00%	0.05%	0.00%	0.00%	0.03%	0.10%	0.03%	0.04%
Pseudomonas	0.01%	0.00%	0.00%	0.00%	0.02%	0.02%	0.01%	0.01%
Psychrilyobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.01%	0.01%
Ralstonia	0.19%	1.11%	0.04%	0.02%	2.95%	0.75%	0.84%	1.02%
Ruminococcaceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.03%	0.06%	0.02%	0.02%
Shewanella	0.00%	0.00%	0.12%	0.00%	0.02%	0.02%	0.03%	0.04%
Sphingomonas	0.01%	0.04%	0.00%	0.00%	0.03%	0.05%	0.02%	0.02%
Staphylococcus	0.03%	0.06%	0.00%	0.00%	0.01%	0.05%	0.03%	0.02%
uncultured	0.04%	0.26%	0.03%	0.38%	0.07%	0.64%	0.23%	0.22%
Vibrionaceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%
Weeksellaceae_unclassified	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%
Weissella	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.01%	0.01%

Table 2.4 (S2). Sequence abundance of top bacterial genera in the distal gut digesta of rainbow trout characterised using titrated 16S rRNA libraries.

Genus	Abundance							
	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Mean	SD
Acinetobacter	0.18%	0.02%	0.00%	0.00%	0.20%	0.07%	0.08%	0.08%
Aeromonas	0.00%	0.01%	0.00%	0.00%	0.02%	0.02%	0.01%	0.01%
Bacillales_unclassified	0.00%	0.00%	0.44%	0.00%	0.01%	0.52%	0.16%	0.23%
Betaproteobacteriales_unclassified	0.00%	0.00%	0.12%	0.00%	0.00%	0.01%	0.02%	0.04%
Bradyrhizobium	0.01%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%
Brevinema	0.71%	60.85%	0.47%	0.04%	8.91%	28.28%	16.54%	22.14%
Brevundimonas	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%
Burkholderia-Caballeronia-Paraburkholderia	0.01%	0.00%	0.01%	0.01%	0.01%	0.08%	0.02%	0.03%
Burkholderiaceae_unclassified	0.01%	0.04%	0.00%	0.00%	0.01%	0.02%	0.02%	0.01%
Candidatus_Bacilloplasma	0.00%	0.00%	0.00%	0.00%	0.00%	1.19%	0.20%	0.44%
Chryseobacterium	0.04%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%
Clostridiaceae_1_unclassified	0.00%	0.00%	0.03%	0.00%	0.00%	20.52%	3.42%	7.64%
Clostridium_sensu_stricto_1	0.00%	0.00%	0.18%	0.00%	0.00%	0.28%	0.08%	0.11%
Cupriavidus	0.00%	0.01%	0.01%	0.00%	0.01%	0.00%	0.01%	0.00%
Deefgea	0.00%	0.00%	8.14%	0.00%	0.00%	0.04%	1.36%	3.03%
Enterobacteriaceae_unclassified	0.02%	0.00%	0.00%	0.00%	0.15%	2.56%	0.45%	0.94%
Flavobacterium	0.01%	0.01%	0.00%	0.00%	0.01%	0.05%	0.01%	0.02%
Fusobacteriaceae_unclassified	0.01%	0.00%	0.00%	0.00%	0.02%	0.06%	0.01%	0.02%
Fusobacterium	0.02%	0.00%	0.00%	0.00%	0.04%	0.19%	0.04%	0.07%
Haematobacter	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%
Halomonas	0.00%	0.00%	0.00%	0.00%	0.05%	0.04%	0.01%	0.02%
Kocuria	0.03%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%
Lachnospiraceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.01%	0.02%
Lactobacillales_unclassified	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.01%	0.01%
Lactobacillus	0.01%	0.00%	0.00%	0.00%	0.02%	0.07%	0.02%	0.02%
Lactococcus	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.02%	0.04%
Lawsonella	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%
Leuconostoc	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.01%	0.03%
Methylobacterium	0.06%	0.00%	0.01%	0.00%	0.15%	0.01%	0.04%	0.05%
Microbacteriaceae_unclassified	0.01%	0.00%	0.00%	0.01%	0.01%	0.07%	0.02%	0.02%
Micrococcaceae_unclassified	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%
Mycoplasma	97.89%	37.74%	90.12%	99.37%	88.78%	41.75%	75.94%	25.90%
Myroides	0.00%	0.00%	0.00%	0.00%	0.03%	0.02%	0.01%	0.01%
Peptostreptococcaceae_unclassified	0.01%	0.00%	0.00%	0.00%	0.06%	0.08%	0.02%	0.03%
Photobacterium	0.02%	0.00%	0.00%	0.00%	0.05%	0.17%	0.04%	0.06%
Planococcaceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%
Polynucleobacter	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%
Pseudoalteromonas	0.05%	0.00%	0.02%	0.01%	0.04%	0.01%	0.02%	0.02%
Pseudomonas	0.02%	0.00%	0.00%	0.00%	0.07%	0.06%	0.03%	0.03%
Psychrilyobacter	0.01%	0.00%	0.00%	0.00%	0.01%	0.07%	0.01%	0.02%
Psychrobacter	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.01%
Ralstonia	0.33%	0.35%	0.34%	0.32%	0.50%	0.52%	0.39%	0.08%
Ruminococcaceae_unclassified	0.00%	0.00%	0.00%	0.00%	0.12%	0.14%	0.04%	0.06%
Shewanella	0.00%	0.00%	0.08%	0.00%	0.08%	0.09%	0.04%	0.04%
Sphingomonas	0.02%	0.00%	0.00%	0.00%	0.01%	0.03%	0.01%	0.01%
Staphylococcus	0.01%	0.00%	0.00%	0.00%	0.02%	0.03%	0.01%	0.01%
Stenotrophomonas	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.01%	0.01%
Streptococcus	0.01%	0.00%	0.00%	0.00%	0.01%	0.06%	0.01%	0.02%
uncultured	0.06%	0.49%	0.02%	0.12%	0.25%	1.79%	0.46%	0.62%
Vibrio	0.00%	0.00%	0.00%	0.00%	0.02%	0.03%	0.01%	0.01%
Vibrionaceae_unclassified	0.01%	0.01%	0.00%	0.00%	0.00%	0.03%	0.01%	0.01%
Weissella	0.00%	0.00%	0.00%	0.00%	0.02%	0.07%	0.01%	0.03%

Table 2.5 (S3). Sequence abundance of top bacterial genera in the negative sequencing control.

Genus	Abundance
67-14_ge	0.34%
Acinetobacter	0.53%
Aeromonas	0.68%
Aliivibrio	0.23%
Aneurinibacillus	0.31%
Bradyrhizobium	0.14%
Brevinema	0.20%
Burkholderia-Caballeronia-Paraburkholderia	0.94%
Burkholderiaceae_unclassified	0.37%
Chitinophagaceae_unclassified	0.17%
Clostridium_sensu_stricto_1	0.34%
Corynebacterium_1	10.47%
Cupriavidus	0.92%
Cutibacterium	0.07%
Deefgea	0.16%
Edwardsiella	0.10%
Empedobacter	0.25%
Enterobacteriaceae_unclassified	0.37%
Flavobacterium	0.30%
Francisella	0.66%
Gordonia	0.03%
Kocuria	0.14%
Lactobacillus	2.67%
Lactococcus	0.06%
Methylobacterium	5.06%
Microbacterium	0.41%
Mycoplasma	4.13%
Paraglaciecola	0.20%
Peptoniphilus	0.11%
Providencia	0.36%
Pseudoalteromonas	2.58%
Psychrobacter	0.13%
Ralstonia	60.59%
Reyranella	0.25%
Rickettsiales_unclassified	0.05%
Sphingomonadaceae_unclassified	0.20%
Sphingomonas	1.46%
Staphylococcus	2.13%
Streptococcus	0.26%
Tumebacillus	0.26%
uncultured	0.76%
Vibrionaceae_unclassified	0.63%

Table 2.6 (S4). Operational taxonomic units (OTU) identified as discriminatory according to library method by Metastats, LEfSe and ISA algorithms in Mothur.

OTU	Phylum	Genus	*p* Value		
			Metastats	LEfSe	ISA
OTU1	Tenericutes	*Mycoplasma*	0.960	-	0.824
OTU5	Proteobacteria	*Ralstonia*	0.731	-	0.308
OTU11	Actinobacteria	*Corynebacterium_1*	**0.015**	**0.013**	**0.044**
OTU66	Firmicutes	*Pediococcus*	**0.009**	-	0.096
OTU109	Actinobacteria	*Microbacterium*	**0.011**	-	0.068
OTU113	Proteobacteria	*Wohlfahrtiimonas*	**0.029**	-	0.16
OTU183	Bacteroidetes	*Chryseobacterium*	**0.008**	-	0.14
OTU196	Proteobacteria	*Vibrio*	**0.009**	-	0.184
OTU206	Actinobacteria	*Corynebacteriaceae_unclassified*	**0.023**	-	0.14
OTU216	Proteobacteria	*Pseudomonas*	**0.047**	-	0.28
OTU229	Planctomycetes	*uncultured*	**0.010**	-	0.152
OTU232	Proteobacteria	*Reyranella*	**0.029**	-	0.176
OTU243	Proteobacteria	*Photobacterium*	**0.021**	-	0.164
OTU253	Firmicutes	*Clostridium_sensu_stricto_1*	**0.038**	-	0.14
OTU261	Tenericutes	*Mycoplasma*	**0.015**	-	0.052
OTU276	Tenericutes	*Mycoplasma*	**0.004**	-	0.096
OTU292	Firmicutes	*Ruminococcaceae_unclassified*	**0.039**	-	0.28
OTU296	Proteobacteria	*Achromobacter*	**0.036**	-	0.28
OTU312	Bacteroidetes	*Pedobacter*	**0.017**	-	0.156
OTU318	Proteobacteria	*Sphingomonas*	**0.016**	-	0.16
OTU341	Bacteroidetes	*Pedobacter*	**0.018**	-	0.168
OTU342	Firmicutes	*Geobacillus*	**0.030**	-	0.16
OTU351	Bacteroidetes	*Chryseobacterium*	**0.034**	-	0.28
OTU361	Proteobacteria	*Burkholderiaceae_unclassified*	**0.015**	-	0.108
OTU402	Bacteroidetes	*Dyadobacter*	**0.011**	-	0.144
OTU453	Firmicutes	*Acidaminococcaceae_unclassified*	**0.016**	-	0.164
OTU467	Proteobacteria	*Methylobacterium*	**0.036**	-	0.292
OTU482	Firmicutes	*Clostridiaceae_1_unclassified*	**0.018**	-	0.1
OTU499	Actinobacteria	*Corynebacterium_1*	**0.022**	-	0.184
OTU500	Firmicutes	*Erysipelotrichaceae_unclassified*	**0.030**	-	0.168
OTU502	Firmicutes	*Family_XI_unclassified*	**0.044**	-	0.284
OTU506	Proteobacteria	*Paracoccus*	**0.015**	-	0.16
OTU537	Bacteroidetes	*Dyadobacter*	**0.036**	-	0.292
OTU548	Proteobacteria	*uncultured*	**0.034**	-	0.28
OTU574	Firmicutes	*Bacillales_unclassified*	**0.039**	-	0.28
OTU591	Proteobacteria	*Rhizobiaceae_unclassified*	**0.041**	-	0.276
OTU656	Spirochaetes	*Brevinema*	**0.027**	-	0.156
OTU665	Firmicutes	*Tissierella*	**0.044**	-	0.28
OTU702	Proteobacteria	*Enterobacteriaceae_unclassified*	**0.044**	-	0.296
OTU714	Firmicutes	*Family_XIII_unclassified*	**0.042**	-	0.284
OTU734	Bacteroidetes	*Hymenobacter*	**0.019**	-	0.144
OTU758	Tenericutes	*Mycoplasma*	**0.034**	-	0.176
OTU787	Tenericutes	*Mycoplasma*	**0.005**	-	0.164

CHAPTER 3. Low-level oxytetracycline treatment disrupts the gut microbiome in rainbow trout (*Oncorhynchus mykiss*)

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3.1. Abstract

The fish intestinal environment is colonised by a complex microbiome community, which plays a critical role in host physiology and health. Antibiotic compounds have previously been reported to induce both short and long-term changes in the gut microbiomes of higher vertebrate animals; however, the influence of commercially licensed antibiotics on the gut microbiome in some farmed fish species remains unclear. The following study explored the effect of oxytetracycline, a broad-spectrum antibiotic licensed for use in UK aquaculture, on the gut microbiome community and inflammatory status in rainbow trout (*Oncorhynchus mykiss*). This was evaluated using a 4-week feeding study, where fish were fed diets with or without oxytetracycline (35 mg kg bodyweight day^{-1}) for 7-days, followed by a 14-day withdrawal period. Distal gut digesta and tissue samples were collected from individual fish in a time series manner (on days 0, 2, 8, 10, 15 and 22). The dynamic changes in the microbiome community before, during and after antibiotic treatment was then profiled from the gut digesta using next generation sequencing of the bacterial 16S rRNA gene. In addition, real-time qPCR methods were used to measure the expression of key pro- (IL-1β) and anti-inflammatory (TGF-β) cytokines in the gut tissue of fish over the same time frame. Results presented here showed that low-level oxytetracycline treatment stimulated rapid changes in the distal gut microbiome community of rainbow trout. Within two days of antibiotic treatment, the Tenericutes phylum which dominated the distal gut in pre-treated fish, was observed to decline in abundance within treated fish, accompanied by an enrichment of Proteobacteria members. After antibiotic treatment, the gut microbiomes of treated fish underwent a series of restructuring events and were not found to stabilise by the end of the study. By 14-days withdrawal from the antibiotic, treated fish displayed microbiomes with higher alpha diversity compared with the control fish. Likewise, the gut microbiome community of treated fish also had a reduction in Firmicutes abundance, accompanied by an enrichment in Cyanobacteria and Proteobacteria, among other bacterial phyla. Oxytetracycline was not found to have an effect on the expression of IL-1β and TGF-β genes within the distal gut tissue of treated fish in this study. However, fish in both treatment groups displayed lower TGF-β gene expression levels by day 22. Results from this study demonstrate that low-level antibiotic treatment can disturb the distal gut microbiome of farmed fish species. However, further work is required to better understand the interaction between oxytetracycline, host physiology and the gut microbiome.

Keywords: aquaculture, rainbow trout, antibiotic, oxytetracycline, bacteria, microbiome, succession

3.2. Introduction

Consumption of fish and fish products are vital for supporting the nutritional requirements for billions of people worldwide (Béné et al., 2015). In recent years, the global aquaculture sector has experienced rapid growth in production, peaking at 80 million tonnes in 2016 and representing 47% of the total global fish production (FAO, 2018). By 2030, it is estimated that this food production sector will need to contribute more than 60% of the seafood demand, to achieve global food and nutritional security for the growing population (Kobayashi et al., 2015). Achieving this volume will likely require further intensification of existing production systems, as well as the cultivation of new, non-native fish species in some countries (Tarkan et al., 2020). Given the existing trends, future intensification may be accompanied by increased disease outbreaks and the emergence of new infectious diseases. In some aquaculture sectors, current disease outbreaks have already been associated with losses surpassing 40% of the global production (Stentiford et al., 2017), resulting in the intensive use of antimicrobials including antibiotics to help control infectious diseases. Consequently, if the industry continues to intensify and alternatives to antibiotics are not available, we may see the increased use and over-reliance of antibiotics to maintain production demands.

Oxytetracycline (OTC) is one of several antibiotics currently licensed for use in aquaculture within the United Kingdom (National Office of Animal Health, 2017), where it is routinely used for the treatment of important bacterial diseases affecting salmonids (Leal *et al.*, 2019). Oxytetracycline belongs to the tetracycline family of aromatic polyketide antibiotics, which has a broad-spectrum activity against both Gram-positive and Gram-negative bacteria through the inhibition of translation and subsequent protein synthesis (Zhou et al., 2018; Yang et al., 2019). Whilst several studies have demonstrated the histopathological, immunosuppressive and genotoxic properties of OTC across several anatomical sites of farmed fish species (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 guts of vertebrate animals are colonised by a diverse and specialised community of microorganisms, or "microbiome" (Colston & Jackson, 2016). This community in fish has been demonstrated to fall under the influence of a range of endogenous and external factors including host genetics, temperature, salinity and diet (Naviner et al., 2006; Sullam et al., 2012; Li et al., 2014). In other vertebrate animals such as humans, non-human primates, and farmed chicken

(*Gallus gallus*), antimicrobial compounds have also been demonstrated to induce short- and long-term changes in the diversity and composition of the gut microbiome community (Jakobsson et al., 2010; Videnska et al., 2013; Vlčková et al., 2016). This is not surprising given that most antibiotics used to treat bacterial diseases often display broad-spectrum activities (Melander et al., 2018), thus in addition to targeting the bacterial pathogens causing the infection, these compounds can also indirectly act upon members of the resident microbiome. Likewise, antibiotic treatments can affect non-target bacteria within the microbiome community due to the exchange in secondary metabolites of target species, or the loss in co-operative microbial interactions or keystone species, which other resident members rely upon for survival (Willing et al., 2011).

Any potential side-effects on the gut microbiome through antibiotic treatment is a concern for farmed fish, as members within the commensal community have been demonstrated to serve numerous microbial-mediated functions. These functions are thought to be vital to the biological and physiological status of the fish host, as they contribute to growth, digestion/metabolism and behaviour (Ni et al., 2014; Borrelli et al., 2016; Giorgia et al., 2018). In addition, the gut microbiome has also been reported to play a key role in the disease resilience of fish by way of modulating immune homeostasis, where members can induce both pro- and anti-inflammatory cytokine pathways through *myD88* signalling (Kelly & Salinas, 2017). In fact, gut microbiome community members have been shown to induce the expression of the key pro-inflammatory cytokine interleukin-1beta (IL-1β) in common carp (*Cyprinus carpi*) (Chi et al., 2014). Likewise, administration of intestinal autochthonous bacteria in European sea bass (*Dicentrarchus labrax*) has been associated with the downregulation of transcription growth factor beta (TGF-β), a major anti-inflammatory cytokine in fish (Picchietti et al., 2009). Therefore, any disruption to the gut microbiome community through antibiotic treatment, may have long-term implications on fish welfare and host fitness.

Several studies have investigated the influence of antibiotics on the gut microbiome of different fish species e.g. Western mosquito fish (*Gambusia affinis*) (Carlson et al., 2017), black molly (*Poecilia sphenops*) (Schmidt et al., 2017), Nile tilapia (*Oreochromis niloticus*) (Limbu et al., 2018), zebrafish (*Danio rerio*) (Zhou et al., 2018) and Atlantic salmon (*Salmo salar*) (Gupta et al., 2019), however, the influence of antibiotics on the gut microbiome in rainbow trout (*Oncorhynchus mykiss*) remains unclear. This is concerning given that this particular production sector relies heavily on the use of antibiotics to maintain production, due to limited prophylactic measures e.g. vaccines being available to prevent infectious diseases (Brudeseth et al., 2013). Therefore, the primary objective of this study was to determine the effect of OTC on the distal gut microbiome community in rainbow trout. As OTC displays a broad-spectrum activity against both Gram-positive and Gram-negative bacteria, we hypothesised that OTC treatment would

reduce the distal gut microbiome diversity in treated fish, and disrupt the microbiome community composition. Furthermore, we wanted to investigate whether the distal gut microbiome community in fish would recover following a single, therapeutic treatment of OTC. Due to the recognised importance of the microbiome in gut health, a secondary aim of this study was to investigate the effect of OTC on the inflammatory status in the distal gut of treated fish. To achieve this, a 16S rRNA amplicon-sequencing and real-time quantitative-PCR (qPCR) approach was applied to profile the changes in (i) microbiome community diversity and composition, and (ii) the gene expression of key pro- (IL-1β) and anti-inflammatory (TGF-β) cytokines, in the distal gut of fish before, during and after antibiotic treatment.

3.3. Methods

3.3.1. Experimental design

The effects of OTC exposure on the distal gut microbiome and inflammatory cytokine gene expression in adult rainbow trout was performed over a 27-day time series feeding study (Figure 3.1). The experimental study was divided into three stages: a 6-day acclimation period (non-medicated diet), a 7-day treatment period (medicated diet) and finally a 14-day withdrawal period (non-medicated feed). A stock of rainbow trout fish were obtained from a local trout farm and transferred to the Niall Bromage Freshwater Research Unit (NBFRU), University of Stirling (UoS) in November 2017. None of the fish had received any antibiotic treatment within nine months prior to the start of the study. Furthermore, all fish were also vaccinated against enteric red mouth using the AquaVac® RELERA vaccine (MSD Animal Health, Buckinghamshire, United Kingdom). The average weight of fish upon arrival at NBFRU was 152.8 ± 8.9 g.

All fish were held in a single tank upon arrival at NBFRU where they received a salt water (Instant Ocean; Aquarium Systems®, France) treatment at 2 g L^{-1} for one hour, followed by two separate Halamid (Tosylchloramide Sodium) (Axcentive SARL®, France) treatments, both at 5 ppm for one hour. 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. Following the recommended treatments, fish were randomly allocated into six 300 L tanks (n = 15 per tank), which were maintained on a flow through system, under a 12:12 hour light:dark cycle and an ambient water temperature of 2.85 ± 0.9°C. Fish were maintained in these conditions throughout the entire trial. Following the salt water and Halamid treatments, fish were acclimated in tanks for six days prior to starting the antibiotic treatment period. After the 6-day acclimation period, each tank was randomly allocated into two treatment groups with three replicate tanks per group (Figure 3.1). During the treatment period, the fish in treatment group one were fed a medicated (OTC) diet which was surface coated with OTC (35 mg kg bodyweight day^{-1}), whereas fish in treatment group two were fed a non-medicated (control) diet. Both diets were administered using an automatic feeder at a feeding rate of 0.7% bodyweight day^{-1}, over a period of eight hours for seven days. After the 7-day treatment period, fish in both treatment groups were fed the control diet at a feeding rate of 0.2% bodyweight day^{-1} for 14 days, after which time the experimental trial was terminated.

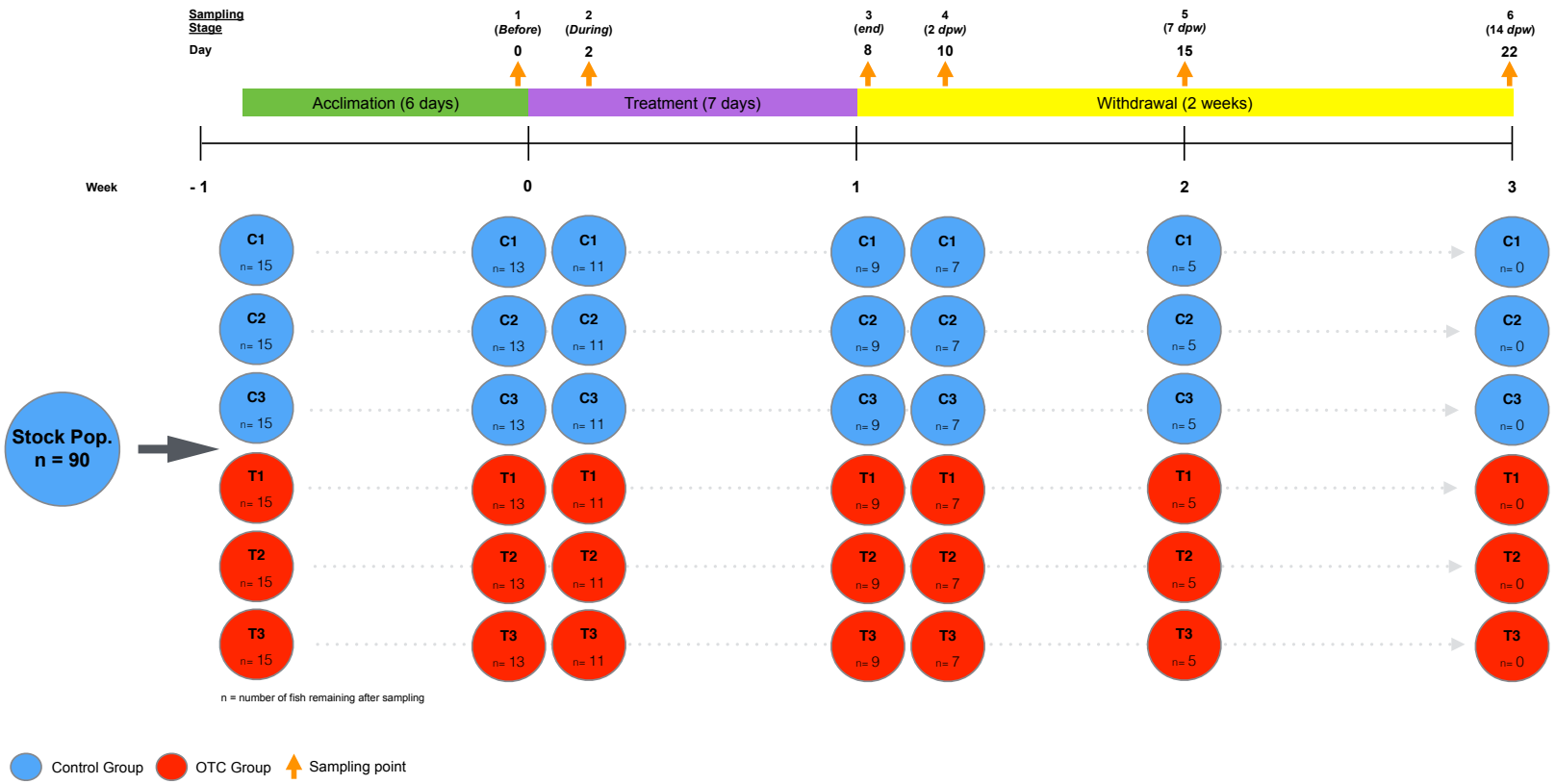

Figure 3.1. Experimental design and sampling strategy. Fish were acclimated to tank conditions at the Niall Bromage Freshwater Research Unit, University of Stirling for six days. Tanks were then randomly assigned to either control (blue) or oxytetracycline (OTC; red) groups in triplicate design. During the 7-day antibiotic treatment, fish in the OTC group received a diet surface-coated with OTC (35 mg kg bodyweight day^{-1}). During the same period, fish in the control group received a control diet void of any antibiotic. Following the antibiotic treatment period, all fish were given the control diet for 14 days to simulate a withdrawal period. Fish were sampled on days 0, 2, 8, 10, 15 and 22 to reflect before, during and after antibiotic treatment. With the exception of day 22, two fish from each tank were randomly sacrificed at each sampling point and sampled for distal gut digesta and tissue. The trial was terminated on day 22, where all remaining fish were sacrificed and sampled. Days post withdrawal; *dpw*.

3.3.2. Diet preparation & in vitro antimicrobial activity

The OTC diet was prepared onsite at the Institute of Aquaculture, UoS (Stirling, UK) using T-Elite FR 4 mm complete trout feed (20% oil content, 39% protein content) (Skretting, France). A pre-weighted volume of pellets was surface coated with OTC hydrochloride (98.2% purity) (Duchefa Biochemie®, Haarlem, the Netherlands) at an inclusion rate of 75 mg kg bodyweight^{-1}, which was homogenised by hand mixing for five minutes. The OTC diet was prepared for an intended feeding rate of 1.5% bodyweight day^{-1}, although fish actually received the OTC diet at a feeding rate of 0.7% bodyweight day^{-1} resulting in an actual dose of 35 mg kg bodyweight day^{-1} for OTC. Following coating of pellets with OTC, cod liver oil (Vitarenew®; Principle Healthcare International Limited, Skipton, UK) was applied as a binding agent at a rate of 20 mL kg diet^{-1}. The control diet was similar in composition to the OTC diet, except it lacked OTC hydrochloride. Both diets were prepared 24 hours prior to commencing the 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.

The OTC diet was tested for the inhibition of OTC-sensitive *Yersinia ruckeri* NCIMB 2194 following Alderman & Smith (2001), to confirm antimicrobial activity. Briefly, one colony of *Y. ruckeri* was incubated in 30 mL sterile tryptone soy broth (TSB; Oxoid®, UK) for 18 hours at 28°C, before being centrifuged at 2,600 x g for 15 minutes at 4°C. The supernatant containing TSB was discarded and the bacterial pellet was resuspended in sterile phosphate buffered saline (pH 7.2) to reach 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 (TSA; Oxoid®, UK) as a bacterial lawn. After five minutes, three pellets from the OTC diet were then aseptically placed onto individual sections of the agar plate. The agar plate was then sealed before incubating at 28°C for 48 hours. The agar plate was checked every 24 hours for bacterial growth and the presence of inhibition zones around the diet pellets. Pellets from the control diet were also tested for comparison, and to confirm this diet was free of any antimicrobial compounds.

3.3.3. Sample collection

The intestinal digesta was aseptically collected from the distal gut of individual fish at six time points, which were as follows: immediately before antibiotic treatment (day 0, baseline), two days into antibiotic treatment (day 2), immediately after the end of the antibiotic treatment (day 8), two days post-treatment withdrawal (day 10), 1-week post-treatment withdrawal (day 15) and lastly at the end of the two week withdrawal period (day 22) (Figure 3.1). At each timepoint, two fish were randomly sampled from each tank giving n=6 fish per treatment group and per time point. This sample size followed international recommendations for RNA-seq experiments (Schurch et

al., 2016), which uses similar molecular methods. Following euthanasia using a lethal dose of the anaesthetic tricaine methanesulfonate (Tricaine 1000 mg g^{-1} powder; Pharmaq®, UK), digesta from the distal gut was collected as described in section 2.3.1, and placed in a sterile 2 mL microcentrifuge tube (Alpha Labs®, Hampshire, UK), containing 1 mL ASL lysis buffer (Qiagen®, Hilden, Germany). No intact feed pellets were observed within the digesta material of any fish sampled. Following collection of digesta, ca 0.2 cm of distal gut tissue was collected in sterile 2 mL microcentrifuge tubes with 1 mL TRI Reagent (Sigma Life Science, UK) for RNA extraction. All tubes were held on dry ice and transported back to the Institute of Aquaculture (ca. 30 minutes), where they were stored at -80°C until DNA or RNA extraction, respectively. A total of ten pellets from each diet and a 10 mL sample of tank water from a representative treatment tank was also collected at each sampling point, and were stored in separate sterile universal tubes. The same tank was sampled at each sampling point to allow for comparison of the tank water over time. Diet and tank water samples were stored at 4°C until required for DNA extraction.

3.3.4. DNA extraction

A total of 150 mg of digesta was processed for total genomic DNA (gDNA) extraction using the QIAamp DNA Stool Mini Kit (Qiagen®, Hilden, Germany), which followed similar modifications as described in section 2.3.1, except final DNA was eluted in 50 µL EB buffer (10mM Tris-HCl, pH 8.5; Qiagen®, Hilden, Germany). Total genomic DNA was also extracted from diet and tank water samples using the same commercial DNA extraction kit and method described previously. Prior to DNA extraction, two pellets from each diet were transferred into separate 2 mL microcentrifuge tubes containing 1 mL buffer ASL. In addition, 1 mL from each individual tank water sample was added into separate 2 mL microcentrifuge tubes containing 0.5 mL of buffer ASL. The final DNA from feed and tank water samples was eluted in 50 µL EB buffer. Following DNA extraction, the concentration and purity of all DNA samples was analysed by spectrophotometry using the Nanodrop® 2000c (Thermo Scientific®, Glasgow, UK). Ten microlitre aliquots were prepared and stored at -20°C until required.

A synthetic microbiome community standard (IoA_MB_STD) was also prepared using the gDNA from five bacterial isolates as listed in Table 3.1. The IoA_MB_STD sample was prepared as described in section 2.3.1, however in this study, the IoA_MB_STD sample was modified to comprise DNA from *Pseudomonas aeruginosa* ATCC 27853 instead of the *Salmonella enterica* subsp. *enterica* isolate. In addition, the final DNA from each isolate was eluted in 200 µL EB buffer. The IoA_MB_STD sample was used as a positive control during the quantification of bacterial DNA step. In an attempt to sequence all potential microbial DNA contaminants introduced during 16S rRNA library preparation, total gDNA was also extracted from a negative sequencing control (NSC) sample. No sample or DNA was added to the NSC, instead 1 mL buffer

ASL was used as the starting material. Genomic DNA extraction for the NSC sample followed that described previously for digesta samples using the QIAamp DNA Stool Mini Kit. After extraction, the concentration and purity of eluted DNA samples was analysed by spectrophotometry using the Nanodrop® 2000c. Following quantification, ten microlitre aliquots were prepared and stored at -20°C until required.

Table 3.1. Composition and biochemical characteristics of bacterial isolates used in the synthetic microbiome community standard.

Bacterial isolate	Gram morphology	Motility	Oxidase	Oxidation/ Fermentation
Aeromonas hydrophila NCIMB 9240	Negative, short straight rods	Motile	Positive	Fermentative
Edwardsiella ictaluri NCIMB 13272	Negative, short straight rods	Non-motile	Negative	Fermentative
Pseudomonas aeruginosa ATCC 27853	Negative, straight rods	Motile	Positive	Oxidative
Vibrio anguillarum NCIMB 6	Negative, curved rods	Motile	Positive	Fermentative
Yersinia ruckeri NCIMB 2194	Negative, straight thin rods	Non-motile	Negative	Fermentative

3.3.5. Illumina MiSeq sequencing of the microbiome

Prior to preparing 16S rRNA Illumina libraries, the 16S rRNA gene concentration was measured in each DNA sample, to determine the amount of bacterial DNA recovered from each of the digesta samples. Quantification was performed by real-time qPCR with absolute quantification, using the primer pair 341F/805R (Eurofins Genomics UK, Wolverhampton, UK) (Table 3.2). This primer set was used to target the V3-4 hypervariable region (length 464 bp) of the bacterial 16S rRNA gene (Huang et al., 2018). Plasmid DNA standards containing the 16S rRNA V3-4 hypervariable region insert, were prepared as described in section 2.3.2, except the IoA_MB_STD sample was used for template gDNA material. In addition, *Escherichia coli* strain XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]) (Agilent Technologies Inc, Cheshire, UK) was used as the competent cell type for transformation. Briefly, the 16S rRNA V3-4 region was amplified using one-step PCR in a 25 µL PCR reaction containing 12.5 µL 2X HS MytaQ master mix (Bioline®, London, UK), 8.5 µL ultrapure water, 2.5 µL of each primer (10 pM) and 2 µL (100 ng) gDNA from the IoA_MB_STD sample. Amplification of the target region was completed using the following PCR conditions: 95°C for two minutes followed by 30 x cycles at 95°C for 20 seconds, 55°C for 30 seconds and 72°C for one minute, after which a final elongation step of 72°C for two minutes was completed. Following PCR amplification, purified PCR products were ligated into the pGEM-T Easy Vector system (Promega Corporation, Madison, USA) and transformed into *E. coli* XL1-Blue cells, following

the transformation protocol provided by Stratagene (now Agilent Technologies Inc). Extraction of plasmid DNA followed that described in section 2.3.2. The extracted plasmid DNA was screened for the presence of the 16S rRNA V3-4 gene insert by one-step PCR using the primers 341F/805R and conditions described previously. A plasmid DNA sample was confirmed as having the 16S rRNA V3-4 gene insert by the presence of a single band with a molecular weight of 464 bp. Finally, the plasmid DNA sample was serially diluted ten-fold in nuclease free water to concentrations containing 9.86 x 10^8 – 9.86 x 10^4 copies μL^{-1}. All plasmid DNA stocks were stored at -20°C until required. Real-time qPCR was performed as described in section 2.3.3, except the primer set 341F/805R was used with an annealing stage of 55°C for 30 seconds. 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 generated using the serially diluted plasmid standards described above. Absolute qPCR runs achieved correlation coefficients and efficiencies greater than 0.95 and 1.73, respectively.

Table 3.2. PCR primers used in this study.

Primer	Nucleotide Sequence (5' – '3)	Product Size	Source
341F	CCTACGGGNGGCWGCAG	464 bp	Huang et al., 2018
805R	GACTACHVGGGTATCTAATCC		
16S_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[341F]	531 bp	Illumina®
16S_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[805R]		
IL-1B_F	AACACCGGGGTTGACATCAG	156 bp	This Study
IL-1B_R	TTAGTTGTGGCGCTGGATGG		
TGF-B_F	AGATAAATCGGAGAGTTGCTGTG	275 bp	Bilen et al., 2016
TGF-B_R	CCTGCTCCACCTTGTGTTGT		

Following 16S rRNA quantification, DNA samples below a minimum threshold of 1 x 10^4 16S rRNA copies μL^{-1} (Rubin et al., 2014) were not processed further. Following the results from chapter 2, Illumina compatible 16S rRNA libraries were prepared following the titration protocol described in section 2.3.4, with minor modifications. Briefly, a total of 6.76 x 10^5 copies of the bacterial 16S rRNA gene from each DNA sample was used in each respective PCR reaction. In this study, the V3-4 hypervariable region of the 16S rRNA was targeted using the 16S_F/ 16S_R primers listed in Table 3.2. All samples were amplified in triplicate 10 µL reactions using the following PCR conditions: 98°C for one minute, followed by 25 x cycles of 98°C for ten seconds, 55°C for 30 seconds and 65°C for 45 seconds. All PCR reactions underwent a final extension stage at 65°C for five minutes. The 16S rRNA V3-4 hypervariable region was also amplified from gDNA in the NSC sample. Due to the low genomic material available, a total of 6 ng of gDNA

was added to respective PCR reactions for the NSC sample. Illumina libraries targeting the same 16S rRNA region were also generated from gDNA from individual diet (30 ng gDNA) and tank water samples (20 ng gDNA), utilising the PCR conditions described above. Due to the poor yield and quality in the baseline tank water sample, this sample was omitted from any further downstream processing.

Final libraries (length ~ 600 bp) which included dual indices, and Illumina® sequencing adapters were quantified fluorometrically using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific®, Glasgow, UK). Libraries were pooled in equal molar concentration (3.6 nM) and the final pooled library was quantified again with Qubit™, using the same high sensitivity dsDNA kit. The final pooled library was denatured in fresh 0.2 M NaOH and diluted to a final concentration of 20 pM in pre-chilled HT1 buffer (Illumina®). To increase the diversity within the final library and provide quality control data, the diluted library was combined with a PhiX control (Illumina®) and an internal control containing DNA from multiple sources, both at 4% of the final library concentration. The final pooled library at 4 pM concentration was sequenced using the Illumina MiSeq® NGS system with the Illumina® MiSeq Reagent Kits v2 (2 x 250 bp; 500-cycle) at the Institute of Aquaculture.

3.3.6. Bioinformatics

Illumina reads underwent demultiplexing with Casava v. 1.8 (Illumina®) and removal of reads representing the PhiX/ internal controls or reads not matching Illumina indices. The open-source program Mothur (Schloss et al., 2009) was used to process sequence read data generated using the commands described in section 2.3.5 with slight modifications. Resulting contig reads, generated using the make.contigs command were quality filtered to remove those which contained ambiguous bases, homopolymers > 8 bp, and reads with sequences < 460 bp or > 500 bp. Remaining sequences were aligned to the SILVA reference database after customising the reference alignment to the specific V3-4 region amplified by the primers used in this study (length = 464 bp), using the pcr.seqs and screen.seqs commands. Sequences were aligned using the default needleman alignment method, and further denoised using the pre.cluster command allowing for up to five nucleotide differences between duplicate sequences. Chimeric and undesirable sequences were filtered out of the final dataset using the chimera.uchime and subsequent remove.lineage commands, with the latter removing sequences assigned to "chloroplast", "mitochondria", "unknown", "archaea" and "eukaryota". Following personal communication with P. Schloss (Mothur creator; March 2019) regarding issues with clustering operational taxonomic units (OTUs) according to DNA sequence, final sequences were assembled into 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, singleton OTUs (those OTUs which only had one sequence across the whole dataset) were removed from the final dataset using the remove.rare command with nseqs set to a value of one. The final dataset was rarefied to the lowest number of sequences per sample (e.g. 8,058) prior to performing any further downstream analysis. The final sample size after rarefaction was n=5 across most treatment groups and time points, except for day 0 and day 8, OTC groups, which had a sample size of n=4.

3.3.7. RNA extraction and cDNA synthesis

Total RNA was extracted from distal gut tissue using TRI-Reagent (Sigma Life Science, UK), following the manufacturer's protocol with minor modifications. Briefly, homogenisation of tissue material was performed using a Mini bead-beater 16 (Biospec®, Bartlesville, OK, USA) at maximum speed (3.5 x 1000 rpm) for four separate cycles of 60 seconds each. Next, following phase separation of RNA, a 1:1 ratio of supernatant:100% isopropanol was performed, followed by an overnight precipitation of RNA at -20°C. Two separate washes of the precipitated RNA pellet were performed in 75% ethanol at 17,000 x g for five and ten minutes, respectively. Final RNA pellets were eluted in 50 µL nuclease-free DEPC treated water (Invitrogen, California, United States). The concentration and quality of extracted RNA was measured using the Nanodrop® 2000c, followed by visualisation of RNA on a 1.5% agarose gel under UV illumination. The presence of two bands with an intensity ratio of 2:1 (28S:18S rRNA), was considered an indication of good quality RNA. Samples free of DNA contamination were confirmed by the lack of visible smears below the 18S rRNA band, along with no material being present within the well. Fifteen hundred nanograms of total RNA was reverse transcribed into single-stranded complimentary DNA (cDNA), using the SuperScript™ III Reverse Transcriptase (Invitrogen, California, United States). Complimentary DNA was synthesised using the supplied oligo$(dT)_{20}$ primers and following the manufacturer's protocol. All cDNA samples were stored at -20°C until required.

3.3.8. real-time qPCR analysis of rainbow trout cytokine gene expression

The mRNA expression of key pro-inflammatory (IL-1β) and anti-inflammatory (TGF-β) cytokines were measured in distal gut tissue using real-time qPCR with absolute quantification, to determine the inflammatory status of the distal gut in rainbow trout before, during and after antibiotic treatment. Real-time qPCR was performed on a Stratagene Mx3005P QPCR System (Agilent Technologies LDS UK Ltd, Cheshire, UK) and was conducted in triplicate reactions for all cDNA samples inside 96-well plates. The qPCR reactions were prepared to a total volume of 20 µL containing 10 µL Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific,

Massachusetts, United States), 7 µL nuclease-free water, 0.5 µL of each forward and reverse primers (0.5 µM) and 2 µL cDNA diluted 1:5 with nuclease-free water. The primer sequences and expected amplicon sizes for each gene are listed in Table 3.2. Each qPCR run also included duplicate no DNA template control reactions to confirm qPCR reactions were free from contamination. Quantification was performed using the following conditions: Uracil-DNA glycosylase inactivation at 50°C for two minutes, an initial denaturation step at 95°C for three minutes, followed by 35 x cycles at 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds. 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 seconds. The number of gene copies per microlitre was calculated from Ct values using a standard curve of serially diluted plasmid, containing the gene insert from 1 x 10^7 to 1 x 10^1 gene copies μL^{-1}. Plasmids were prepared in *E. coli* strain DH5α as described in section 2.3.2, except 25 ng of cDNA from the distal gut tissue of an adult rainbow trout was used as the template material to amplify cytokine genes for cloning. In addition, transformed cells were incubated in Luria-Bertani broth + 0.001% (v/v) ampicillin at a concentration of 50 mg mL^{-1} for 18 hours at 37°C prior to plasmid DNA extraction. All qPCR runs conducted achieved correlation coefficients greater than 0.92 and efficiencies of 1.87.

3.3.9. Statistical analysis

Differences in the final mean length and weight of fish across treatment and time was evaluated with two-way analysis of variance (ANOVA) ($p < 0.05$) using JMP® version 14. Rarefaction curves were generated to assess sequencing depth and were visualised using the ggplot2 (Wickham, 2011) and reshape2 (Wickham, 2007) packages within RStudio Version 1.1.419. To evaluate the impact of OTC on distal gut microbiome communities in rainbow trout, alpha diversity as measured by Chao1 richness, Inverse Simpson and Shannon Diversity indices was calculated for each individual fish. Alpha diversity data was first $\log_{10}$ transformed to improve normality prior to performing statistical analysis. Differences in mean alpha diversity were then analysed by 2-way ANOVA (JMP®) with treatment and time as factors ($p < 0.05$). In addition, the community membership and structure in each of the samples was calculated by creating a distance matrix of beta diversity. Distance matrices were generated for thetaYC coefficient (Yue & Clayton, 2005) and Bray-Curtis dissimilarity (Bray & Curtis, 1957), using the dist.shared algorithm in Mothur. The generated distance matrices were visualised using non-metric multidimensional scaling ordination methods. For all distance measures, PERMANOVA (vegan; adonis function) (Anderson, 2001) was used to test for differences in beta diversity according to sample type designated as fish (distal gut), tank water, diet or NSC ($p < 0.05$). This same test was also applied to evaluate the influence of treatment and time on inter-sample distances of gut

microbiome communities ($p < 0.05$). In all tests, PERMANOVA was conducted using 10,000 permutations. The composition of the top most abundant bacterial phyla and genera in the distal gut of individual fish was profiled using the phyloseq (McMurdie & Holmes, 2013) and ggplot2 packages in RStudio. Shared and unique OTUs between treatment groups at different time points were calculated using the UpSetR package (Conway et al., 2017) in RStudio. Again, the ggplot2 package was used to profile the composition of unique OTUs within OTC treated groups at different time points. Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) and Metatstats (White et al., 2009) analyses were performed to determine whether any OTUs were differentially abundant/ represented between control and treatment groups, respectively. Differential sequence abundance of OTUs between treatment groups was considered significant at $p < 0.05$. Log2 fold changes in the sequence abundance of indicator OTUs between days 2 and 22 were visualised using the ggplot2 package. Finally, following $\log_{10}$ transformation of qPCR data, cytokine gene expression was assessed for differences between treatment groups and time by 2-way ANOVA ($p < 0.05$) and post-hoc analysis (Tukey HSD) using IBM SPSS Statistics version 25.

3.4. Results

3.4.1. Fish

All fish consumed both diets readily however, feeding rate was lower than expected at 0.7% bodyweight day^{-1} and 0.2% bodyweight day^{-1} for the treatment and withdrawal periods, respectively. Short-term antibiotic treatment with oxytetracycline was not found to have an effect on the growth performance parameters tested, as 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 3.3.

Table 3.3. Final mean (+SD) length and weight measurements for control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment.

Treatment	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

No mortalities or internal clinical signs of disease were observed in either treatment group throughout the study. Externally, some fish in both treatment groups presented erosion of the dorsal fins. In addition, there was also a low incidence of superficial lesions on the pectoral and anal fins, as well as the mouth. On day 2, one fish from each of the OTC diet and control 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 seven days.

3.4.2. in vitro antimicrobial testing of prepared diets

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 hours incubation.

3.4.3. Sequence data and diversity analysis

A total of 14,393,819 reads were obtained from the Illumina MiSeq system. Following quality filtering, a total of 10,238,421 sequences remained in the final dataset, which were clustered into 899 aligned OTUs for analysis. Of these OTUs, 442 were observed in samples originating from fish digesta, whereas 344, 596 and 105 OTUs were detected in the diet, tank water or the NSC samples, respectively. All samples reached a Good's coverage estimate of > 99% suggesting that most OTUs present within these communities were detected. This was confirmed following rarefaction analysis, which indicated high sequence coverage in fish samples as all curves reached saturation phase (Figure 3.2). Rarefaction analysis also showed increased microbial diversity in the distal gut of OTC-treated fish, particularly at day 22 (Figure 3.2), as this group was found to have a greater number of OTUs compared with the control group and other time points (Figure 3.3). This finding was reflected in Chao1 richness estimates as microbial richness on average was significantly higher in the distal gut of OTC treated fish compared with control fish ($F = 4.2893$, $p = 0.0436$) (Figure 3.3). Furthermore, community richness in OTC treated fish also increased significantly over time ($F = 7.9191$, $p = 0.0070$) (Figure 3.3). However, OTC treatment was not found to have an effect on microbial diversity and evenness within these communities, as both Inverse Simpson ($F = 0.3243$, $p = 0.5717$) and Shannon Diversity ($F = 0.5950$, $p = 0.4442$) indices were not found to be significantly different between treatment groups (Figure 3.3). In all alpha diversity measures investigated, time was found to significantly influence the alpha diversity of microbiome communities in both control and treated fish ($p < 0.05$) (Figure 3.3).

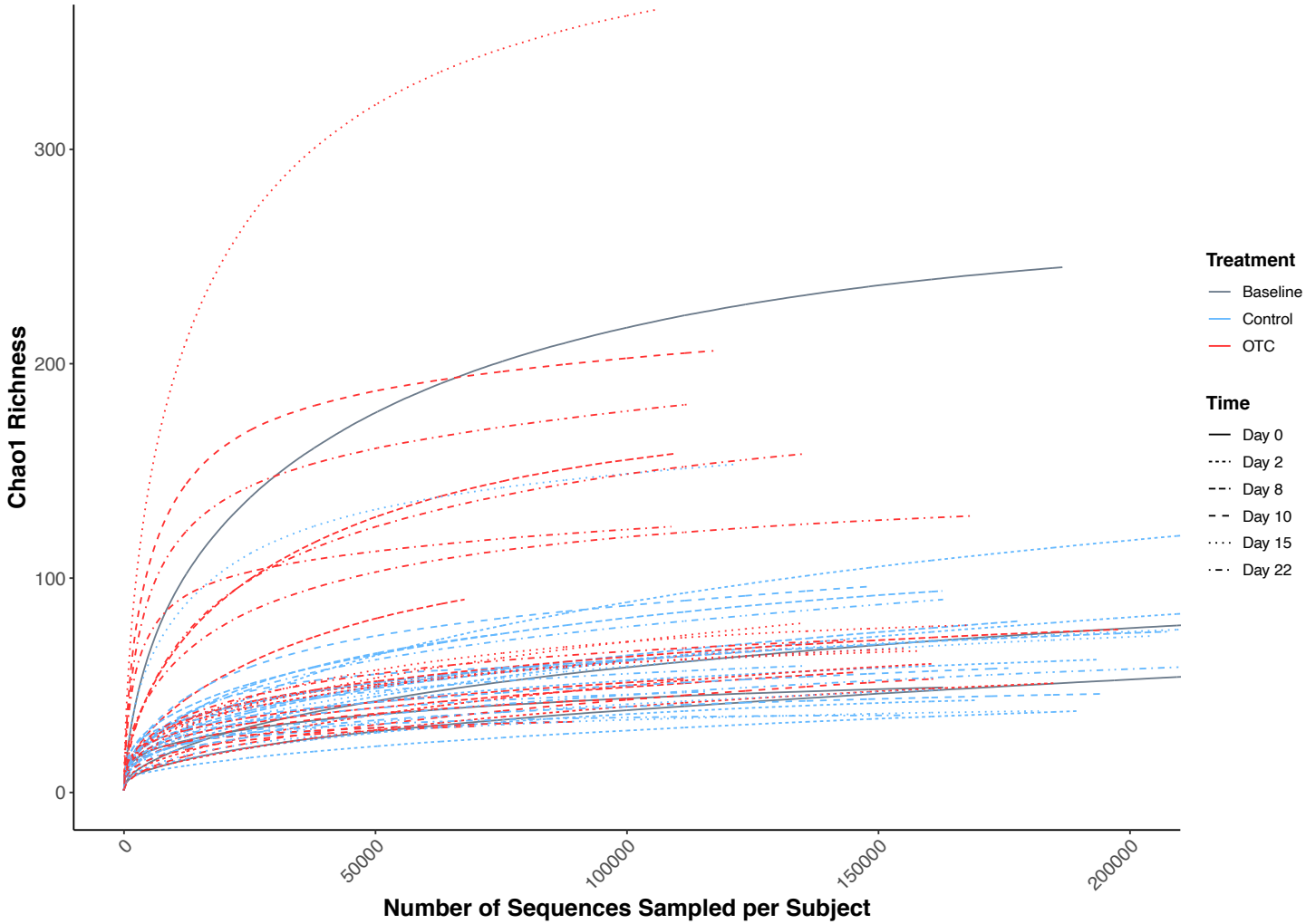

Figure 3.2. Rarefaction curves for each individual fish sampled (*n*=53). Curves represent the Chao1 richness observed per sample as a function of the sequencing effort. Colour of line indicates treatment group and line shape indicates time of sampling.

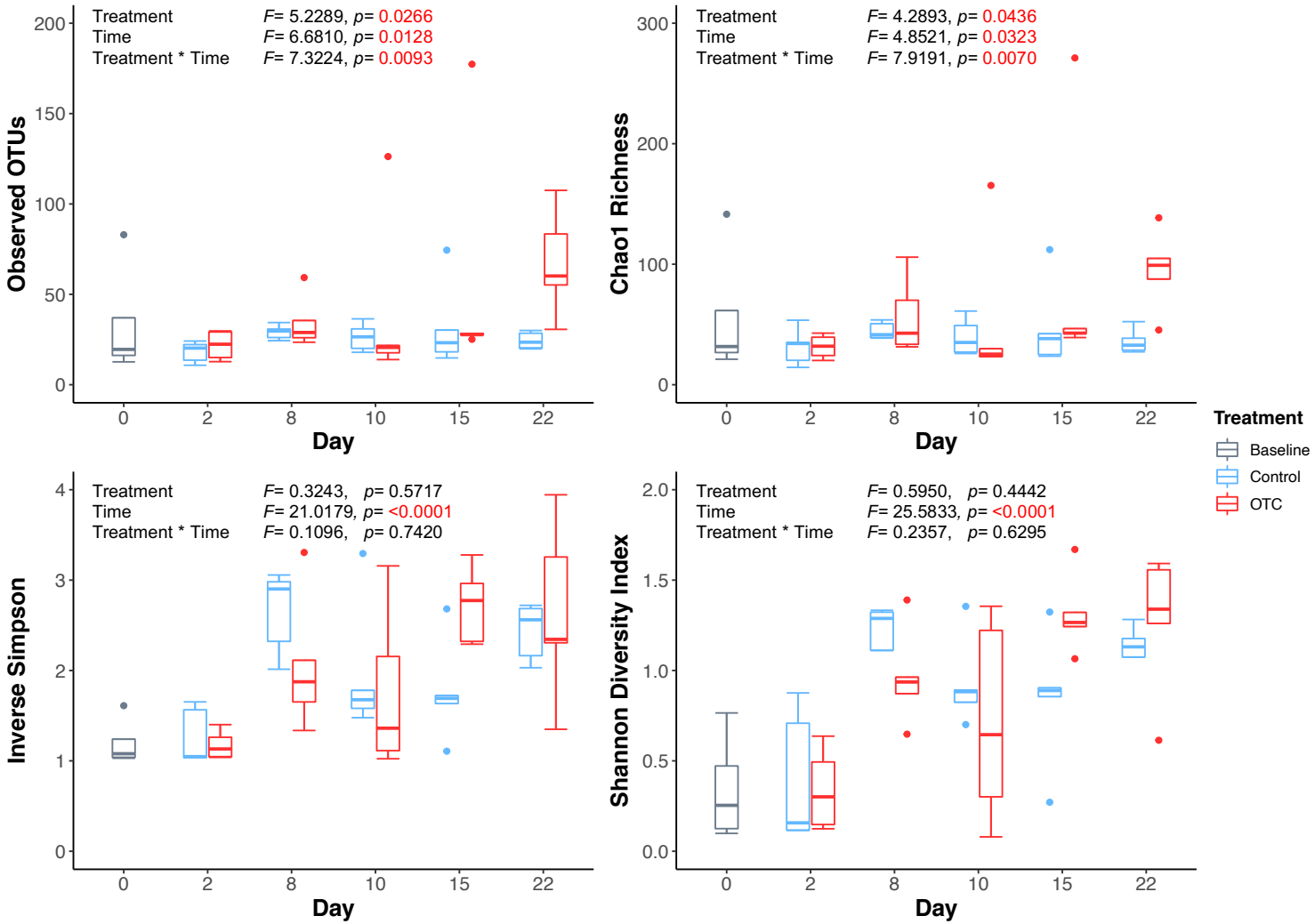

Figure 3.3. Alpha diversity measures of microbiome communities in the distal gut of control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

Time also appeared to be a significant effector in the community structure of distal gut microbiome communities in fish, as samples clustered according to sampling day in terms of community membership (ThetaYC; PERMANOVA F = 1.94, p = 0.054) and composition (Bray-Curtis; PERMANOVA F = 2.16, p = 0.026). More specifically, microbiome communities at day 0 and 2 were found to cluster together in community membership, and to some extent, composition (Figure 3.4 B & D). However, high inter-individual variability was observed between samples in the later time points of the study (Figure 3.4 B & D). In contrast, treatment group was only found to be a significant effector in microbiome community composition (PERMANOVA F = 2.37, p = 0.041) where distal gut microbiome communities were found cluster for baseline fish (Figure 3.4 D). Individual variability in community composition was noted for the control group, however these samples were found to cluster closer together compared with OTC-treated fish, where higher variation was found (Figure 3.4 D). When ThetaYC distances were explored, microbial community membership was indistinguishable by treatment group (PERMANOVA F = 2.06, p = 0.072) (Figure 3.4 B). No interaction was observed between treatment group and time (PERMANOVA $p > 0.1$). Furthermore, the microbiome communities in the distal gut of fish were also not influenced by the microbiome communities present in the diet or tank water, as samples visibly clustered according to sample type in terms of membership and composition (PERMANOVA $p < 0.001$) (Figure 3.4 A & C).

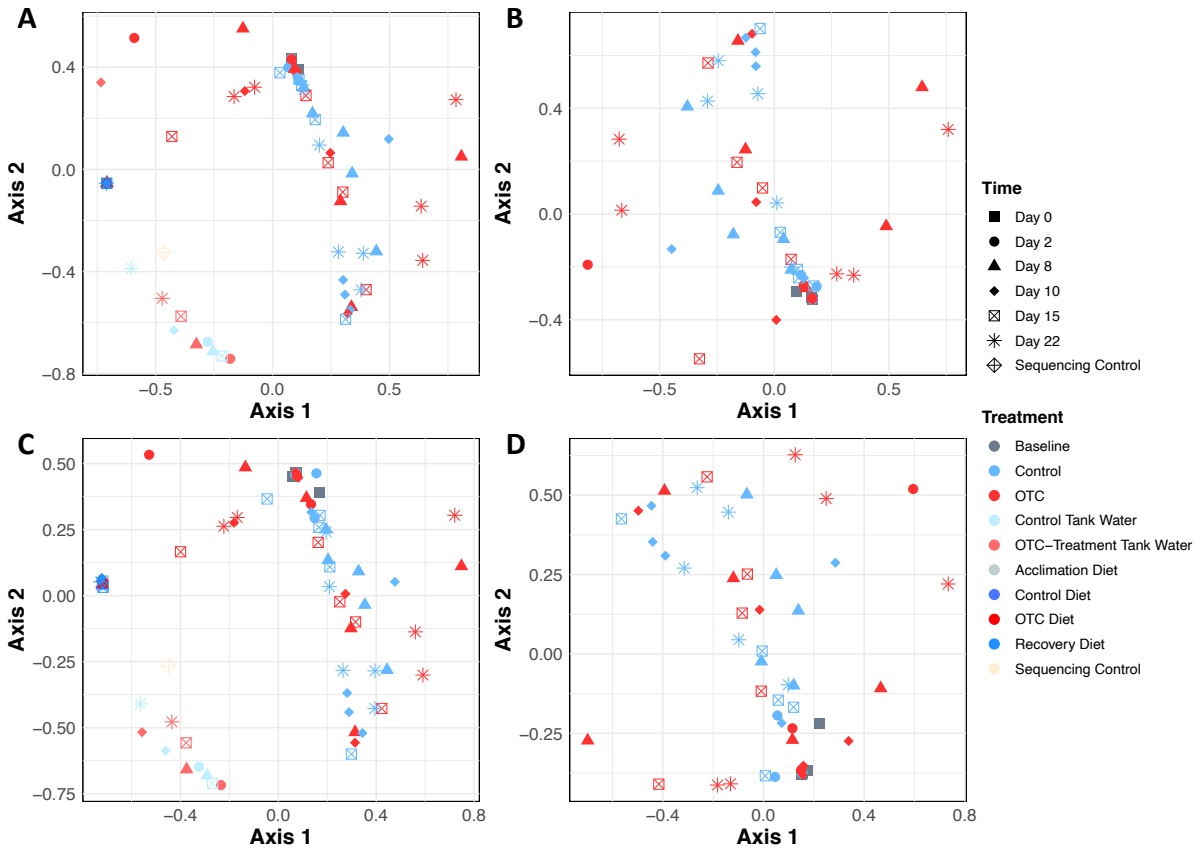

Figure 3.4. Non-multidimensional scaling of ThetaYC (A & B) and Bray-Curtis (C &D) distances. Distances illustrate differences in the microbiome community membership and composition of samples across time and exposure to oxytetracycline (OTC). Distances were generated for the complete dataset including diet, tank water and NSC samples (A &C), and within the distal gut of rainbow trout alone (B & D).

3.4.4. Microbial community composition and influence of oxytetracycline

The distribution of OTUs at phylum and genus level in the distal gut of fish across time and treatment group is illustrated in Figure 3.5. The mean (+SD) abundance of the top bacterial phyla and genera (Table 3.4 (S1)) are available as supplementary information (section 3.12). A total of 23 bacterial phyla and 411 bacterial genera were detected in the distal gut of fish, although 10 and 25 appeared to dominate these communities at varied sequence abundance across time points and in response to OTC treatment, respectively (Table 3.4 (S1)). At day 0, the distal gut microbiome communities of pre-treated fish were dominated by Tenericutes, followed by Proteobacteria, Firmicutes and Spirochaetes (Figure 3.5 A; Table 3.4 (S1)). At the genus level, *Mycoplasma* dominated distal gut microbiome communities of fish, followed by *Deefgea*, *Brevinema* and *Bacillus* (Figure 3.5 B; Table 3.4 (S1)).

The microbiome communities in the distal gut of rainbow trout were found to shift throughout the 27-day study period, reflecting the significant time effect observed in distal gut microbiome community structure (Figure 3.4). This was largely associated with OTUs assigned to Firmicutes, Proteobacteria and Tenericutes, which dominated the distal guts of fish in both treatment groups (Figure 3.5 A; Table 3.4 (S1)). At the genus level, these shifts were largely attributed to *Mycoplasma*, which was observed to dominate distal gut communities of fish at the start of the trial, but reduced in prevalence in both treatment groups over time (Figure 3.5 B). In contrast, the Firmicutes genera *Bacillus* and *Clostridium_sensu_stricto_1,* had higher sequence abundance in both treatment groups after day 8 compared with fish at earlier time points of the study (Figure 3.5 B; Table 3.4 (S1)). Likewise, a number of genera within the Proteobacteria phylum were found to increase in representation by day 22 in both treatment groups. These genera included *Aeromonas* and *Deefgea* (Figure 3.5 B; Table 3.4 (S1)).

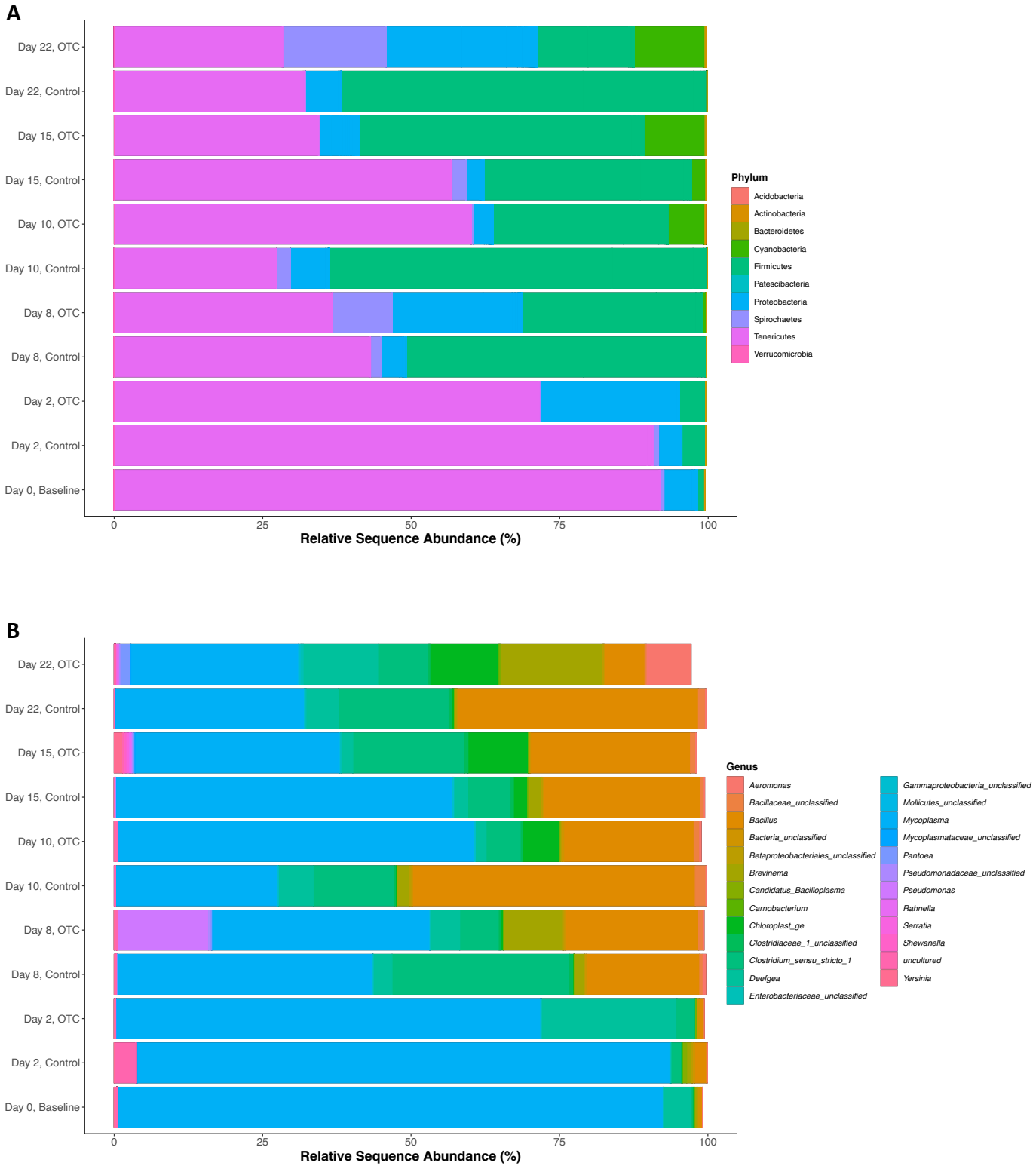

Figure 3.5. Mean relative sequence abundance (%) of the top 10 bacterial phyla (A) and top 25 bacterial genera (B) in the distal gut of control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment.

Oxytetracycline treatment also induced considerable changes in the distal gut microbiome of rainbow trout. This was associated with a reduced prevalence of Tenericutes in treated fish, compared with the control group (Figure 3.5 A). On closer inspection, this was found to arise from a declining trend in the sequence abundance of dominating OTUs assigned to *Mycoplasma*, at days 2 and 8 (Figure 3.5 B). Likewise, the abundance of Firmicutes was also found to be reduced in the distal gut of treated fish compared with the control group at days 8, 10 and 22 (Figure 3.5 A). However, within the Firmicutes phylum, the effect of antibiotic treatment was not universal across all genera, as *Clostridium_sensu_stricto_1* became enriched within the distal gut of treated fish compared with the control group at day 2, and again at one week following antibiotic withdrawal on day 15 (Figure 3.5 B). Oxytetracycline treatment was also observed to induce shifts in the abundance of Proteobacteria, both during the antibiotic treatment and then throughout the withdrawal period (Figure 3.5 A). At the genus level, *Deefgea* were found to have increased representation in the distal guts of treated fish immediately in response to OTC at day 2 (Figure 3.5 B). Similar patterns were also observed for less abundant genera including *Aeromonas* and *Shewanella* (Figure 3.5 B; Table 3.4 (S1)). In the case of *Shewanella*, this genus continued to increase in abundance throughout the antibiotic treatment period and withdrawal period, reaching their highest abundance in treated fish at day 15 (Table 3.4 (S1)). Some Proteobacteria members including *Pseudomonas* also displayed an increase in sequence abundance in response to OTC, albeit delayed, and became considerably elevated in the distal gut of treated fish by day 8 (Figure 3.5 B; Table 3.4 (S1)). However, the enrichment of *Pseudomonas* was observed to be unstable, as the abundance of this genus reduced following termination of the antibiotic treatment (Figure 3.5 B; Table 3.4 (S1)). A number of Proteobacteria genera also became enriched in the distal guts of treated fish following the withdrawal of OTC. Indeed, the bacterial genera *Aeromonas, Pantoea, Rahnella, Serratia, Shewanella* and *Yersinia* all reached their highest sequence abundance between days 15 and 22 (Figure 3.5 B; Table 3.4 (S1)), following the trend of increased microbial richness at these time points in treated fish (Figure 3.3).

Oxytetracycline also induced shifts in other bacterial taxa of the distal gut microbiome community in rainbow trout, albeit after treatment had stopped. For example, sequence abundance for Actinobacteria was found to be higher in treated fish compared with the control group at day 10, and further increased in abundance by day 22 (Table 3.4 (S1)). Likewise, Bacteroidetes was frequently detected in higher abundances within treated fish compared with the control group, and steadily increased in sequence abundance from day 8 to 22 (Table 3.4 (S1)). *Chloroplast_ge*, within the Cyanobacteria phylum was also among the taxa whose sequence abundance became enriched in the distal gut microbiome of treated fish following withdrawal of OTC (Figure 3.5 B). The proportion of *Chloroplast_ge* was observed to increase within the distal gut of treated

fish immediately following termination of OTC at day 8, and became further enriched in treated fish through days 10 to 22 (Figure 3.5 B; Table 3.4 (S1)). The observed enrichment in *Chloroplast_ge* was associated with a single OTU (OTU0005), which also dominated the microbiome communities associated with the feed pellets given during the trial (Figure 3.6). Further analysis of microbial communities associated with the diets revealed similar microbial communities across treatment group and time, supporting results from beta diversity analysis (Figure 3.4). Analysis of OTUs detected in the microbial communities within tank water revealed similar membership across treatment groups and time, although abundances between microbial genera varied (Figure 3.7). Overall, distinct microbiome communities were detected between the diet and tank water samples, following the trends observed in microbiome community structure (Figure 3.4). On closer inspection however, the microbial communities within the tank water samples did display similar composition to the NSC used in this study (Figure 3.7), and as such were not analysed further.

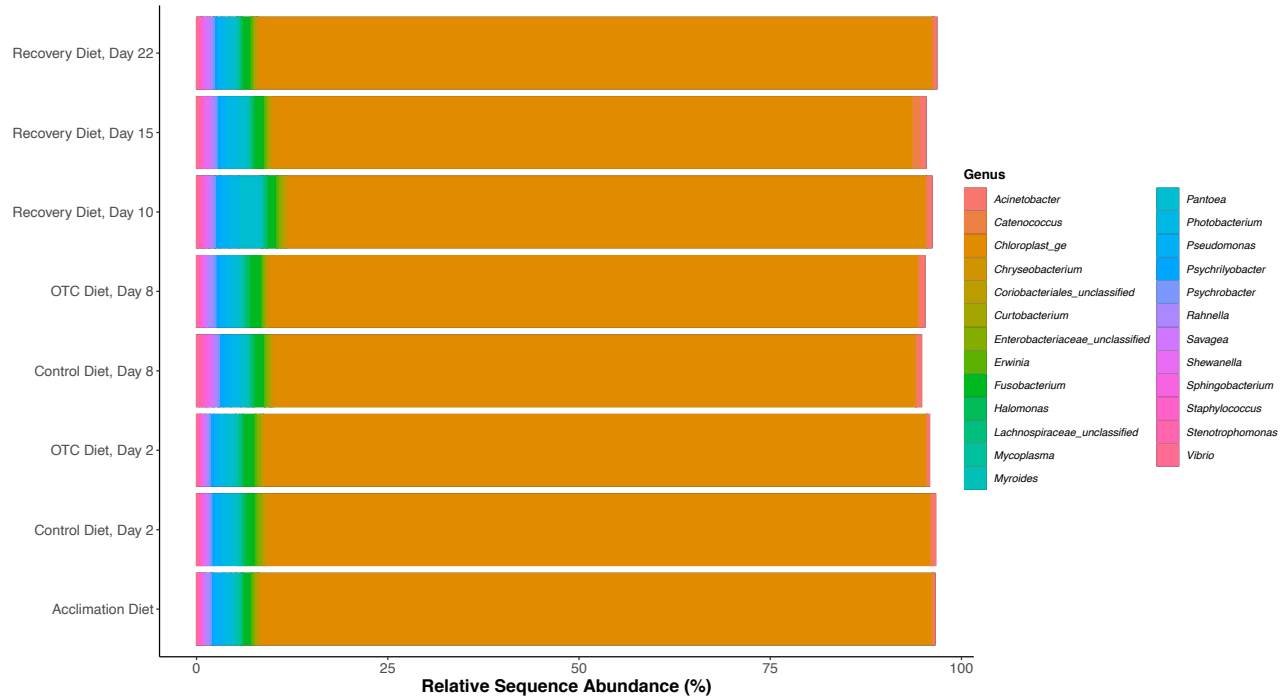

Figure 3.6. Relative sequence abundance (%) of the top 25 bacterial genera in feed pellets across time and treatment.

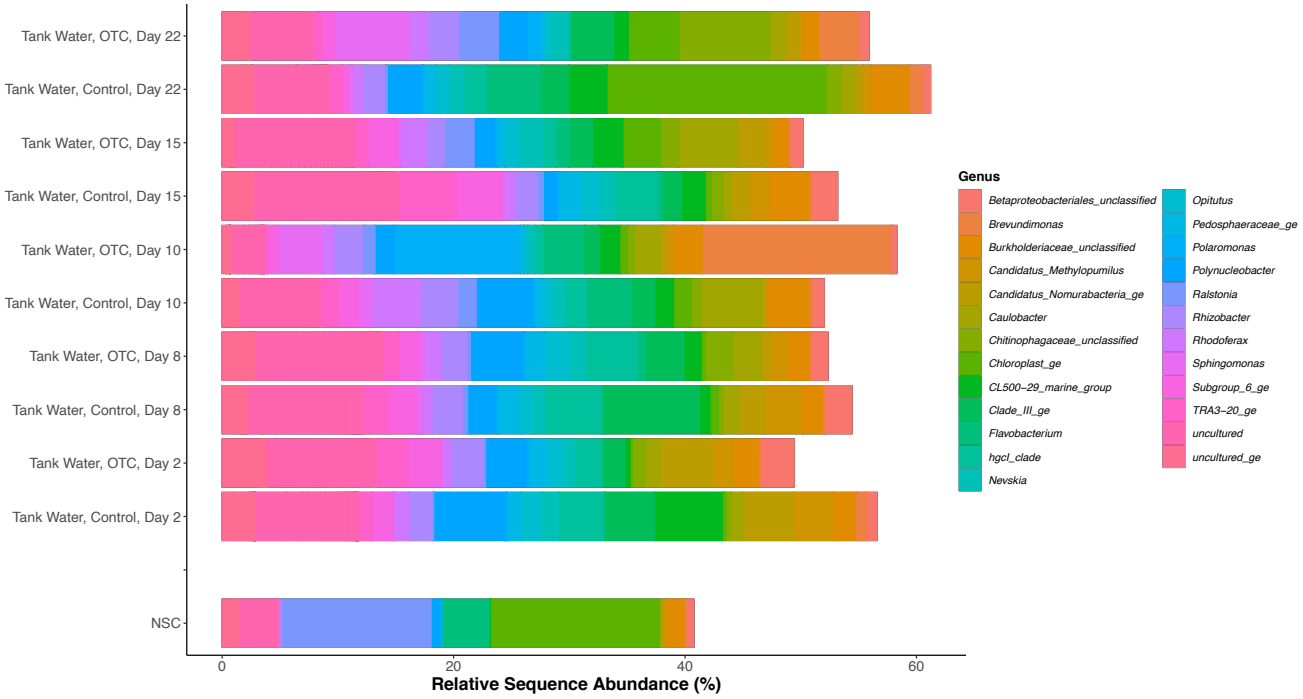

Figure 3.7. Relative sequence abundance (%) of the top 25 bacterial genera in the negative sequencing control (NSC) and tank water samples across time and treatment.

3.4.5. Comparison of OTUs and microbiome communities between control and treated fish

To better understand the influence of OTC on bacterial membership within the distal gut, the shared and distinct OTUs in fish across treatment and time were analysed (Figure 3.8). Out of a total of 442 OTUs observed in fish, only 19 were shared across all treatment groups and time points. As expected from community profiles, these OTUs were assigned to Cyanobacteria, Firmicutes, Proteobacteria, Tenericutes and Spirochaetes. Unique OTUs were primarily observed more frequently in treated fish (Figure 3.8 A), following the trend for increased microbial richness in the distal gut of fish in this treatment group compared with the control group (Figure 3.3). Likewise, unique OTUs were also observed between time points within the distal gut of fish exposed to OTC, with the number of unique OTUs and associated bacterial phyla observed to increase over time (Figure 3.8 A). Again, this followed the trend of increased microbial diversity over time observed in the distal gut of fish in the OTC treatment group (Figure 3.3). During OTC treatment, the distinct microbiome community within the distal gut of treated fish at day 2 was dominated by Firmicutes (one OTU) and represented 95% of all reads within this community (Figure 3.8 B; Table 3.5 (S2)). At the end of OTC treatment however, the distinct microbiome community in treated fish had shifted, with 50% of all reads assigned to Proteobacteria (five OTUs) at day 8 (Figure 3.8 B; Table 3.5 (S2)). Following treatment with OTC, distinct microbiome communities within the distal gut shifted towards a dominance of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, although distribution of these phyla varied over time (Figure 3.8 B; Table 3.5 (S2)). Whilst Actinobacteria dominated distinct microbiome communities in the distal gut of treated fish at day 10 (9 OTUs), and represented 36% of reads assigned in this community, this phylum became less abundant at days 15 (9 OTUs) and 22 (9 OTUs) with only 11% and 18% of reads assigned, respectively (Figure 3.8 B; Table 3.5 (S2)). Instead, the number of reads assigned to Proteobacteria became elevated at day 15 (22 OTUs) when it comprised 41% of all reads within this community, further increasing to 52% by day 22 (26 OTUs) (Figure 3.8 B; Table 3.5 (S2)). Whilst Firmicutes disappeared from the distinct microbiome community in the distal gut of treated fish at the end of OTC treatment, this phylum reappeared at day 10 (six OTUs) where it comprised 16% of all reads within this community (Figure 3.8 B; Table 3.5 (S2)). Furthermore, this phylum continued to increase in abundance by day 15 (11 OTUs) in treated fish, with 21% of all reads assigned to this phylum. However, Firmicutes became depleted again at day 22 (4 OTUs) when it represented only 4% of the distinct microbiome community within treated fish (Figure 3.8 B; Table 3.5 (S2)).

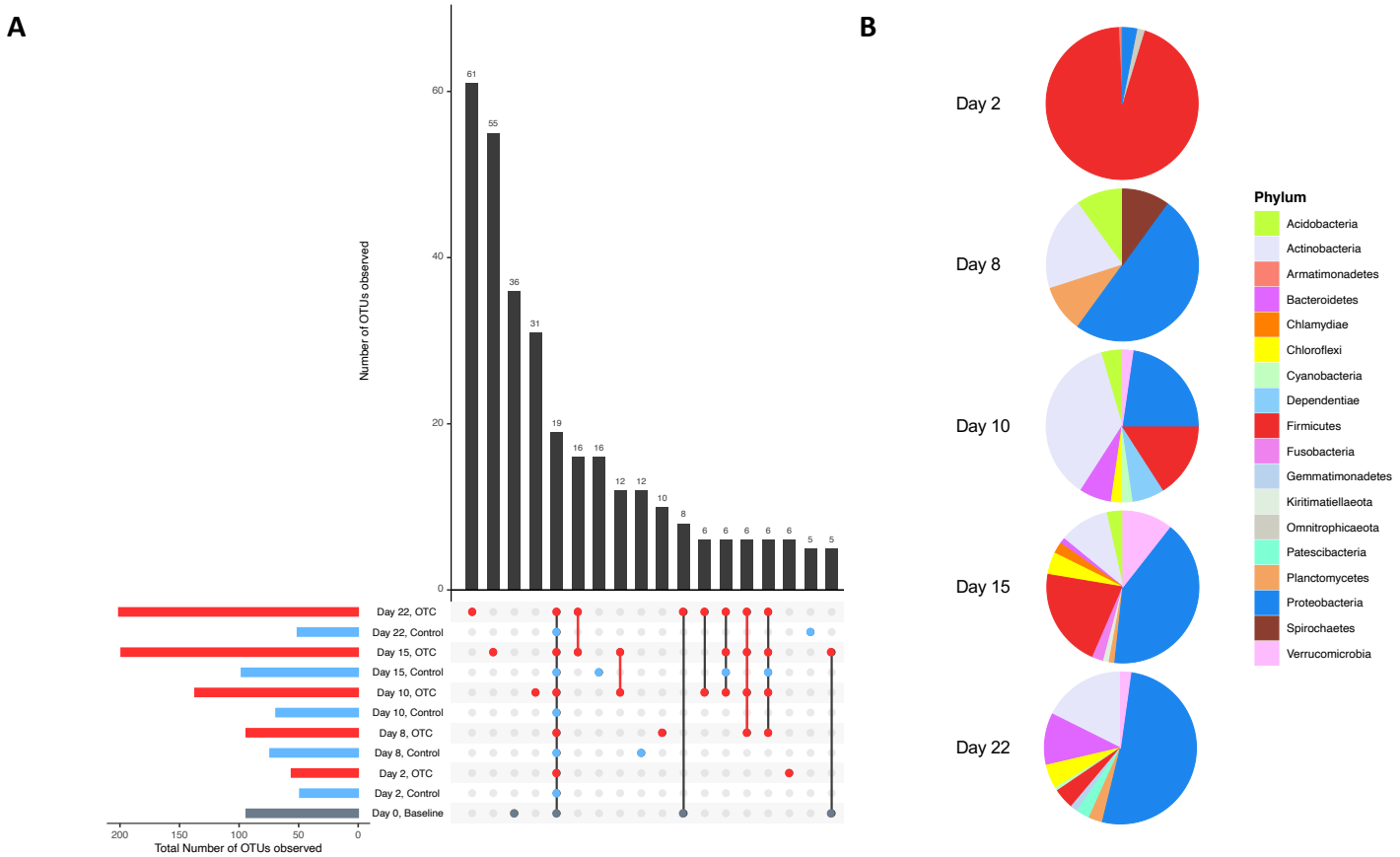

Figure 3.8. An UpsetR plot of core operational taxonomic units (OTUs) across treatment group and time (A), and the composition of unique OTUs assigned to phylum level in the distal gut of treated fish at days 2, 8, 10, 15 and 22 (B). Total number of OTUs observed in the distal gut of fish across treatment group and time was plotted to the left of the upsetR plot. Coloured circles on upsetR plot indicate core microbiome present in all samples within a particular treatment group. Connecting bar indicates multiple overlapping treatment groups and/or time points. Connecting bar coloured according to treatment groups which share OTUs; all groups (black), oxytetracycline group only (red). Circles coloured according to treatment; baseline (grey), control diet (blue) and oxytetracycline (OTC)-coated diet (red).

Differentially abundant OTUs within the distal gut of fish were also determined between control and OTC diet groups across time (Figure 3.9, Table 3.6 (S3)). In general, OTC treatment had the greatest impacts on Proteobacteria and Firmicutes bacteria. Following the trends observed in community profiles, a number of OTUs assigned to Proteobacteria were observed to become enriched in the distal gut of treated fish at selected time points (Figure 3.9). In contrast, differentially abundant OTUs assigned to Firmicutes were frequently observed to be depleted in the distal guts of treated fish across the study period (Figure 3.9). During antibiotic treatment, at days 2 and 8, OTC treatment was associated with significant reductions in the sequence abundance of Firmicutes OTUs assigned to several genera including *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_4* (Table 3.6 (S3)). However, the same OTU assigned to *Clostridium_sensu_stricto_1* was observed to significantly increase in abundance within the distal gut of treated fish following 1-week withdrawal of OTC, supporting the trend observed in taxonomic profiles (Figure 3.5). Likewise, several OTUs assigned to the Proteobacteria genera *Aeromonas* as well as *Reyranella* were also found to significantly increase in representation in the distal gut of treated fish following OTC treatment at day 10 (Table 3.6 (S3)), following findings observed in taxonomic profiles (Figure 3.5). Following the two-week withdrawal period, all differentially abundant Firmicutes OTUs were observed to be depleted in the distal gut microbiome communities of treated fish (Figure 3.9), supporting the observed trend in this phylum within taxonomic profiles (Figure 3.5). These OTUs were assigned to several genera including *Bacillus* (Table 3.6 (S3)). Further analysis of these OTUs revealed that one particular OTU assigned to an unclassified group of Clostridiaceae (OTU0007), had not recovered since becoming depleted in treated fish at the end of OTC treatment on day 8 (Table 3.6 (S3)). In contrast, OTUs assigned to the phyla Actinobacteria, Bacteroidetes, as well as a number of Proteobacteria OTUs, were observed to become enriched in the microbial communities present within the distal gut of treated fish (Figure 3.9). Again, this trend followed the patterns observed in community profiles (Figure 3.5). On closer inspection, these Proteobacteria OTUs were assigned to *Legionella, Polynucleobacter, Pseudomonas* and *Undibacterium* (Table 3.6 (S3)).

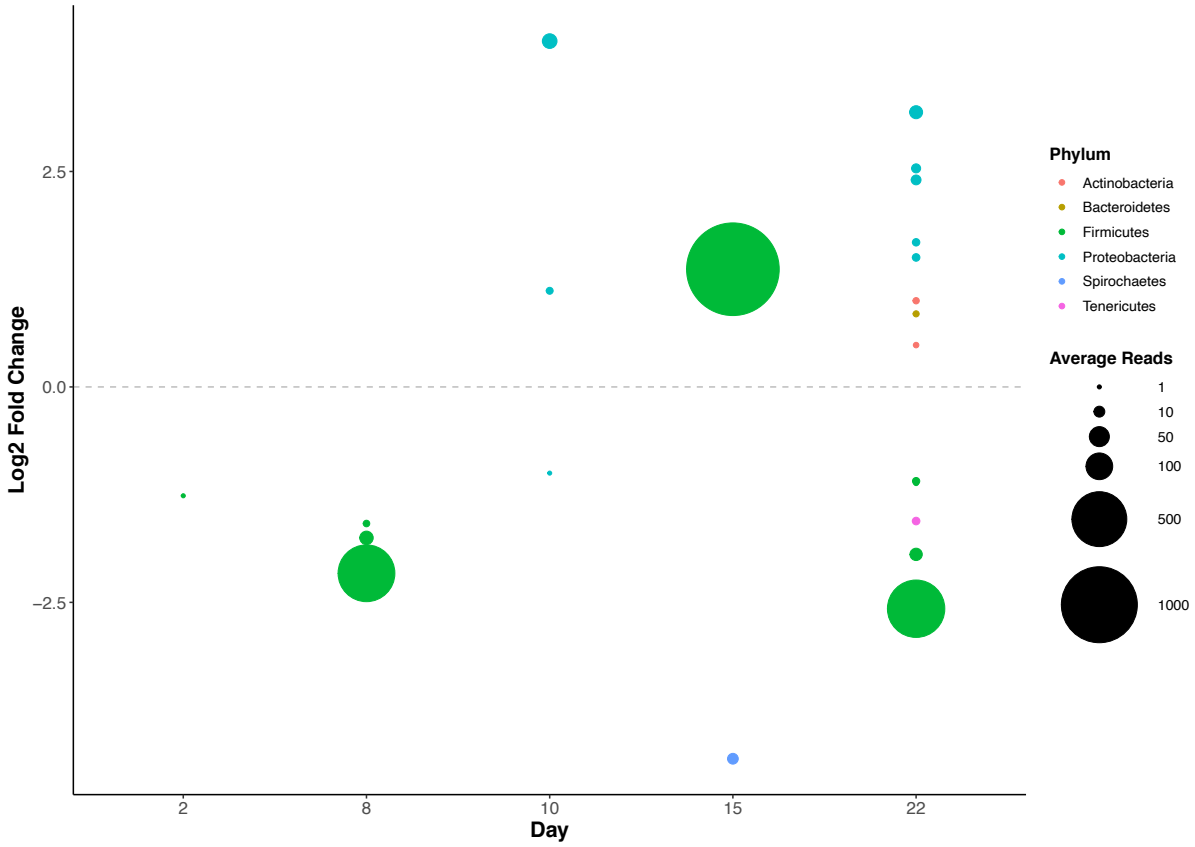

Figure 3.9. Plot of operational taxonomic units (OTUs) that had significantly different abundance ($p < 0.05$) in the distal gut of rainbow trout during and after antibiotic treatment, compared with control fish. Effect size is represented as the log2 fold-change of each OTU observed in rainbow trout from the oxytetracycline diet treatment group compared with fish fed the control diet. Each circle represents a single OTU and is coloured according to the phylum to which the OTU originates. Circle size is proportional to the mean read abundance of each OTU.

3.4.6. Influence of oxytetracycline on immune gene expression

The mRNA expression of the inflammatory cytokines IL-1β and TGF-β was also measured in the distal gut tissue of fish in this study (Figure 3.10). The expression of immune-related genes was measured for fish at days 0, 2 and 22 as a preliminary exploration of the influence of OTC on inflammatory status within the distal gut of fish over time. The mean expression of the pro-inflammatory cytokine IL-1β, was frequently found to be higher within the distal gut tissue of OTC-treated fish compared with control fish (Figure 3.10 A) however, no significant difference was found between treatment groups in this study ($p > 0.05$). Whilst the expression of IL-1β was found to decline over time in both treatment groups (Figure 3.10 A), this pattern was not found to be significant ($p > 0.05$). The mean expression of the anti-inflammatory cytokine TGF-β within the distal gut tissue of fish was also not found to be significantly different between control and treated groups in this study ($p > 0.05$), as seen in Figure 3.10 B. However, time was found to have a significant effect on the expression of TGF-β in fish from both treatment groups ($F = 8.93$, $p = 0.006$). On further post-hoc analysis, the mean expression of TGF-β was observed to become significantly reduced in the distal gut tissue of fish at days 2 and 22, compared with fish at day 0 ($p < 0.01$) (Figure 3.10 B). Furthermore, TGF-β expression was found to significantly increase in the distal gut tissue of fish from both treatment groups between day 2 and 22 ($p = 0.016$) (Figure 3.10 B).

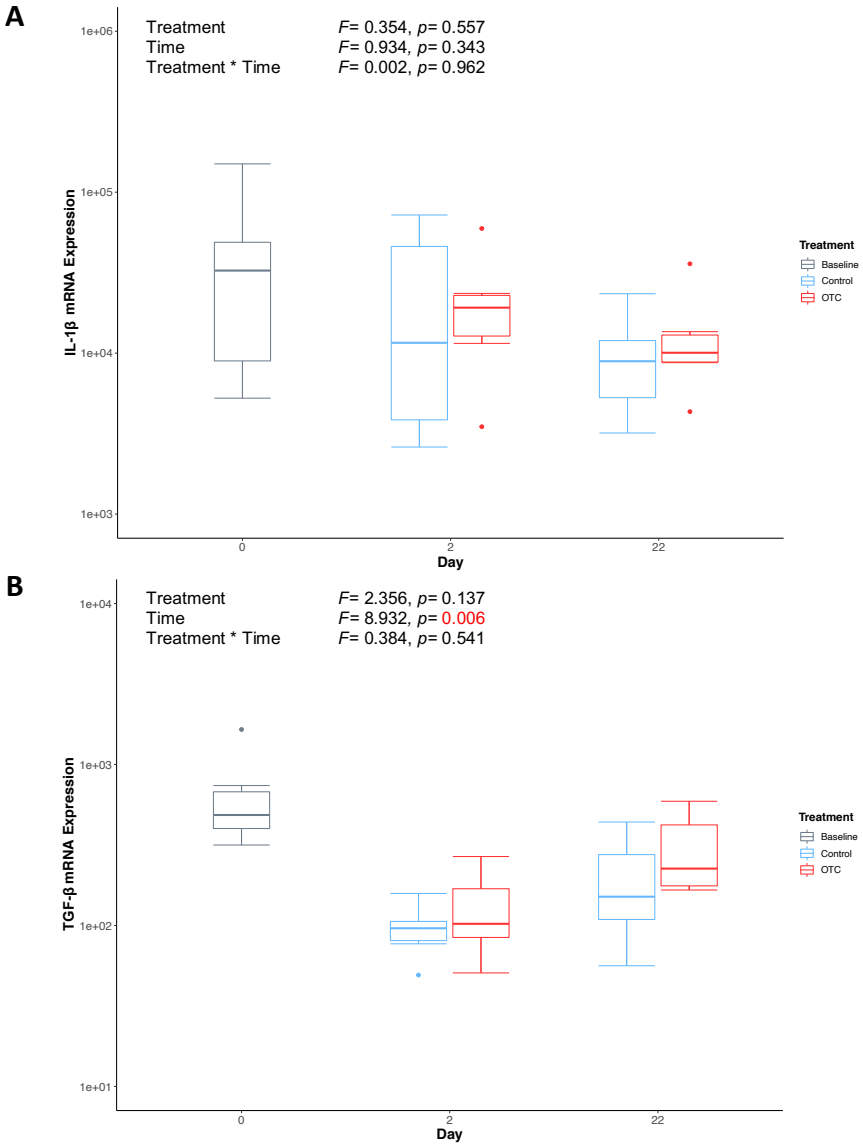

Figure 3.10. Box and whisker plot of IL-1β (A) and TGF-β (B) expression in the distal gut tissue of control or OTC-treated rainbow trout before, during and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

3.5. Discussion

Findings from this study showed that short-term low-level exposure to OTC rapidly induced disruption in the distal gut microbiome of rainbow trout. This disruption lead to alterations in the diversity and taxonomic profiles of microbiome communities in treated fish, and agrees with reports from previous studies on antibiotic administration in other fish species (Carlson et al., 2017; Limbu et al., 2018). In this study, an attempt was made to deliver a therapeutic dose of OTC (75 mg kg bodyweight day^{-1}), similar to what would be provided on a fish farm during an antibiotic treatment. Following the guidance from the National Office of Animal Health (2017), antibiotic dosage was therefore calculated on an expected feeding rate of 1.5% bodyweight day^{-1}. However, during this study, the low ambient water temperature resulted in a lower feeding response in fish. As a result, fish in the OTC treatment group actually received the antibiotic at a feeding rate of 0.7% bodyweight day^{-1} resulting in a lower dose of 35 mg kg bodyweight day^{-1}. Despite this, the subtherapeutic dose of OTC was sufficient to induce changes in the bacterial composition within the distal gut of fish exposed to the antibiotic. In this study, the abundance of Tenericutes and *Mycoplasma* in particular, decreased in the distal gut microbiome of treated fish within 48 hours of antibiotic treatment. It has previously been demonstrated that the abundance of Tenericutes also decreases in the guts of farmed piglets (*Sus scrofa*) exposed to OTC, when given in combination with other antibiotic compounds (Mu et al., 2017). These findings are not surprising given that the tetracycline group of antibiotics are often used in treating a range of diseases in humans and poultry caused by *Mycoplasma* bacterial pathogens (Brown, 2009; Kleven, 2017). As Tenericutes lack a cell wall, this cellular property results in a reduced protection against antibiotic compounds which target intracellular mechanisms of the bacteria, such as OTC which inhibits translation and protein synthesis by blocking ribosomal acceptor sites (Chopra & Robers, 2001). In this study, the reduced cellular protection of Tenericutes bacteria like *Mycoplasma*, may have allowed OTC to penetrate members of this group more easily, leading to the rapid decline of this phylum and genus within the distal guts of treated fish.

Oral exposure of OTC at low levels was also associated with the proliferation of Proteobacteria in treated fish, and follows reports in other fish species such as Atlantic salmon and Senegalese sole (*Solea senegalensis*) (Navarrete et al., 2008; Tapia-Paniagua et al., 2015). Proliferation of Proteobacteria in response to OTC treatment could result through a number of different pathways. The first being resistance to tetracycline compounds through the acquirement and expression of *tet* and *otr* genes, respectively (Chopra & Robers, 2001; Roberts, 2005). Variants of these genes have been described in both Gram-positive and Gram-negative bacteria. In addition, Gram-negative bacteria are reported to display a greater capacity to carry these genes, as *tet* genes found in Gram-positive bacteria have been demonstrated to successfully transfer into Gram-negative

organisms (Chopra & Robers, 2001). Several *tet* genes have been detected in a range of Gram-negative Proteobacteria, including those belonging to *Aeromonas, Pseudomonas, Serratia* and *Shewanella* (Roberts, 2005; Dang *et al.*, 2008). All of these genera were found in the microbial communities colonising the distal guts of treated fish, thus the potential presence of these genes may explain their increased prevalence, either during antibiotic treatment or the withdrawal period, potentially through selection pressures. As the abundance of antimicrobial resistance (AMR) genes were not quantified in this study, this theory was further explored in chapter 4 by investigating the change in abundance of genes related to tetracycline resistance in the distal gut of Nile tilapia following OTC treatment.

The expansion of Proteobacteria in the distal gut microbiome may have also occurred indirectly through OTC-induced changes in the immune functioning of treated fish. In higher vertebrates, immunoglobulin (Ig) A-mediated regulation of Proteobacteria in the gut microbiome has been demonstrated (Mirpuri et al., 2014). In fish, the immune system can also shape the gut microbiome community through exclusion and neutralisation pathways, such as secretory IgT which coat intestinal bacteria members (Zhang et al., 2010; Llewellyn et al., 2014; Kelly et al., 2017). Therefore, changes in immune pathways e.g. IgT levels, through OTC treatment, may have indirectly influenced the microbiome community through changes in taxa-specific regulation, which ultimately lead to the proliferation of Proteobacteria observed in this study. This seems likely as OTC has been reported to have immunosuppressive effects on immune parameters such as leucocyte counts, Ig levels and phagocytic activity in rainbow trout (Lundén et al., 1998; Enis Yonar et al., 2011). In this study, preliminary exploration of key pro- and anti-inflammatory cytokine gene expression, revealed that OTC did not have an observable effect on the inflammatory status within the distal gut of treated fish either during, or after antibiotic treatment. However, fish in both treatment groups did exhibit lower mRNA expression of IL-1β and TGF-β genes at day 22, compared with day 0. The gene expression profiles, particularly of IL-1β, followed that of the ambient water temperature recorded at NBFRU, which was also observed to decline from 4°C at day 0, to 1.6°C by day 22. These results would suggest a potential negative interaction between distal gut immunity of fish and water temperature in this study, and follows the current understanding surrounding the negative effect of low water temperature on fish physiology including the immune system (Abram et al., 2017). In fact, similar findings have been reported in rainbow trout previously with the expression of both pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokines becoming impaired at water temperatures below 5°C (Zou et al., 2000; Raida & Buchmann, 2007). As a result of the suboptimal water temperatures, the immune system of treated fish e.g., inflammatory components, could have exhibited a slower response to the OTC treatment or the observed changes in microbiome community, which were not able to be detected due to the short duration of treatment and withdrawal periods in this study.

Therefore, future studies which are conducted at optimal temperatures for host immune functioning, would be required to further investigate the interaction between OTC, the immune response and the gut microbiome in fish. In addition, due to the complexity of the immune system and its interaction with the gut microbiome in fish, it would also be of benefit to investigate a wider variety of immune-related genes which encompass the entire immune response pathway. These were addressed in the study described in chapter 4 with Nile tilapia reared at optimal water temperatures (El-Sayed & Kawanna, 2008), along with the exploration of changes in the expression of genes involved in immune perception e.g., pattern recognition receptors, as well as cell signalling and the induction of immune responses within the distal gut of treated fish.

Both time and OTC treatment were found to have significant effects on alpha diversity within the distal gut microbiome communities of fish in this study. Indeed, time was found to be a significant factor for community richness, diversity and evenness in fish from both treatment groups, reflecting the temporal variability observed in community structure and taxonomic profiles. Temporal changes in the gut microbiome community have been previously demonstrated in various fish species including Atlantic cod (*Gadus morhua*), fathead minnow (*Pimephales promelas*) and Southern catfish (*Silurus meridionalis*) (Narrowe et al., 2015; Zhang et al., 2017; Keating et al., 2021). In this study, temporal variation may have arisen through changes in tank water temperature described previously, as temperature-induced shifts in the gut microbiome have been demonstrated in both Atlantic salmon and rainbow trout (Naviner et al., 2006; Zarkasi et al., 2014; Huyben et al., 2018). These temperature-induced changes in the gut microbiome community of fish are likely associated with the ectothermic nature of fish (Coutant, 1976), resulting in the internal gut temperature reflecting that of the external environment. Therefore, changes in the internal body temperature of fish over time may have led to shifts in the microbiome community, as a result of the different thermal ranges of bacteria within the microbiome (Großkopf & Soyer, 2016) or changes in the activity of particular physiological systems, which are reported to regulate the fish gut microbiome community e.g. immune system (Kelly & Salinas, 2017). In addition, changes in stocking density within tanks following each sampling point, may have also played a role in the temporal changes in the distal gut microbiome community of fish observed in this study. In the present study, stocking density reduced from n=15 fish $tank^{-1}$ at day 0, to n=5 fish $tank^{-1}$ at day 15. In fact, the gut microbiome has been demonstrated to shift in response to different stocking densities in other fish species such as blunt snout bream (*Megalobrama amblycephala*) and gilthead sea bream (*Sparus aurata*) (Du et al., 2019; Parma et al., 2020). These shifts in microbiome community diversity and composition may arise directly through the release of cortisol as part of the primary stress response, as this has previously been demonstrated in Atlantic salmon in response to confinement stress (Uren Webster et al., 2020). It could be hypothesised therefore, that the continued reduction in stocking density

over time in the present study may have induced stress responses in fish, leading to elevated cortisol levels or potential adaptive physiological changes in systems known to influence the gut microbiome community e.g. immunity and digestion/metabolism. Indeed, low stocking densities (e.g. 10 kg m^{-3}) have previously been associated with elevated cortisol levels in rainbow trout (North et al., 2006), therefore this hypothesis is likely.

Oxytetracycline was also found to significantly affect microbiome community richness within the distal guts of treated fish. Following antibiotic treatment, the distal gut microbial communities in treated fish were observed to experience a period of instability and restructuring, leading to microbiomes with higher community richness. Whilst this finding contradicts previous studies in other fish species e.g. Atlantic salmon (Navarrete et al., 2008), increases in microbial richness as seen in this study, have been observed in Nile tilapia and fathead minnow exposed to low levels of OTC and triclosan, respectively (Narrowe et al., 2015; Limbu et al., 2018). These findings would suggest there is a potential intensity-dependant response in microbial ecosystems towards OTC and other antimicrobial compounds. This evidence supports that found in macroecosystems according to the intermediate disturbance hypothesis (Connell J.H., 1978). Within this theory, it is thought that the intermediate frequency or intensity of a disturbance can lead to increases in diversity within an ecosystem, as both colonisers and competitors can co-exist. However, during low or high intensity, only the competitors or colonisers can exist, respectively. This can be seen in riparian forests which show increased species richness and evenness after intermediate flooding (Giehl & Jarenkow, 2015). It is important to note that this study utilised a low number of animals, therefore any inferences relating to larger macroecosystems are met with caution, and further experimentation employing a larger number of animals should be conducted to strengthen this theory.

One particular advantage of increased richness in the gut microbiome following antibiotic disturbance in fish, may be an improved resilience of the community against future perturbations. Microbiomes are thought to share similar features to macroecosystems in that species-rich communities are considered more resilient to future disturbances. This is thought to occur as established species are more specialised for their occupied niche, and better able to use limiting resources more efficiently (Lozupone et al., 2012). In fact, this can be seen in soil microbiomes as more diverse soil-associated communities have been demonstrated to display increased resilience against exposure to pollutants (Girvan et al., 2005). Furthermore, higher bacterial diversity and improved community stability following antibiotic treatment, may also lead to improved functional resistance in the fish host following repeated disturbances. One microbial-mediated function in fish where this may be beneficial is immune homeostasis (Kelly & Salinas, 2017). Recent published evidence suggests that depletion of bacteria within the microbiome e.g.

through antibiotic treatment, can result in immune suppression (Khosravi et al., 2014). Improved functional resistance in immunity through better microbiome resilience may therefore reduce the risk of susceptibility to opportunistic pathogens following repeated antibiotic treatments.

Similar to other ecosystems, the gut microbiome communities of vertebrate animals can undergo successional events following a disturbance (Kriss et al., 2018). In the study presented, the distal gut microbiome communities of OTC-treated fish underwent successional changes throughout the recovery period, leading to the final community observed at day 22. In macroecosystems e.g. tropical or temperate forests, successional changes in the community following disturbances can take place over many years (Clebsch & Busing, 1989), owing to the generation time for the large multicellular organisms which colonise these habitats. In contrast, given that bacteria display much faster generation times, it is possible that disturbed microbial communities experience rapid successional events and reach the final state (or "climax community") in a shorter time frame. In the present study, the richness of distal gut microbiome communities in OTC-treated fish continued to increase throughout the withdrawal period and had still not stabilised by day 22. These findings would suggest that communities in OTC-treated fish had not reached their final state following the two-week withdrawal period. In other vertebrate animals, recovery of post-antibiotic communities has been reported to vary considerably. For example, in farmed chicken and humans, recovery of the gut microbiome can range from 12 days to more than four 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 beneficial services to support the health and physiology of the fish host. These microbial-mediated functions can include the breakdown of cellulose in the guts of herbivorous fish species (Liu et al., 2016), or the production of short chain fatty acids involved in energy synthesis and disease resilience (Kihara & Sakata, 1997, 2002). Likewise, the gut microbiome is also pivotal in modulating the immune response of fish through numerous pathways and host-microbiome interactions (Rawls et al., 2004, 2006; Kanther et al., 2011). Therefore, during microbiome recovery, if particular communities have not yet re-established or been replaced by bacteria which perform similar roles, certain functions may not be provided to the fish host during this process. 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. The recovery of microbiome communities in fish following antibiotic treatment could be influenced by factors relating to host genetics and physiology, as well as environmental conditions, as these have been demonstrated to influence fish microbiome communities previously (Li et al., 2012; Wong & Rawls, 2012). In the present study, the low

water temperature could have influenced the time taken for distal gut microbiome communities to recover in antibiotic treated fish. Due to the exothermic nature of fish (Coutant, 1976), water temperature can directly alter host physiological processes, as observed in the cytokine expression within the distal gut tissue of fish in this study. As certain physiological systems are thought to shape the gut microbiome community e.g. immune system (Llewellyn et al., 2014; Kelly et al., 2017), water temperature could also indirectly influence the recovery of the gut microbiome community of fish following a disturbance. Therefore, it is possible that at higher water temperatures, gut microbiome recovery in antibiotic treated fish may be faster, as fish will likely have improved physiological performance. Future experiments should therefore be conducted over longer time frames to build on evidence from this study, and to determine the length of the recovery process following antibiotic treatment. Furthermore, investigating how different environmental factors affect the recovery process in fish, will greatly improve our understanding of how microbiome recovery following antibiotic treatment can be supported through the production environment.

In this study, Cyanobacteria became a dominant bacterial phylum within the distal gut microbiome of treated fish following withdrawal of the OTC treatment. Within this phylum, the same genus (*Chloroplast_ge*) and OTU were also observed within the NSC sample and dominated the microbial community associated with feed pellets. The 16S rRNA gene sequences generated in this study were clustered into OTUs based on taxonomic similarity (phylotype-based) rather than DNA sequence (OTU-based). One problem identified with phylotype-binning approaches, is that there are many organisms within taxonomic databases that have similar phenotypes but belong to different taxonomic lineages (Schloss & Westcott, 2011). Therefore, in the present study, it was difficult to identify whether the reads assigned to the same OTU originated from the same organism across fish, environment and control samples. However, given that utilising fishmeal alternatives in aquafeeds has become a top priority for the global aquaculture sector, it was not surprising that Cyanobacteria were found to dominate feed pellets, as they are often included in commercial aquafeeds where they provide high levels of crude protein as well as polysaccharides and pigment components (Liang et al., 2015). Likewise, since this same OTU comprised less than 0.1% abundance within the distal guts of control fish in this study, it is unlikely that the OTU sequence abundance in the treated fish were solely derived from contaminant microbial DNA found in the NSC, as it would be expected to result in similar abundances across all fish and diet samples, respectively. Instead, considering that dietary influences on the gut microbiome have previously been reported in numerous fish species including rainbow trout (Desai et al., 2012; Sullam et al., 2012; Ingerslev et al., 2014), it is possible that the diet-associated microflora, including Cyanobacteria members, were able to colonise the distal gut of treated fish following antibiotic disruption. This supports previous

findings from Schmidt et al., (2017) who demonstrated successful colonisation of the probiotic strains *Phaeobacter inhibens* S4Sm and *Bacillus pumilus* RI06-95Sm in the recovered gut microbiome of black molly, following treatment with the antibiotic streptomycin sulfate. This study therefore provides important preliminary evidence which strengthens the link between nutrition and the influence on gut microbiome communities in fish. However, evidence from this study does warrant further research, to investigate how environmental microbial communities can be utilised to manipulate the recovery of post-antibiotic microbiomes in farmed fish. Furthermore, as antibiotic treatment is a common husbandry practice on many fish farms, understanding how these manipulated post-antibiotic communities may function to support or impair the health in these animals, would be of huge benefit to the aquaculture industry.

Disturbances in the distal gut microbiome community in response to low concentrations of OTC, as observed in this study, may have implications for the fish host and aquaculture production. In particular, the recovered microbiome community at day 22 has the potential for both short- and long-term side effects on fish health through further microbiome community changes. For example, imbalances in the fish gut microbiome through antibiotic treatment, may facilitate in the development of opportunistic infections. In this study, *Aeromonas, Pseudomonas* and *Yersinia* were all detected within the distal gut of fish, and were observed to increase in sequence abundance either during or following OTC treatment in treated fish. Whilst members of these genera can form part of the commensal microbial community associated with the fish gut (Austin, 2006); certain members within these genera can also be pathogenic (Austin & Austin, 2012). Successional changes and the establishment of opportunistic pathogens may arise indirectly through the establishment of Cyanobacteria, as Cyanobacterial metabolites have previously been shown to induce changes in the microbiome communities of Medaka (*Oryzias latipes*) (Duperron et al., 2019). Proliferation of pathogenic bacteria may also result directly through disruptions in community networks, which in healthy individuals promote stability of the microbiome community and colonisation resistance against pathogenic invaders (Bäumler & Sperandio, 2016). In non-healthy hosts, these networks are often less complex with fewer co-operative interspecies interactions, allowing for opportunistic bacteria to integrate into communities and become established. This is supported by recent evidence in Ayu (*Plecoglossus altivelis*), which displayed perturbed network dynamics with diminished complexity and co-operative interspecies interactions when infected with the bacterial pathogen *Vibrio anguillarum* (Nie et al., 2017). Finally, as the gut microbiome is thought to provide beneficial services to the fish host, disruptions in functionally important communities may lead to compromised health and physiology in farmed fish. In particular, Firmicutes members are considered to play important roles in fish immunity with many strains of *Bacillus* often used as probiotics to improve disease resilience in farmed fish (Gómez & Balcázar, 2008). Throughout this study, and particularly at

day 22, OTUs assigned to the Firmicutes phylum, and *Bacillus* and *Clostridium* in particular, became significantly depleted within the distal guts of treated fish. If these OTUs do not recover or are not replaced by other members serving a similar function, these disturbances in microbiome communities in fish following antibiotic treatment may have a long-term negative effect on host fitness, with potential consequences for production.

3.6. Conclusions

Overall, findings from this study showed that one-week exposure to a low-level treatment with a commercially licensed antibiotic, resulted in a significant shift in the distal gut microbiome community of rainbow trout. Furthermore, the gut microbiome of treated fish displayed similar patterns to other bacterial and terrestrial macroecosystems. As in other systems, the distal gut microbiome of treated fish was observed to undergo successional changes following low-level OTC exposure, leading to microbiome communities which were more diverse. However, this increase in community richness was accompanied by an enrichment in potential opportunistic pathogens and OTC-resistant populations. These findings therefore warrant further research efforts into the role of the resident microbiome community as an AMR reservoir in fish, and its implications for future treatments. Likewise, the establishment of diet-associated microbial communities, highlights the importance of nutritional support during the recovery of the gut microbiome following antibiotic disturbance. The suboptimal environmental conditions present at NBFRU were associated with the potential suppression of immune markers within the distal guts of fish in this study, therefore further work is required to evaluate how recovered microbiome communities may impact on host-microbial interactions and long-term host fitness.

3.7. Acknowledgments

This study was funded by the Fisheries Society of the British Isles as part of their PhD Studentship program. The authors would like to thank aquarium staff at NBFRU for their technical assistance during the feeding trial, and to Dr. J. Taggart and Mrs. J. Ireland for their expertise and guidance during the molecular phase of this research. We also appreciate the help provided by Mr. A. Pargana during sampling.

3.8. Author Contributions

CJP and MC developed the study concept and design. CJP collected stool samples from fish and performed all laboratory work. CJP analysed all data with input from JT, SM and MC.

3.9. Conflict of interest

The authors declare that they have no competing interests.

3.10. Ethical Approval

All work carried out was approved by the Animal Welfare and Ethical Review Body (AWERB) at UoS (AWERB (17 18) 006 New ASPA (B)) and followed guidelines set out by the UK Home Office Animals (Scientific Procedures) Act 1986.

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3.12. Supplementary Information

Table 3.4 (S1). Mean (± SD) abundance of top bacterial phyla and genera in the distal gut of control or oxytetracycline (OTC)-treated rainbow trout before, during and after antibiotic treatment.

Phylum	Genus	Day 0	Day 2		Day 8		Day 10		Day 15		Day 22	
		Baseline	Control	OTC	Control	OTC	Control	OTC	Control	OTC	Control	OTC
Acidobacteria	**All Genera (%)**	0.15 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.04 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Actinobacteria	**All Genera (%)**	0.01 ±0.02	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.02	0.01 ± 0.01	0.09 ± 0.20	0.03 ± 0.07	0.09 ± 0.17	0.00 ± 0.01	0.17 ± 0.16
Bacteria_unclassified	**All Genera (%)**	0.50 ± 0.07	0.41 ± 0.12	0.41 ± 0.18	0.26 ± 0.11	0.23 ± 0.16	0.14 ± 0.09	0.35 ± 0.31	0.31 ± 0.19	0.19 ± 0.11	0.19 ± 0.11	0.19 ± 0.09
	Bacteria_unclassified (%)	0.50 ± 0.07	0.41 ± 0.12	0.41 ± 0.18	0.26 ± 0.11	0.23 ± 0.16	0.14 ± 0.09	0.35 ± 0.31	0.31 ± 0.19	0.19 ± 0.11	0.19 ± 0.11	0.19 ± 0.09
Bacteroidetes	**All Genera (%)**	0.01 ± 0.02	0.02 ± 0.03	0.01 ± 0.02	0.01 ± 0.01	0.08 ± 0.12	0.04 ± 0.08	0.04 ± 0.09	0.02 ± 0.04	0.20 ± 0.29	0.00 ± 0.01	0.25 ± 0.16
Chlamydiae	**All Genera (%)**	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.03	0.01 ± 0.01	0.02 ± 0.04	0.01 ± 0.02	0.04 ± 0.08	0.01 ± 0.02	0.03 ± 0.04
Cyanobacteria	**All Genera (%)**	0.06 ± 0.07	0.06 ± 0.13	0.02 ± 0.02	0.01 ± 0.01	0.56 ± 1.15	0.01 ± 0.01	6.19 ± 13.83	2.45 ± 5.48	10.20 ± 22.72	0.00 ± 0.01	11.62 ± 16.92
	Chloroplast_ge (%)	0.06 ± 0.07	0.06 ± 0.13	0.02 ± 0.02	0.01 ± 0.01	0.55 ± 1.15	0.01 ± 0.01	6.18 ± 13.82	2.44 ± 5.46	10.18 ± 22.68	0.00 ± 0.01	11.60 ± 16.28
Firmicutes	**All Genera (%)**	1.15 ± 1.25	3.91 ± 8.05	4.36 ± 6.11	50.54± 23.28	30.29 ± 37.47	63.52 ± 35.72	29.38 ± 41.89	34.92 ± 37.98	47.80 ± 34.87	61.49 ± 29.11	16.23 ± 20.53
	Bacillaceae_unclassified (%)	0.01 ± 0.01	0.08 ± 0.12	0.02 ± 0.02	0.56 ± 0.27	0.81 ± 1.05	1.81 ± 1.62	0.94 ± 1.42	0.67 ± 0.96	0.88 ± 0.63	1.16 ± 0.58	0.20 ± 0.21
	Bacillus (%)	0.32 ± 0.61	2.01 ± 4.05	0.73 ± 0.44	19.14 ± 12.37	22.50 ± 31.36	47.60 ± 40.21	21.96 ± 37.06	26.36 ± 39.12	26.96 ± 22.17	40.63 ± 21.33	6.82 ± 8.44
	Carnobacterium (%)	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02	0.04 ± 0.08	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.02	0.00 ± 0.01	0.03 ± 0.07	0.25 ± 0.55	0.19 ± 0.36
	Clostridiaceae_1_unclassified (%)	0.14 ± 0.25	0.04 ± 0.09	0.09 ± 0.17	0.74 ± 0.15	0.21 ± 0.35	0.38 ± 0.28	0.26 ± 0.48	0.40 ± 0.48	0.58 ± 0.40	0.67 ± 0.51	0.16 ± 0.23
	Clostridium_sensu_stricto_1 (%)	0.19 ± 0.28	1.76 ± 3.76	3.07 ± 5.86	29.89 ± 14.18	6.67 ± 9.20	13.61 ± 13.19	5.90 ± 11.14	7.29 ± 6.63	18.81 ± 12.92	18.60 ± 13.95	8.46 ± 11.73
Fusobacteria	**All Genera (%)**	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.09 ± 0.20	0.03 ± 0.06	0.09 ± 0.21	0.00 ± 0.00	0.13 ± 0.19
Patescibacteria	**All Genera (%)**	0.01 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.03	0.00 ± 0.01	0.01 ± 0.03	0.02 ± 0.04	0.02 ± 0.05

Table 3.4 (S1). *Continued.*

Phylum	Genus	Day 0	Day 2		Day 8		Day 10		Day 15		Day 22	
		Baseline	Control	OTC	Control	OTC	Control	OTC	Control	OTC	Control	OTC
Proteobacteria	**All Genera (%)**	5.64 ± 9.58	3.93 ± 7.38	23.43 ± 45.10	4.27 ± 4.09	21.98 ± 34.93	6.62 ± 13.97	3.32 ± 2.06	3 ± 3.62	6.73 ± 5.57	6.07 ± 6.69	25.52 ± 25.92
	Aeromonas	0.01 ± 0.01	0.00 ± 0.01	0.12 ± 0.15	0.47 ± 0.76	0.09 ± 0.09	0.00 ± 0.01	0.27 ± 0.27	0.06 ± 0.13	0.06 ± 0.13	0.12 ± 0.27	7.57 ± 16.89
	Betaproteobacteriales_unclassified (%)	0.12 ± 0.25	0.00 ± 0.01	0.05 ±0.06	0.03 ± 0.04	0.07 ± 0.12	0.33 ± 0.74	0.01 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.03
	Deefgea (%)	4.76 ± 9.49	0.16 ± 0.34	22.84 ± 45.02	3.19 ± 3.62	4.91 ± 8.39	5.98 ± 12.92	1.86 ± 1.99	2.33 ± 3.75	1.92 ± 3.29	5.50 ± 6.59	12.73 ± 21.65
	Enterobacteriaceae_unclassified (%)	0.01 ± 0.02	0.00 ± 0.01	0.02 ± 0.05	0.03 ± 0.05	0.01 ± 0.02	0.01 ± 0.02	0.04 ± 0.08	0.12 ± 0.23	0.28 ± 0.46	0.25 ± 0.55	0.34 ± 0.37
	Gammaproteobacteria_unclassified (%)	0.02 ± 0.02	0.00 ± 0.01	0.04 ± 0.08	0.03 ± 0.03	0.11 ± 0.19	0.00 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.07 ± 0.09	0.01 ± 0.02	0.37 ± 0.68
	Pantoea (%)	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.03	0.00 ± 0.00	0.10 ± 0.23	0.02 ± 0.04	0.37 ± 0.83	0.00 ± 0.00	1.87 ± 3.64
	Pseudomonadaceae_unclassified (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 1.09	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.01
	Pseudomonas (%)	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.00	15.30 ± 34.22	0.01 ± 0.02	0.04 ± 0.09	0.06 ± 0.14	0.31 ± 0.68	0.01 ± 0.02	0.19 ± 0.18
	Rahnella (%)	0.00 ± 0.00	0.00 ± 0.01	0.01 ± 0.02	0.04 ± 0.09	0.00 ± 0.01	0.06 ± 0.14	0.02 ± 0.04	0.02 ± 0.03	0.26 ± 0.59	0.00 ± 0.00	0.15 ± 0.25
	Serratia (%)	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.02	0.01 ± 0.02	0.58 ± 1.29	0.00 ± 0.00	0.30 ± 0.68
	Shewanella (%)	0.09 ± 0.16	0.01 ± 0.02	0.05 ± 0.09	0.01 ± 0.03	0.08 ± 0.10	0.00 ± 0.00	0.04 ± 0.09	0.02 ± 0.04	0.39 ± 0.88	0.00 ± 0.00	0.12 ± 0.22
	Yersinia (%)	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.03	0.01 ± 0.02	0.07 ± 0.17	0.00 ± 0.00	0.00 ± 0.01	1.44 ± 3.21	0.01 ± 0.02	0.00 ± 0.00
Spirochaetes	**All Genera (%)**	0.52 ± 0.39	0.94 ± 1.48	0.11 ± 0.12	1.71 ± 3.19	10.02 ± 20.90	2.22 ± 2.95	0.36 ± 0.31	2.36 ± 2.95	0.11 ± 0.17	0.07 ± 0.10	17.40 ± 37.97
	Brevinema (%)	0.44 ± 0.40	0.94 ± 1.48	0.11 ± 0.12	1.71 ± 3.19	10.02 ± 20.89	2.22 ± 2.95	0.36 ± 0.31	2.36 ± 2.95	0.11 ± 0.17	0.07 ± 0.10	17.40 ± 37.97
Tenericutes	**All Genera (%)**	92 ± 10.39	90.70 ± 10.44	71.62 ± 44.03	43.15 ±23.01	36.76 ± 33.37	27.39 ± 30.77	60.08 ± 39.94	56.86 ± 32.42	34.43 ± 22.74	32.15 ± 27.10	28.33 ± 27.65
	Candidatus_Bacilloplasma (%)	0.00 ± 0.00	0.74 ± 1.66	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.43	0.00 ± 0.00
	Mollicutes_unclassified (%)	0.07 ± 0.04	0.06 ± 0.03	0.06 ± 0.04	0.03 ± 0.02	0.04 ±0.04	0.01 ± 0.01	0.04 ± 0.04	0.04 ± 0.03	0.05 ± 0.06	0.02 ± 0.03	0.03 ± 0.04
	Mycoplasma (%)	91.76 ± 10.41	89.71 ± 11.43	71.45 ± 43.94	43.04 ± 22.93	36.66 ± 33.28	27.31 ± 30.72	59.93 ± 39.88	56.74 ± 32.35	34.31 ± 22.66	31.83 ± 27.30	28.27 ± 27.59
	Mycoplasmataceae_unclassified (%)	0.17 ± 0.03	0.18 ± 0.04	0.12 ± 0.08	0.08 ± 0.08	0.06 ± 0.06	0.05 ± 0.07	0.11 ± 0.11	0.08 ± 0.05	0.07 ± 0.06	0.11 ± 0.06	0.03 ± 0.03
Uncultured	*uncultured* (%)	0.43 ± 0.50	3.67 ± 7.41	0.17 ± 0.19	0.32 ± 0.21	0.50 ± 0.61	0.06 ± 0.06	0.42 ± 0.66	0.10 ± 0.10	0.14 ± 0.13	0.06 ± 0.08	0.13 ± 0.11
Verrucomicrobia	**All Genera (%)**	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.02	0.02 ± 0.04	0.00 ± 0.01	0.04 ± 0.10	0.00 ± 0.00	0.01 ± 0.03

Table 3.5 (S2). Unique operational taxonomic units (OTU) observed in oxytetracycline-treated fish before, during and after antibiotic treatment.

Day	OTU	Phylum	Genus	Cumulative Reads
2	OTU0174	Armatimonadetes	*Fimbriimonadaceae_ge*	1
	OTU0156	Firmicutes	*Ruminococcaceae_unclassified*	120
	OTU0367	Omnitrophicaeota	*Omnitrophicaeota_ge*	2
	OTU0032	Proteobacteria	*Rhizobacter*	1
	OTU0462	Proteobacteria	*Unknown_Family_ge*	2
	OTU0797	Proteobacteria	*EV818SWSAP88_ge*	1
8	OTU0613	Acidobacteria	*DS-100_ge*	1
	OTU0792	Actinobacteria	*Marmoricola*	1
	OTU0851	Actinobacteria	*Pseudarthrobacter*	1
	OTU0181	Planctomycetes	*Schlesneria*	1
	OTU0114	Proteobacteria	*Moraxellaceae_unclassified*	1
	OTU0375	Proteobacteria	*Pedomicrobium*	1
	OTU0427	Proteobacteria	*uncultured_ge*	1
	OTU0520	Proteobacteria	*OM60(NOR5)_clade*	1
	OTU0983	Proteobacteria	*Lelliottia*	1
	OTU0597	Spirochaetes	*Spirochaetia_unclassified*	1
10	OTU0239	Acidobacteria	*Paludibaculum*	1
	OTU0356	Acidobacteria	*Subgroup_2_ge*	1
	OTU0507	Actinobacteria	*Aeromicrobium*	1
	OTU0562	Actinobacteria	*Corynebacterium*	1
	OTU0569	Actinobacteria	*Gordonia*	2
	OTU0617	Actinobacteria	*Streptomyces*	5
	OTU0649	Actinobacteria	*MB-A2-108_ge*	1
	OTU0660	Actinobacteria	*Frondihabitans*	1
	OTU0699	Actinobacteria	*Propionibacterium*	1
	OTU0717	Actinobacteria	*Ilumatobacter*	2
	OTU0742	Actinobacteria	*Modestobacter*	2
	OTU0611	Bacteroidetes	*Ulvibacter*	1
	OTU0846	Bacteroidetes	*Capnocytophaga*	2
	OTU0833	Chloroflexi	*TK10_ge*	1
	OTU0655	Cyanobacteria	*uncultured*	1
	OTU0752	Dependentiae	*Babeliaceae_ge*	3
	OTU0207	Firmicutes	*Erysipelotrichaceae_unclassified*	1
	OTU0377	Firmicutes	*Ureibacillus*	2
	OTU0593	Firmicutes	*Aerosphaera*	1
	OTU0601	Firmicutes	*Novibacillus*	1
	OTU0685	Firmicutes	*Nosocomiicoccus*	1
	OTU0778	Firmicutes	*Hathewaya*	1
	OTU0206	Proteobacteria	*Legionellaceae_unclassified*	1
	OTU0231	Proteobacteria	*Rhodobacteraceae_unclassified*	1
	OTU0357	Proteobacteria	*MBNT15_ge*	1
	OTU0525	Proteobacteria	*Dokdonella*	1
	OTU0595	Proteobacteria	*Pseudoalteromonas*	3
	OTU0633	Proteobacteria	*Oleiagrimonas*	1
	OTU0698	Proteobacteria	*Xenophilus*	1
	OTU1137	Proteobacteria	*Xanthobacter*	1
	OTU0798	Verrucomicrobia	*Chthoniobacterales_unclassified*	1
15	OTU0116	Acidobacteria	*Candidatus_Solibacter*	1
	OTU0529	Acidobacteria	*RB41*	2
	OTU0285	Actinobacteria	*uncultured_ge*	1
	OTU0334	Actinobacteria	*Corynebacteriales_unclassified*	1
	OTU0349	Actinobacteria	*Corynebacteriaceae_unclassified*	1
	OTU0644	Actinobacteria	*67-14_ge*	1
	OTU0694	Actinobacteria	*Candidatus_Aquiluna*	1
	OTU0695	Actinobacteria	*Williamsia*	1
	OTU0763	Actinobacteria	*Geodermatophilaceae_unclassified*	1
	OTU0850	Actinobacteria	*Friedmanniella*	1
	OTU1033	Actinobacteria	*Eggerthellaceae_unclassified*	1
	OTU0138	Bacteroidetes	*KD3-93_ge*	1
	OTU0432	Chlamydiae	*uncultured*	2
	OTU0449	Chloroflexi	*KD4-96_ge*	1
	OTU0590	Chloroflexi	*SBR1031_ge*	1
	OTU1002	Chloroflexi	*Dehalococcoidia_unclassified*	2

Table 3.5 (S2). *Continued.*

Day	OTU	Phylum	Genus	Cumulative Reads
15	OTU0330	Firmicutes	*Family_XIII_unclassified*	1
	OTU0457	Firmicutes	*Anoxybacillus*	5
	OTU0561	Firmicutes	*Allofustis*	3
	OTU0573	Firmicutes	*Calditerricola*	1
	OTU0643	Firmicutes	*Ruminococcaceae_ge*	1
	OTU0683	Firmicutes	*Peptostreptococcaceae_unclassified*	1
	OTU0735	Firmicutes	*Christensenellaceae_unclassified*	1
	OTU0949	Firmicutes	*Cellulosilyticum*	1
	OTU0979	Firmicutes	*Ruminococcaceae_UCG-002*	1
	OTU1001	Firmicutes	*Limnochordaceae_ge*	1
	OTU1031	Firmicutes	*Caldicellulosiruptor*	2
	OTU0072	Fusobacteria	*Fusobacteriaceae_unclassified*	2
	OTU0279	Kiritimatiellaeota	*WCHB1-41_ge*	1
	OTU0415	Planctomycetes	*Zavarzinella*	1
	OTU0262	Proteobacteria	*Wohlfahrtiimonas*	7
	OTU0358	Proteobacteria	*Novosphingobium*	3
	OTU0388	Proteobacteria	*Rhodanobacteraceae_unclassified*	1
	OTU0411	Proteobacteria	*Devosia*	1
	OTU0475	Proteobacteria	*Morganella*	1
	OTU0487	Proteobacteria	*Uruburuella*	1
	OTU0547	Proteobacteria	*Rhodobacter*	1
	OTU0549	Proteobacteria	*Candidatus_Paracaedibacter*	2
	OTU0553	Proteobacteria	*Pasteurellaceae_unclassified*	2
	OTU0559	Proteobacteria	*Candidatus_Alysiosphaera*	1
	OTU0560	Proteobacteria	*Pasteurella*	1
	OTU0568	Proteobacteria	*Desulfobacca*	1
	OTU0584	Proteobacteria	*Noviherbaspirillum*	2
	OTU0626	Proteobacteria	*Polymorphobacter*	1
	OTU0630	Proteobacteria	*Kosakonia*	1
	OTU0677	Proteobacteria	*Pseudorhodobacter*	2
	OTU0706	Proteobacteria	*Roseiarcus*	1
	OTU0812	Proteobacteria	*Izhakiella*	1
	OTU0954	Proteobacteria	*Citrobacter*	1
	OTU1096	Proteobacteria	*Acidibacter*	1
	OTU1190	Proteobacteria	*uncultured_ge*	1
	OTU1191	Proteobacteria	*Azospirillaceae_unclassified*	2
	OTU0040	Verrucomicrobia	*Prosthecobacter*	1
	OTU0407	Verrucomicrobia	*Candidatus_Udaeobacter*	6
	OTU0614	Verrucomicrobia	*Candidatus_Xiphinematobacter*	2
22	OTU0093	Actinobacteria	*Candidatus_Planktophila*	4
	OTU0121	Actinobacteria	*hgcI_clade*	5
	OTU0602	Actinobacteria	*Lawsonella*	3
	OTU0665	Actinobacteria	*MWH-Ta3*	1
	OTU0701	Actinobacteria	*Frigoribacterium*	1
	OTU0783	Actinobacteria	*Flaviflexus*	1
	OTU0842	Actinobacteria	*Actinotalea*	4
	OTU0895	Actinobacteria	*Galbitalea*	3
	OTU0959	Actinobacteria	*Leifsonia*	2
	OTU0086	Bacteroidetes	*uncultured*	1
	OTU0089	Bacteroidetes	*NS11-12_marine_group_ge*	4
	OTU0102	Bacteroidetes	*Sediminibacterium*	2
	OTU0254	Bacteroidetes	*uncultured*	1
	OTU0374	Bacteroidetes	*Muribaculaceae_ge*	1
	OTU0418	Bacteroidetes	*OPB56_ge*	1
	OTU0428	Bacteroidetes	*Dinghuibacter*	1
	OTU0550	Bacteroidetes	*Mucilaginibacter*	2
	OTU0697	Bacteroidetes	*Dysgonomonas*	1
	OTU1030	Bacteroidetes	*Pseudarcicella*	1
	OTU0211	Chloroflexi	*SL56_marine_group_ge*	5
	OTU0950	Chloroflexi	*Chloronema*	2
	OTU0420	Cyanobacteria	*Halotia_CENA158*	1
	OTU0291	Firmicutes	*uncultured*	1
	OTU0303	Firmicutes	*Veillonellaceae_unclassified*	1
	OTU0563	Firmicutes	*Granulicatella*	2
	OTU0696	Firmicutes	*Aerococcaceae_unclassified*	2
	OTU0129	Gemmatimonadetes	*uncultured*	1
	OTU0228	Gemmatimonadetes	*Gemmatimonas*	1

Table 3.5 (S2). *Continued.*

Day	OTU	Phylum	Genus	Cumulative Reads
22	OTU0117	Patescibacteria	*Parcubacteria_ge*	1
	OTU0157	Patescibacteria	*Candidatus_Kaiserbacteria_ge*	2
	OTU0368	Patescibacteria	*Candidatus_Peribacteria_ge*	1
	OTU0248	Planctomycetes	*vadinHA49_ge*	1
	OTU0495	Planctomycetes	*uncultured*	3
	OTU0030	Proteobacteria	*Candidatus_Methylopumilus*	7
	OTU0036	Proteobacteria	*Clade_III_ge*	2
	OTU0065	Proteobacteria	*Caulobacter*	1
	OTU0092	Proteobacteria	*Methylotenera*	3
	OTU0148	Proteobacteria	*Phenylobacterium*	1
	OTU0166	Proteobacteria	*Edwardsiella*	2
	OTU0167	Proteobacteria	*Limnohabitans*	2
	OTU0175	Proteobacteria	*T34_ge*	6
	OTU0195	Proteobacteria	*OM27_clade*	2
	OTU0200	Proteobacteria	*Halomonadaceae_unclassified*	1
	OTU0286	Proteobacteria	*Aeromonadaceae_unclassified*	4
	OTU0329	Proteobacteria	*uncultured*	3
	OTU0384	Proteobacteria	*R7C24_ge*	3
	OTU0458	Proteobacteria	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	5
	OTU0478	Proteobacteria	*Aureimonas*	2
	OTU0506	Proteobacteria	*uncultured*	11
	OTU0532	Proteobacteria	*Sphingobium*	3
	OTU0536	Proteobacteria	*Paracoccus*	1
	OTU0580	Proteobacteria	*Luteimonas*	2
	OTU0604	Proteobacteria	*Geobacter*	1
	OTU0689	Proteobacteria	*Salmonella*	1
	OTU0740	Proteobacteria	*Arenimonas*	1
	OTU0751	Proteobacteria	*uncultured_ge*	3
	OTU0830	Proteobacteria	*Schlegelella*	1
	OTU1048	Proteobacteria	*Blastomonas*	1
	OTU1136	Proteobacteria	*Nordella*	1
	OTU0068	Verrucomicrobia	*Opitutus*	1
	OTU0378	Verrucomicrobia	*Luteolibacter*	1
	OTU0422	Verrucomicrobia	*uncultured*	1

Table 3.6 (S3). Operational taxonomic units (OTU) identified as discriminatory according to oxytetracycline exposure by Metastats and LEfSe algorithms in Mothur.

Day	Phylotype			*p* value	
	OTU	Phylum	Genus	metastats	LEfSe
2	OTU0019	Firmicutes	*Bacillales_unclassified*	0.013	-
8	OTU0007	Firmicutes	*Clostridiaceae_1_unclassified*	0.006	-
	OTU0021	Firmicutes	*Clostridium_sensu_stricto_4*	0.009	-
	OTU0031	Firmicutes	*Clostridium_sensu_stricto_1*	0.006	-
10	OTU0013	Proteobacteria	*Gammaproteobacteria_unclassified*	0.007	-
	OTU0017	Proteobacteria	*Aeromonas*	0.012	-
	OTU0080	Proteobacteria	*Reyranella*	0.044	-
15	OTU0031	Firmicutes	*Clostridium_sensu_stricto_1*	0.032	-
	OTU0008	Spirochaetes	*Brevinema*	0.035	-
22	OTU0033	Actinobacteria	*Sporichthyaceae_unclassified*	-	0.044
	OTU0097	Actinobacteria	*Microbacteriaceae_unclassified*	0.017	-
	OTU0089	Bacteroidetes	*NS11-12_marine_group_ge*	-	0.002
	OTU0002	Firmicutes	*Bacillus*	0.002	-
	OTU0003	Firmicutes	*Bacillaceae_unclassified*	0.002	-
	OTU0007	Firmicutes	*Clostridiaceae_1_unclassified*	0.025	-
	OTU0019	Firmicutes	*Bacillales_unclassified*	0.037	-
	OTU0051	Firmicutes	*Bacilli_unclassified*	0.003	-
	OTU0010	Proteobacteria	*Burkholderiaceae_unclassified*	0.002	-
	OTU0011	Proteobacteria	*Pseudomonas*	0.016	-
	OTU0020	Proteobacteria	*Polynucleobacter*	0.020	-
	OTU0046	Proteobacteria	*Legionella*	0.021	-
	OTU0153	Proteobacteria	*Undibacterium*	0.015	-
	OTU0009	Tenericutes	*Mycoplasmataceae_unclassified*	0.006	-

Statistical significance was accepted at $p < 0.05$.

CHAPTER 4. Oxytetracycline treatment can affect gut health of Nile tilapia (*Oreochromis niloticus*) through changes in the gut microbiome

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4.1. Abstract

Antibiotics play a vital role in aquaculture where they are commonly used to treat a wide range of bacterial infections. Data from other vertebrate species has shown that oral administration of antibiotics can cause dysbiosis within the gut microbiome community. Similar findings have now been reported for farmed fish species however, how these changes in microbial populations influence gut health over time remains to be fully addressed. In this study, Nile tilapia (*Oreochromis niloticus*) were fed a single, high dose treatment of oxytetracycline (100mg kg bodyweight^{-1}) for eight days, followed by a 14-day withdrawal period. To profile the changes in the microbiome and gut health; digesta and tissue samples from the distal guts of individual fish were taken before, and after antibiotic treatment, as well as throughout the two-week withdrawal period. Changes in the distal gut microbiome community were measured using high-throughput 16S rRNA sequencing. In addition, the abundance of antimicrobial resistance genes was quantified using real-time qPCR methods. Likewise, similar quantitative methods were also used to profile the expression of genes related to immunity, metabolism and gut barrier integrity as biomarkers for gut health. Oxytetracycline treatment decreased the microbial diversity within the distal gut microbiome of treated fish over time, causing a shift in the microbiome community. However, whilst oxytetracycline was associated with a decline in most bacterial taxa, several bacterial genera including *Plesiomonas* and *Nocardia*, increased in prevalence in response to antibiotic treatment. Furthermore, the increased abundance of these genera was correlated with a higher detection of the antimicrobial resistance genes *tetA*, *tetM* and *tetX*. Finally, whilst the expression of host-related genes did not significantly differ between treatment groups, oxytetracycline-induced shifts in the distal gut microbiome community were found to be strongly associated with the expression of several genes involved in immunity. Findings from this study revealed that oxytetracycline treatment can lead to considerable changes in the distal gut microbiome communities of Nile tilapia, which can potentially have downstream detrimental effects on gut health through shifts in host-microbiome interactions. Moreover, results also demonstrate that antibiotic treatment can exert selective pressures on the gut microbiome of fish, in favour of resistant populations which may have long-term impacts on fish health.

Keywords: aquaculture, Nile tilapia, antibiotic, oxytetracycline, microbiome, antimicrobial resistance, gut health

4.2. Introduction

Nile tilapia (*Oreochromis niloticus*) is considered one of the most extensively farmed fish species worldwide, with a projected production value exceeding 6.6 million tonnes by 2030 (Omasaki et al., 2017). Like many farmed fish species, production of Nile tilapia is expected to intensify in the coming decades, in order to meet the growing demand for aquatic animal protein and global food security (Kobayashi et al., 2015). However, expansion of this sector is currently hampered by a number of challenges including infectious disease outbreaks which can limit production. Presently, farmed Nile tilapia have been reported to be susceptible to a number of bacterial diseases caused by Gram-negative bacteria including *Aeromonas hydrophila* (Ibrahem et al., 2008), *Aeromonas shubertii* (Liu et al., 2018), *Edwardsiella ictaluri* (Soto et al., 2012), *Flavobacterium columnare* (Wonmongkol et al., 2018) and *Francisella noatunensis* subsp. *orientalis* (Leal et al., 2014). Likewise, Gram-positive bacteria such as *Lactococcus garvieae* (Anshary et al., 2014), *Streptococcus agalactiae* (Jantrakajorn et al., 2014) and *Streptococcus iniae* (Figueiredo et al., 2012), have also been reported to cause significant economic losses through disease outbreaks in Nile tilapia. Due to a lack of available vaccines and other efficacious prevention tools, the tilapia sector relies heavily on the use of antibiotics to treat or prevent infectious disease outbreaks, with a limited understanding of how they may impact overall fish health.

There is an increasing awareness that the fish microbiome plays a pivotal role in host physiology and health. In the gut, members of the microbiome community provide beneficial services in several metabolic pathways (Falcinelli et al., 2015) including glucose metabolism (Falcinelli et al., 2016). Likewise, the gut microbiome has also been demonstrated to regulate gut barrier integrity and permeability (Hoseinifar *et al.*, 2019). In addition, recent studies have reported that a functional and stable gut microbiome is also critical for promoting disease resilience in fish, through the prevention of opportunistic pathogen establishment (Li et al., 2016; Safari et al., 2016; Nie et al., 2017). In healthy fish with a stable gut microbiome, colonisation resistance can arise directly through microbiome-pathogen interactions, including niche occupation and toxic secondary metabolite production, as demonstrated previously in zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) (Kihara & Sakata, 2002; Rendueles et al., 2012). Furthermore, disease resilience can be promoted by the microbiome indirectly through host-microbiome interactions and modulation of the host's immune response (Galindo-Villegas et al., 2012). These interactions are thought to originate following detection of the commensal bacteria by pattern

recognition receptors, such as toll-like receptors or nucleotide-binding oligomerization domain (NOD)-like receptors, which recognise various microbe-associated ligands. Following detection, signalling cascades such as NF-κB transcription factor are activated resulting in specific innate immune responses, such as the induction of inflammatory cytokines and the activation of various antimicrobial mechanisms (Li *et al.*, 2017). Such host-microbiome interactions include the microbial-mediated regulation of the pro- and anti-inflammatory cytokines; interleukin-1beta (IL-1β) and transcription growth factor beta (TGF-β), which have been demonstrated in common carp and European sea bass (*Dicentrarchus labrax*), respectively (Picchietti et al., 2009; Chi et al., 2014). Likewise, members of the gut microbiome in hybrid grouper (*Epinephelus lanceolatus*♂ × *E. fuscoguttatus*♀) have also been reported to modulate the expression of piscidin, an important host-derived antimicrobial peptide (Li et al., 2019). Furthermore, members of the gut microbiome are also thought to interact with components of the adaptive immune system in fish, similar to what is observed in the gill and skin of rainbow trout (*Oncorhynchus mykiss*) with the immunoglobulins IgM and IgT (Sepahi *et al.*, 2016). Specifically, secretory IgT, an important mucosal immunoglobulin, has been demonstrated to play a pivotal role in gut microbiome homeostasis, where it is thought to coat commensal bacteria and aid in immune exclusion (Kelly & Salinas, 2017).

Recently, the fish gut microbiome community has been demonstrated to be influenced by a range of factors commonly associated with aquaculture husbandry practices. These include dietary changes (Parata et al., 2019), as well as suboptimal levels of pH (Sylvain et al., 2016), salinity (Zhang et al., 2016) and temperature (Guerreiro et al., 2016). Likewise, recent evidence demonstrates that gut microbiome diversity and composition in fish can also be altered through exposure to a wide range of antibiotics, including oxytetracycline (OTC) (Navarrete et al., 2008; Carlson et al., 2017; Zhou et al., 2018a). Oxytetracycline is one of the most extensively used antibiotics across the aquaculture industry, where it has broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Yang et al., 2019). The diverse range of target organisms is attributed to the non-specific, intracellular mode-of-action of OTC, whereby on entry into the bacterial cell it interferes with ribosome functioning, resulting in the inhibition of translation and downstream protein synthesis (Zhou et al., 2018a). This broad nature of activity makes OTC particularly effective at treating a range of bacterial diseases when administered on the fish farm. However, the lack of specificity exhibited by OTC means it can also act on non-target organisms, resulting in the direct alteration of the commensal microbiome community. As members within the gut microbiome serve important biological functions, any alteration or dysbiosis of this community e.g. through antibiotics, may have detrimental effects on the physiological status of the fish host. In fact, disruption of the gut microbiome has already been linked with the onset, or increased susceptibility of disease in a number of fish species,

including black molly (*Poecilia sphenops*), gilthead sea bream (*Sparus aurata*) and zebrafish (Piazzon et al., 2017; Schmidt et al., 2017; Zhou et al., 2018a). Furthermore, in addition to eradicating the commensal and beneficial bacterial populations; there are also concerns that antibiotic treatment may exert selective pressures on the fish gut microbiome, favourably supporting the development of antibiotic resistant communities (Navarrete et al., 2008). In the case of OTC, antimicrobial resistance (AMR) within microbiome community members could be mediated through various mechanisms, such as efflux pumps, ribosomal protection proteins or enzymatic inactivation of the antibiotic compound, acquired from mobile genetic elements and other extrachromosomal material (Leal *et al.*, 2019).

The recent drive to reduce antimicrobial use in aquaculture remains challenging, especially as antibiotic use is still extremely high in some fish farming countries. It is postulated that the reliance and continued application of antibiotics in aquaculture will continue to climb, likely driven by financial reasons (Mo et al., 2017), as well as perceived barriers to alternative strategies e.g. vaccination, and a lack of understanding surrounding AMR. Previous studies have explored the impacts of OTC on the gut microbiome community in a number of fish species (Navarrete et al., 2008; López Nadal et al., 2018), including Nile tilapia (Limbu et al., 2018). However, the changes in gut microbiome dynamics over time following OTC treatment, and how this relates to the gut health of this farmed fish species has not been fully explored. Therefore, the primary objective of this study was to determine the effect of a single, high dose treatment of OTC on the gut health of Nile tilapia overtime. To achieve this, high-throughput 16S rRNA amplicon-sequencing and quantitative-PCR (qPCR) methods were applied to profile the changes in (i) the microbiome community; (ii) the abundance of AMR genes; and (iii) the expression of immune, digestive and barrier-integrity related genes in the distal gut before and after antibiotic treatment.

4.3. Methods

4.3.1. Experimental design

The effects of OTC exposure on the gut health in Nile tilapia was performed over a 36-day time series feeding trial, which took place within the Tropical Aquarium (TA), Institute of Aquaculture (Stirling, UK). The trial was divided into three stages: a 14-day acclimation period (non-medicated diet), an 8-day treatment period (medicated diet) and a 14-day withdrawal period (non-medicated feed). A total of 42 mixed sex, apparently healthy Nile tilapia (mean individual weight and lengths were 48.33 ± 7.26 g and 13.69 ± 0.76 cm) were obtained from a single full-sib stock population held onsite at the TA. None of the fish had received any antibiotic treatment or vaccination prior to the start of the trial. The fish were randomly allocated into individual 19 L tanks, which were maintained on a recirculation system, at a flow rate of 1.2 L min^{-1}, under a 12:12 hour light:dark cycle and ambient water temperature of 27 ± 0.5°C. Fish were maintained in these conditions throughout the entire trial. Following a 14-day acclimation period, tanks were randomly allocated into two treatment groups (n=18 per treatment). During the treatment period; fish in treatment group one were fed a medicated diet which was surface coated with OTC and fish in treatment group two were fed a non-medicated (control) diet. Both diets were delivered into respective tanks by hand, over two feeding periods at a rate of 1.5% bodyweight day^{-1} for eight days. After the 8-day treatment period, fish in both treatment groups were fed the control diet at a feeding rate of 1.5% bodyweight day^{-1} for fourteen days, after which time the experimental trial was terminated (Figure 4.1).

4.3.2. Diet preparation & in vitro antimicrobial activity

The OTC diet was prepared onsite at the Institute of Aquaculture using Standard Expanded Floating Pellet 3 mm (8% oil content, 40% protein content) (Skretting, UK). Preparation of the diet followed that described in section 3.3.2, except OTC hydrochloride (98.2% purity) (Duchefa Biochemie®, Haarlem, the Netherlands) was added at an inclusion rate of 100 mg kg $bodyweight^{-1}$. The control diet was similar in composition to the OTC diet except it lacked OTC hydrochloride. Both diets were prepared 24 hours prior to commencing the treatment period and were stored at 4°C until use. Twelve hours prior to daily feeding; both diets were distributed into sterile universal tubes according to the required daily volume of feed per tank, which were then stored at room temperature. The antimicrobial activity of prepared diets were tested following the steps described in section 3.3.2, except the OTC-sensitive *A. hydrophila* strain NCIMB 9240 was used.

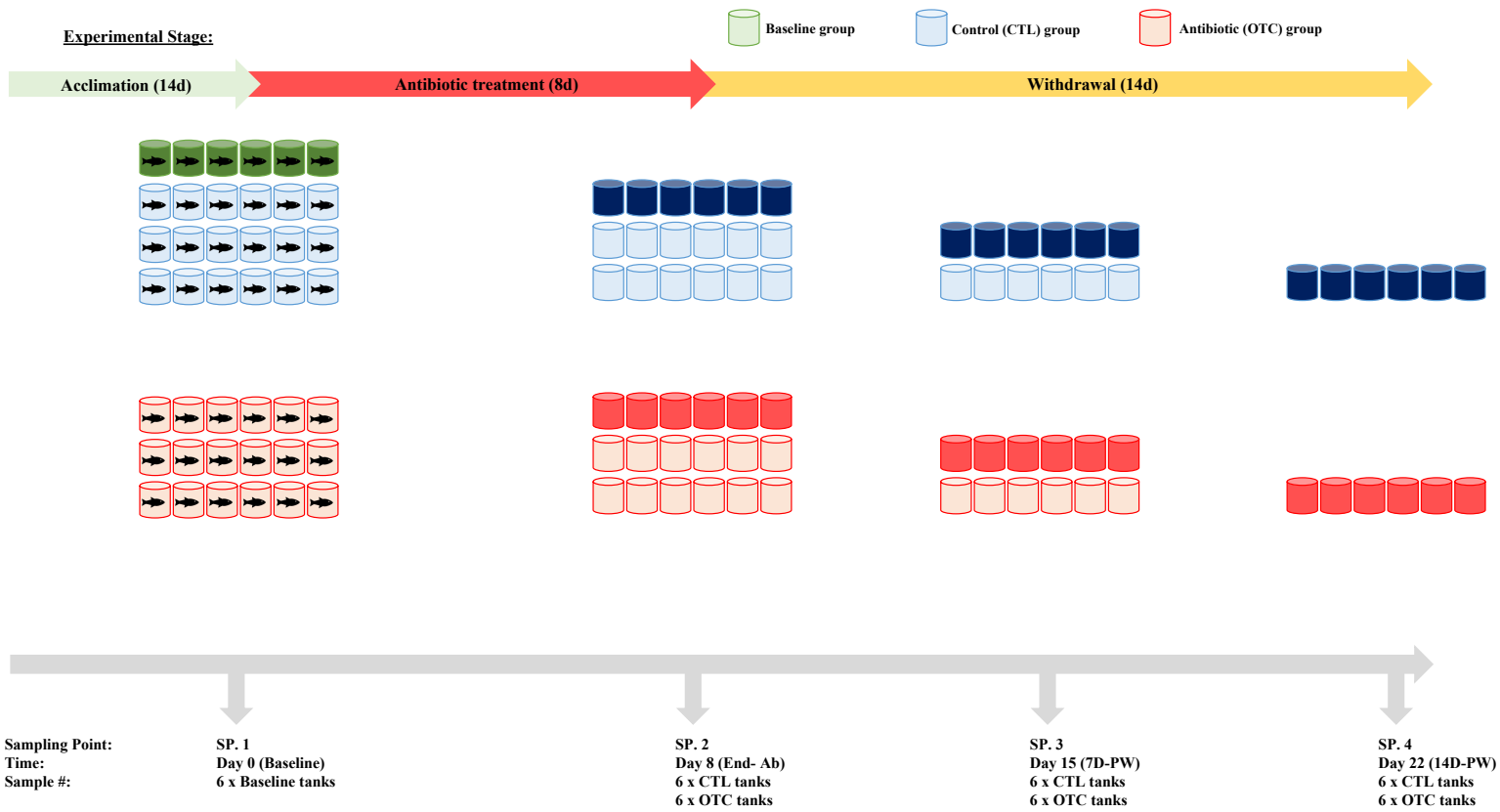

Figure 4.1. Experimental design and sampling strategy. Fish were stocked into individual tanks and acclimated to tank conditions at the Tropical Aquarium, University of Stirling for 14 days. Tanks were then randomly assigned to either control (blue) or oxytetracycline (OTC; red) groups. During the 8-day antibiotic treatment, fish in the OTC group received a diet surface-coated with OTC (100 mg kg bodyweight day^{-1}). During the same period, fish in the control group received a control diet void of any antibiotic. Following the antibiotic treatment period, all fish were given the control diet for 14 days to simulate a withdrawal period. Fish were sampled on days 0, 8, 15 and 22 to reflect before and after antibiotic treatment. At each sampling point; fish from six tanks for each treatment group were randomly sacrificed and sampled for distal gut digesta and tissue. The trial was terminated on day 22, where all remaining fish were sacrificed and sampled. Days post withdrawal; D-PW.

4.3.3. Sample collection

Gut digesta was aseptically collected from individual fish at four time points, which were as follows: immediately before antibiotic treatment (day 0; baseline), at the end of the antibiotic treatment (day 8), one-week post-treatment withdrawal (day 15) and at the end of the two-week withdrawal period (day 22). At each timepoint, individual fish from six tanks were randomly sampled from each treatment group giving n=6 fish per treatment group and per time point (Figure 4.1). This sample size followed international recommendations for RNA-seq experiments (Schurch et al., 2016), which uses similar molecular methods. Following euthanasia by a lethal dose of tricaine methanesulfonate (1000 mg g^{-1}; Pharmaq®, UK), the digesta from individual fish was aseptically collected from the distal portion of the gut (distal point of midgut to ~2 cm before the vent) following the steps outlined in section 2.3.1, except gut digesta was stored in empty 2 mL microcentrifuge tubes (Alpha Labs®, Hampshire, UK). Following collection of digesta, ca 0.2 cm of distal gut tissue was collected in sterile 2 mL microcentrifuge tubes and stored in 1 mL TRI Reagent (Sigma Life Science, UK) for RNA extraction. In addition to gut samples, a total of ten pellets from each diet (stored in sterile 7 mL bijou containers), and biofilm samples from the main filtration unit as well as a respective tank for each treatment/time point, were also collected. Biofilm samples were collected using a sterile swab (VWR International, Pennsylvania, United States), placed just below the water line and moved around each side of the tank/filtration unit for ca 20 seconds. Biofilm samples were stored in 2 mL microcentrifuge tubes as described for digesta samples. At each sampling point, all samples were held on ice until sampling was complete. Following sampling, samples were moved to -80°C storage (within three hours), where they remained until sample processing.

4.3.4. Microbiome analysis

A total of 162.9 ± 77.7 mg of gut digesta was processed for total genomic DNA extraction, following the protocol described by Knudsen et al., (2016) using the QIAamp Fast DNA Stool Mini Kit (Qiagen®, Hilden, Germany) and 0.7 mm garnet beads (PowerBead Tubes, Qiagen®). Total genomic DNA was extracted from diet (80 mg; ca six pellets) and biofilm samples using the same commercial DNA extraction kit and method described previously. Total genomic DNA was also extracted for a group of negative sequencing controls (NSCs), in an attempt to track all sources of microbial DNA contamination in 16S rRNA libraries. Total genomic DNA was extracted using the commercial DNA extraction kit and protocol described previously. No sample or DNA was added to NSC samples, instead, the inhibitEX buffer supplied in the DNA extraction kit was used as the starting material. The NSC samples were generated for all starting material types including digesta (NSC_Fish), diet (NSC_Diet) and biofilm (NSC_Tank) samples, respectively. The NSC_Tank sample included a sterile swab similar to that used in the original

sampling. The final DNA extracted from all microbiome and NSC samples was eluted in 35 µL EB buffer (10 mM Tris-HCl, pH 8.5; Qiagen®, Hilden, Germany). A synthetic microbiome community standard designated as IoA_MB_STD was also prepared as described in section 3.3.4, and used as a positive control for real-time qPCR and to calculate sequencing error following Illumina MiSeq sequencing.

Prior to preparing 16S rRNA Illumina libraries, the bacterial DNA yield recovered from the microbiome and NSC samples was quantified using TaqMan real-time qPCR methods, and the primer/probe combination listed in Table 4.1. The primer set 341F/805R was used to target the V3-4 hypervariable region of the bacterial 16S rRNA gene (Eurofins Genomics UK, Wolverhampton, UK) (Huang et al., 2018). The TaqMan probe was designed to target the template (+) strand of the V3 hypervariable region (Eurofins Genomics UK, Wolverhampton, UK) (Kiruthiga et al., 2018), and included FAM and MGBEQ reporter and quencher dyes, respectively. Real-time qPCR with absolute quantification was performed on a Stratagene Mx3005P QPCR System (Agilent Technologies LDS UK Ltd, Cheshire, UK) using 96-well plates. Quantitative analysis of the 16S rRNA gene copy number was performed in triplicate 20 µL reactions containing: 10 µL SensiFAST™ Probe Lo-ROX mastermix (Bioline Reagents Limited, London, UK), 0.4 µL of each forward and reverse primer (0.2 µM), 0.1 µL probe (0.05 µM), 7.1 µL nuclease-free water and 2 µL DNA (< 50 ng μL^{-1}). The IoA_MB_STD sample was included as a positive control and was added in respective qPCR reactions at a concentration of 5 ng μL^{-1}. Duplicate no DNA template control (NTC) reactions were also included in every qPCR run. The purpose of the NTC reactions was to confirm qPCR reagents were free from microbial DNA contamination. Real-time qPCR conditions were as follows: an initial denaturation step at 95°C for ten minutes, followed by 40 x cycles at 95°C for 30 seconds and 60°C for one minute. The number of 16S rRNA genes per microlitre of DNA sample was calculated from the final Ct values in each qPCR reaction using a standard curve. The standard curve was generated using plasmid standards containing the 16S rRNA V3-4 hypervariable region insert as described in section 3.3.5. The plasmid standards were ten-fold serially diluted to concentrations of 1 x 10^8 to 1 x 10^3 16S rRNA gene copies μL^{-1}. The qPCR efficiencies and R^2 values are detailed in Table 4.1.

Table 4.1. Primer sets used in this study.

Primer	Target	Sequence (5' – '3)	Size (bp)	Ta°C	Eff. (%)	R^2	Application	Source (Accession)
341F 805R Probe	16S rRNA (V3-4)	CCTACGGGNGGCWGCAG GACTACHVGGGTATCTAATCC FAM-ATTACCGCGGCTGCTGG-MGBEQ	464	60	114.37	0.99	16S rRNA qPCR	(Huang et al., 2018; Kiruthiga et al., 2018)
16S_V4F 16S_V4R Cocktail (R1, R2, R4 & R4)	16S rRNA (V4)	[Illumina adapter]-AYTGGGYDTAAAGNG [Illumina adapter] TACCRGGGTHTCTAATCC [Illumina adapter]-TACCAGAGTATCTAATTC [Illumina adapter]-CTACDSRGGTMTCTAATC [Illumina adapter]-TACNVGGGTATCTAATC	245	54	N/A	N/A	Illumina Libraries	(Ma et al., 2017)
intI1_F intI1_R	Class 1 integrase protein	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	63	101	0.99	AMR-related gene qPCR	(Huang et al., 2017)
tetA_F tetA_R	Tetracycline efflux pump	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	64	98	0.99	AMR-related gene qPCR	(Huang et al., 2017)
tetM_F tetM_R	Tetracycline resistance ribosomal protection	AGTGGAGAAATCCCTGCTCGGT TGACTATTTGGACGACGGGGCT	149	66	107	0.99	AMR-related gene qPCR	(Huang et al., 2017)
tetX_F tetX_R	Enzymatic modification of tetracycline	GAAAGAGACAACGACCGAGAG ACACCCATTGGTAAGGCTAAG	131	63	99	0.99	AMR-related gene qPCR	(Huang et al., 2017)

Table 4.1. *Continued.*

Primer	Target	Sequence (5' – '3)	Size (bp)	Ta°C	Eff. (%)	R^2	Application	Source (Accession)
b-ActinF b-ActinR	β-Actin	GCTACTCCTTCACCACCACAG CGTCAGGCAGCTCGTAACTC	144	61	N/A	N/A	cDNA quality control	(Ritchuay, 2020)
slc2a6_Nt_F slc2a6_Nt_R	Solute carrier family 2, facilitated glucose transporter member 8 (Carbohydrate Digestion)	GCATGTGATGAGCAGGGCTCTA TCACTGACGCCAGGTCACTT	187	64	106	0.98	Host-related gene qPCR	*This study* (XM_025896869.1)
atp1b1_Nt_F atp1b1_Nt_R	Na/K-transporting ATPase subunit (Osmoregulation)	AGGAGTTTCTGGGGCGCACT TGTGTGACAGACCTGGGGGAG	174	61	104	0.98	Host-related gene qPCR	*This study* (XM_003444220.5)
IL1B_Nt_F IL1B_Nt_R	IL-1β (Cytokine)	TGAGAGCCTACTTTAGGATTCTGC GCGGCTATTACAACCAATGCT	150	59	97	0.99	Host-related gene qPCR	(Ritchuay, 2020)
TGF-B_Nt_F TGF-B_Nt_R	TGF-β (Cytokine)	GAGATCCCTGCCAACTTGCT TCCCCGACGTTACTCCGTAT	230	60	100	0.99	Host-related gene qPCR	(Ritchuay, 2020)
sIgT_Nt_F sIgT_Nt_R	Secretory IgT	TGACCAGAAATGGCGAAGTATG GTTACAGTCACATTCTCTGGAATTACC	163	54	98	0.99	Host-related gene qPCR	(Velázquez et al., 2018)
TP4_Nt_F TP4_Nt_R	Moronecidin /Piscidin 4 (Antimicrobial peptide)	CGATGGTCGTCCTCATGGCT ACGTCGTATGAGGCGATGGA	101	59	104	0.99	Host-related gene qPCR	*This study* (XM_003456613.3)
TLR21_Nt_F TLR21_Nt_R	Toll-like receptor 21 (PRR)	AACGGACTCACCGTTTTACCA GGAGAAGTTCTGAATGCCCAT	242	56	103	0.99	Host-related gene qPCR	(Pang et al., 2017)
nod1_Nt_F nod1_Nt_R	Nod-like receptor 1 (PRR)	GCATCTGGCCAACGCCATAA GCCGACAGACTGAGGTTGGTA	141	68	70	0.97	Host-related gene qPCR	*This study* (XM_003446199.5)
scarb1_Nt_F scarb1_Nt_R	Scavenger receptor- Class B (PRR)	TGATCTGGTTTGAGGAGAACGG TCCATCCGGTAGGTTGCTTT	321	61	105	0.99	Host-related gene qPCR	*This study* (XM_005449474.4)
nkap_Nt_F nkap_Nt_R	NF-kβ activating protein	CGATCGACCTTTGGATTTCGGTC GCACAGCCTCCATACGACGA	188	68	104	0.99	Host-related gene qPCR	*This study* (XM_025910841.1)

PRR; Pattern recognition receptor

DNA samples that were found to have a 16S rRNA concentration below 1 x 10^4 16S rRNA copies μL^{-1} (Rubin et al., 2014) were not processed further. The remaining DNA samples were then used to generate 16S rRNA libraries following the protocol described in section 2.3.4 for titrated libraries, with minor modifications. Briefly, the bacterial 16S rRNA V4 hypervariable region was amplified using the 16S_V4F and 16S_V4R cocktail primers listed in Table 4.1 (Ma et al., 2017). All samples were amplified in triplicate 10 µL reactions using 5 µL 2X NEBNext Ultra II Q5 mastermix New England Biolabs (UK) Ltd, Herts, UK), 0.4 µL of each primer (0.2 µM), 0.2 µL nuclease-free water and 4 µL DNA (1.49 x 10^4 16S rRNA copies μL^{-1}). Amplification was conducted in a Tgradient thermal cycler (Biometra GmbH, Göttingen, Germany) under the following conditions: 98°C for two minutes, followed by 30 x cycles of 98°C for 15 seconds, 54°C for 30 seconds and 65°C for 45 seconds. All PCR reactions underwent a final extension stage at 65°C for ten minutes. The 16S rRNA V4 region was also amplified from DNA in the IoA_MB_STD sample, as well as for all NSC samples. The IoA_MB_STD sample was added to respective PCR reactions at the same concentration as used for the microbiome samples. However, a total of 4 µL DNA was added to respective PCR reactions for each NSC sample due to low genomic material being available. The indexing PCR was performed as described in section 2.3.4 however, a total of 7 µL of the product from the first PCR was used as template material in this PCR to achieve sufficient amplification for sequencing. Dual indexing of each library was conducted using similar PCR conditions to that described for the first round of PCR, except the cycle number was reduced to eight.

Final libraries (length ~ 381 bp) which included dual indices and Illumina® sequencing adapters, were quantified fluorometrically using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific®, Glasgow, UK). Libraries were pooled in equal concentration (1.2 ng) and the final pooled library was quantified again with Qubit™, using the same high sensitivity dsDNA kit. The final pooled library was processed for sequencing as described in section 2.3.4, and sequenced using the Illumina MiSeq® NGS system with the Illumina® MiSeq Reagent Kits v2 (2 x 250 bp; 500-cycle) at the Institute of Aquaculture, University of Stirling (UoS), UK.

4.3.5. Bioinformatics

Illumina reads underwent demultiplexing with Casava v. 1.8 (Illumina®) and removal of reads representing the PhiX/ internal controls, or reads not matching Illumina indices. The open-source program Mothur (Schloss et al., 2009) was used to process sequence read data generated using the commands described in section 2.3.5 with slight modifications. The reads containing ambiguous bases, > 8 bp homopolymers and lengths < 235 bp or > 250 bp were discarded. The reads were further denoised, allowing for < 2 bp differences between duplicate sequences. The

reads were assessed for chimeric sequences (Edgar et al., 2011), which were then discarded before the remaining reads were aligned to the SILVA-based bacterial reference alignment [Release 132, December 2017] (Quast et al., 2013). Any reads assigned to undesired lineages including "chloroplast", "mitochondria", "archaea", "eukaryota" or "unknown" were later discarded. The sequence reads associated with the IoA_MB_STD sample were used to calculate sequence error rate using the seq.error command and then removed from the final dataset. Operational taxonomic units (OTUs) were selected using the cluster.split command and a sequence cut-off of 97%, and classified using the SILVA database described above. Finally, singleton OTUs were removed from the final dataset using the remove.rare command with nseqs set to a value of one. The final dataset was then rarefied to the lowest number of sequences per sample (i.e. 18,145) prior to performing any further downstream analysis. The final sample size after rarefaction was n=5 for day 0, baseline; n=4, n=3 for control and OTC groups at day 8; n=4, n=5 for control and OTC groups at day 15; and n=6, n=4 for control and OTC groups at day 22, respectively.

4.3.6. RNA extraction and cDNA synthesis

Total RNA was extracted from distal gut tissue using the protocol described in section 3.3.7, except final RNA pellets were eluted in 25-50 µL nuclease-free DEPC treated water (Invitrogen, California, United States). Four hundred nanograms of total RNA was reverse transcribed into single-stranded complimentary DNA (cDNA), using the nanoScript 2 enzyme and oligo$(dT)_{20}$ primers supplied in the Precision nanoscript™ 2 Reverse Transcription Kit (Primerdesign, Southampton, UK). Complimentary DNA was synthesised following the manufacturers recommended conditions of 65°C for five minutes, 42°C for 20 minutes and 72°C for ten minutes. The resulting cDNA was quality controlled using one-step PCR of the β-Actin housekeeping gene using the primers listed in Table 4.1. Each cDNA sample was diluted 1:3 with nuclease-free water before being added to 10 µL PCR reactions, containing 5 µL 2X HS mytaQ mastermix (Bioline Reagents Limited, London, UK), 0.5 µL of each forward and reverse primer (0.5 µM) and 3 µL nuclease-free water. Amplification of the housekeeping gene was performed in the Tgradient thermal cycler described previously at the following conditions: 95°C for two minutes, followed by 30 x cycles of 95°C for 20 seconds, 61°C for 30 seconds and 72°C for one minute, and a final extension at 72°C for two minutes. Following amplification, PCR products were visualised on a 1.5% agarose gel under UV illumination. Good quality cDNA was confirmed by the presence of a single band with a molecular weight of 144 bp, and even band intensity across all cDNA samples. All cDNA samples were stored at -20°C until required.

4.3.7. qPCR analysis of host and antimicrobial resistance related genes

Real-time qPCR with absolute quantification was used to measure the mRNA expression of ten host-related genes within the distal gut tissue of fish before and after antibiotic treatment. Target genes included those involved in immunity (*IL-1β, TGF-β, sIgT, TP4, TLR21, nod1, scarb1* and *nkap*), metabolism (*slc2a6*) and gut barrier integrity (*atp1b1*) as listed in Table 4.1. Real-time qPCR was performed on a LightCycler® 480 II (Roche Holding AG, Basel, Switzerland) and was conducted in triplicate reactions for all cDNA samples inside 384-well plates. The qPCR reactions were prepared to a total volume of 10 µL containing 5 µL Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific, Massachusetts, United States), 3 µL nuclease-free water, 0.5 µL of each forward and reverse primers (0.5 µM) and 1 µL cDNA diluted 1:10 with nuclease-free water. The primer sequences, annealing temperatures and expected amplicon sizes for each gene are listed in Table 4.1. To confirm reagents and subsequent qPCR reactions were free from contamination; duplicate NTC reactions were included in every qPCR run. Quantification was performed using the following conditions: Uracil-DNA glycosylase inactivation at 50°C for two minutes, an initial denaturation step at 95°C for ten minutes, followed by 40 x cycles at 95°C for 15 seconds, n°C for 30 seconds and 72°C for 30 seconds. Finally, a gradient of 0.11°C per second and five reads per °C from 72°C to 95°C, was performed for melt-curve analysis to confirm the specificity of the amplified qPCR products. The number of gene copies per microlitre was calculated from Ct values using a standard curve of serially diluted plasmid, which contained the respective gene insert from 1 x 10^8 to 1 x 10^1 gene copies μL^{-1}. Plasmids were prepared in *Escherichia coli* strain DH5α as described in section 2.3.2, except a cDNA pool generated from day 0, baseline fish and diluted 1:3 with nuclease-free water was used as the template material. In addition, plasmid DNA was extracted from transformed cells in 5 mL Luria-Bertani broth + 0.001% (v/v) ampicillin at a concentration of 50 mg mL^{-1}, which had been incubating for 18 hours at 100 rpm and 37°C. The efficiencies and R^2 values for each qPCR assay are detailed in Table 4.1.

Real-time qPCR with absolute quantification was also used to measure the abundance of four target genes, which have previously been used to monitor antimicrobial resistance within DNA samples derived from aquatic environments (Huang et al., 2017). Target genes included a class 1 intregrase protein (*intI1*), as well as three tetracycline resistance genes (*tetA*, *tetM* and *tetX*) (Table 4.1). The abundance of each gene was quantified in the same DNA samples derived from the distal gut digesta, tank water and diet, which had previously been used to generate 16S rRNA libraries. Quantification was performed in triplicate reactions for each DNA sample on the LightCycler® 480 II platform. Each qPCR reaction was prepared using the same volumes as described for host-related genes, except 1 µL of DNA was used as the template material. The

primer sequences, annealing temperatures and expected amplicon sizes for each gene are listed in Table 4.1. Quantification was performed following an initial denaturation step at 95°C for ten minutes, then 40 x cycles at 95°C for 15 seconds, n°C for 30 seconds and 72°C for 30 seconds. A melt-curve analysis was performed to confirm the specificity of the amplified qPCR products, and the qPCR efficiencies as well as R^2 values for each qPCR assay are provided in Table 4.1. The number of gene copies per microlitre of DNA sample were calculated from the final Ct values of each reaction, using a standard curve of serially diluted plasmid containing the respective gene insert from 1 x 10^8 to 1 x 10^1 gene copies μL^{-1}. Again, plasmids were prepared in *E. coli* strain DH5α as described for host-related genes, except using a pool of DNA from the OTC tank biofilms at days 8, 15 and 22 as the template material. The gene copies for each sample were normalised to the 16S rRNA gene copy number of the same sample. Gene copies were used as an indicator for the relative levels of AMR genes within microbiome communities before and after antibiotic treatment.

4.3.8. Statistical analysis

Data visualisation and statistical analysis were conducted in JMP® version 14 and Rstudio Version 1.1.419, using ggplot2 (Wickham, 2011), phyloseq (McMurdie & Holmes, 2013), reshape2 (Wickham, 2007) and vegan (Oksanen, 2007) packages, respectively. Differences in the final mean length, weight and growth rate (g day^{-1}) of fish across treatment group and time was evaluated using 2-way factorial ANOVA ($p < 0.05$). Likewise, differences in alpha diversity values (observed OTUs, Chao1 Richness, Inverse Simpson Index & Shannon Diversity) were also evaluated using 2-way ANOVA analysis with treatment and time as factors. However, prior to performing analysis, all alpha diversity data was transformed (log_{10}) to improve the normality of data distribution. Differences/interactions were considered significant between factors when $p < 0.05$. Distance matrices of beta diversity were generated using the thetaYC coefficient (Yue & Clayton, 2005) and Bray-Curtis dissimilarity (Bray & Curtis, 1957) calculators on Mothur. For all distance measures; PERMANOVA (vegan; adonis function) (Anderson, 2001) was first used to test differences in beta diversity according to sample type, designated as fish (distal gut), tank biofilm, biofilter, diet or NSCs ($p < 0.05$). Following this, PERMANOVA was used to further test the influence of treatment and time on inter-sample distances of distal gut microbiome communities ($p < 0.05$). PERMANOVA was conducted using 10,000 permutations. To identify differentially abundant OTUs within the distal guts of fish across treatment and time, Metastats (White et al., 2009) and LEfSe (Segata et al., 2011) analyses were performed in Mothur, with $p < 0.05$ indicating significantly differential sequence abundance according to treatment. Following log_{10} transformation, qPCR data for AMR genes were evaluated via 2-way ANOVA, using a critical value of $p < 0.05$ to assess the statistical significance between the distal gut

microbial communities of control and treated fish over time. Likewise, host gene qPCR data was assessed for differences between treatment groups and time using 2-way ANOVA and a significance value of $p < 0.05$. With the exception of *scarb1* and *slc2a6* gene expression data, which were $\log_{10}$ transformed prior to statistical analysis to improve normality, qPCR data for most host genes displayed normal distribution. Correlations between microbiome diversity and the abundance of AMR genes or expression of host-related genes were calculated using Pearson's correlation coefficient (R). Furthermore, the degree of interaction and correlation between individual OTUs and AMR or host-related genes, was further assessed with Pearson's correlation coefficient (r) using the rcorr.adjust algorithm provided in the RcmdrMisc package (Fox et al., 2018). Significance (*p* values) of correlations were corrected for multiple inference using Holm's method.

4.4. Results

4.4.1. Fish

No mortalities were observed in either treatment group. Of the 42 fish used in the trial, 25 (59% of total population) were identified as female. Furthermore, 19 fish (45% of total population) were observed to spawn at least once during the trial, followed by a period of at least 1.5 days before normal feeding behaviour resumed. Short-term antibiotic treatment with oxytetracycline was not found to have an effect on the growth performance parameters tested, as no statistically significant difference was found in the final mean length, weight and growth rate of fish between treatment groups and time ($p = 0.61$, $p = 0.80$ and $p = 0.63$, respectively) (Table 4.2). However, in general, OTC-treated fish displayed lower growth rates compared with control fish.

Table 4.2. Final mean (+SD) length and weight measurements for control or oxytetracycline (OTC)-treated Nile tilapia before, during and after antibiotic treatment.

Treatment	Day	Length (cm)		Weight (g)		Growth Rate (g day^{-1})	
		Mean	SD	Mean	SD	Mean	SD
Baseline	0	15.00	0.83	57.75	8.39	0.37	0.20
Control	8	14.63	0.95	51.88	8.29	0.33	0.37
OTC	8	14.95	1.41	54.22	14.12	0.31	0.25
Control	15	15.65	1.18	61.15	14.00	0.45	0.27
OTC	15	14.72	1.09	54.20	12.03	0.27	0.18
Control	22	15.65	1.04	62.65	12.90	0.32	0.22
OTC	22	15.17	0.71	56.37	8.42	0.28	0.18

4.4.2. in vitro antimicrobial testing of prepared diets

Zones of inhibition in bacterial growth surrounding dietary pellets were only observed on the *A. hydrophila* bacterial lawn exposed to the OTC- coated pellets. These were measured at diameters of > 25mm. Control pellets which lacked OTC, produced no inhibition zones after 48 hours incubation.

4.4.3. Sequence data and diversity analysis

A total of 12,733,648 reads were obtained from the Illumina MiSeq system. Following quality filtering, a total of 7,583,066 sequences remained in the final dataset and were clustered into 4,450 aligned OTUs for analysis. Of these, 2,070 were observed in samples originating from fish distal gut digesta material. In addition, a total of 420, 1,686, 1,661 and 852 OTUs were observed in either the diet, TA biofilter, tank biofilms or NSC's samples, respectively. Sequencing error was calculated at 0.0105%. Rarefaction analysis indicated high sequence coverage in distal gut digesta samples as all curves reached saturation phase (Figure 4.2). In addition, all samples reached a Good's coverage estimate of > 99%, suggesting that most OTUs present within these communities were detected. Rarefaction curves also showed a greater level of microbial diversity in the distal guts of fish in the control treatment group, particularly at day 22 (Figure 4.2), as this group was found to have a greater mean number of OTUs compared with fish in the OTC group and other time points (Figure 4.3). This was reflected in the alpha diversity of microbiome communities, as the distal guts of fish who consumed the OTC-coated pellets had lower microbial richness, diversity and evenness compared with the control fish group at the end of the antibiotic treatment (Figure 4.3). Furthermore, with the exception of Chao1 richness which increased in the OTC group at day 15, all alpha diversity measures were found to decrease throughout the withdrawal period in the distal gut microbiome communities of treated fish (Figure 4.3). However, the overall level of microbial diversity was not found to be significantly different between treatment group or time in any alpha diversity measure investigated ($p > 0.05$).

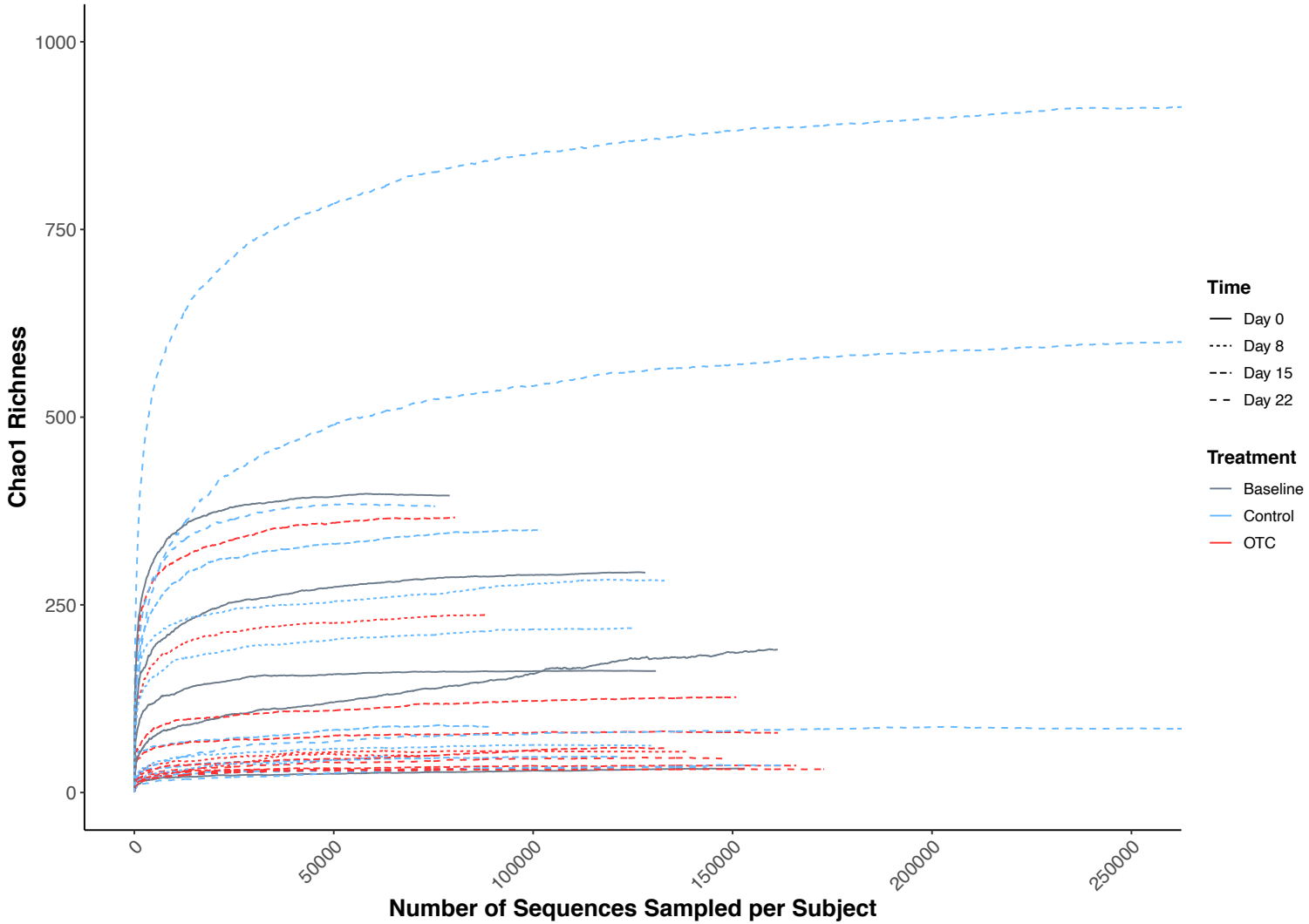

Figure 4.2. Rarefaction curves for each individual fish sampled (*n*=31). Curves represent the Chao1 richness observed per sample as a function of the sequencing effort. Colour of line indicates treatment group and line shape indicates time of sampling.

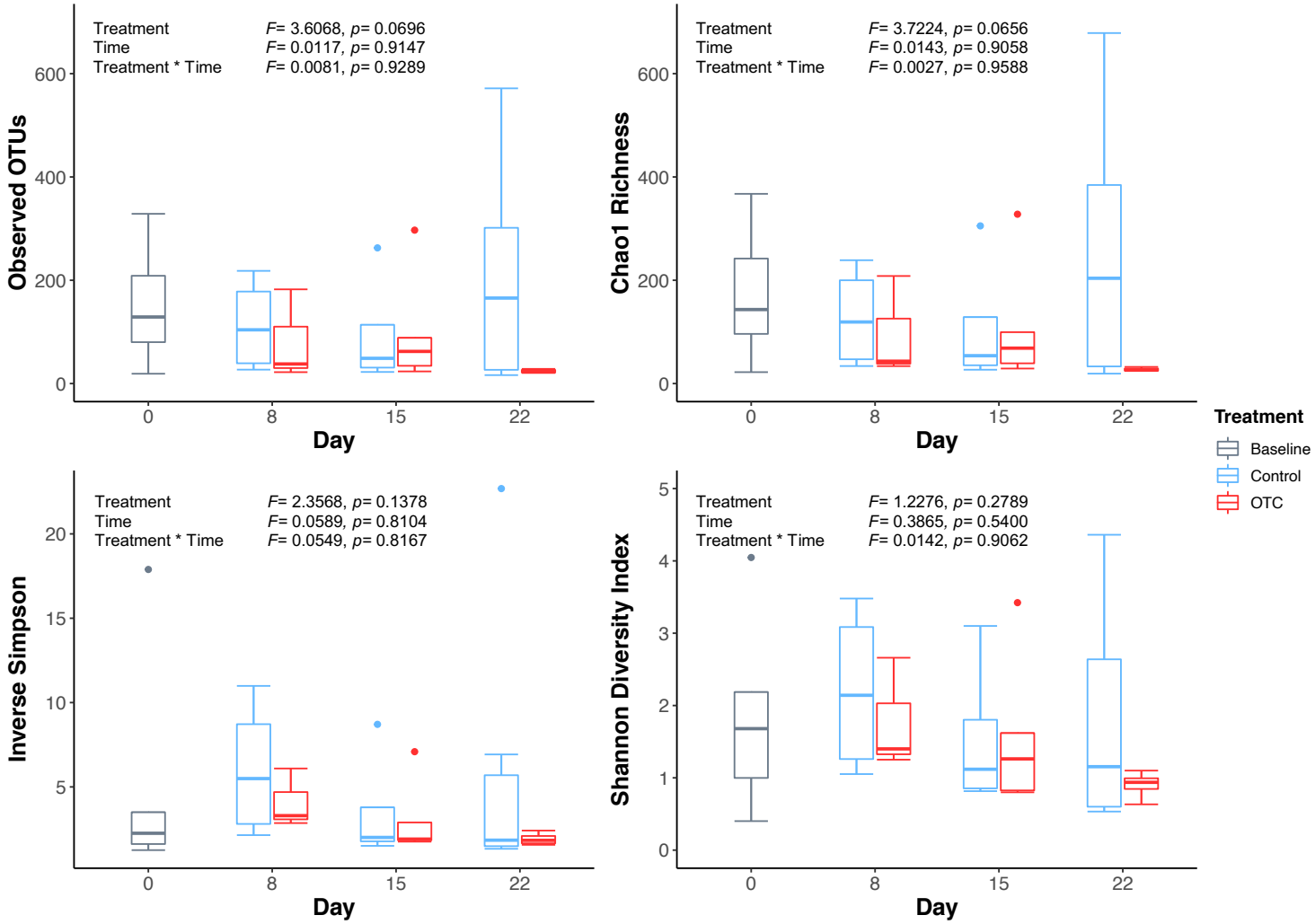

Figure 4.3. Alpha diversity measures of distal gut microbiome communities in control or OTC-treated Nile tilapia before and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles respectively. Circles indicate outliers from the dataset.

In order to test the influence of OTC exposure on the distal gut microbiome community structure over time, non-metric multidimensional scaling ordination was performed on ThetaYC and Bray-Curtis distances, respectively (Figure 4.4). For both microbial community membership (ThetaYC) and composition (Bray-Curtis), samples visibly clustered according to sample type with the distal gut microbiome communities of fish clustering distinctly from all other sample types (Figure 4.4. A & C). In addition, there was also a clear separation in the clusters of samples originating from the diet, TA biofilter, tank biofilms, and NSC samples, respectively (Figure 4.4 A & C). This was confirmed following PERMANOVA analysis (ThetaYC: $F = 5.15$, $p < 0.001$; Bray-Curtis: $F = 4.74$, $p < 0.001$). However, when the distal gut microbiome structure of control and OTC-treated fish was explored further, distances between samples were observed to be indistinguishable by treatment e.g. control and OTC diet (PERMANOVA; ThetaYC: $F = 0.69$, $p = 0.71$; Bray-Curtis: $F = 0.59$, $p = 0.87$) and time e.g. day 0, 8, etc. (PERMANOVA; ThetaYC: $F = 0.66$, $p = 0.75$; Bray-Curtis: $F = 1.15$, $p = 0.30$) (Figure 4.4 B & D). A higher level of inter-individual variability in microbiome community membership and composition, was observed between fish samples compared with other sample types (Figure 4.4. A & C). Furthermore, when fish samples were explored alone, higher variability in the structure of microbiome communities within the distal gut was found between fish within the control group compared with the OTC group (Figure 4.4. B & D).

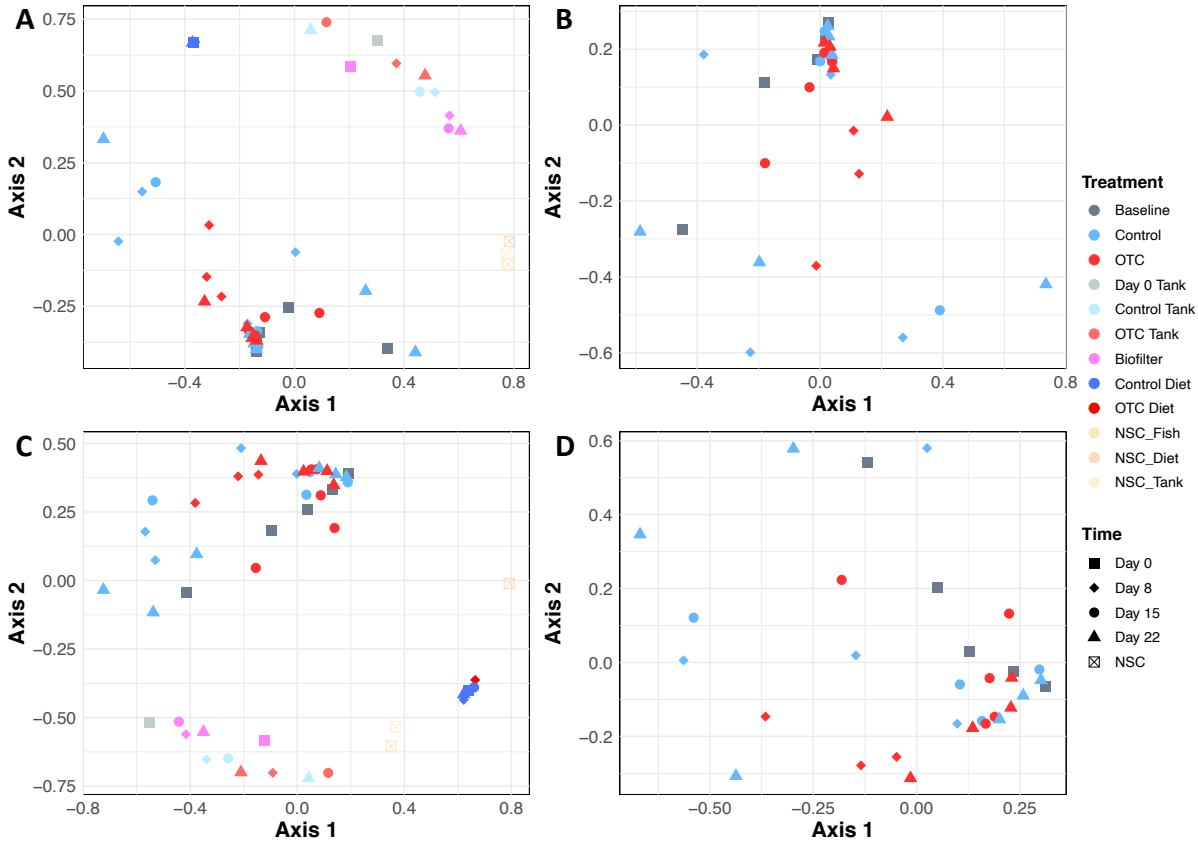

Figure 4.4. Non-multidimensional scaling of ThetaYC (A & B) and Bray-Curtis (C &D) distances. Distances illustrate differences in the microbiome community membership and composition of samples across time and exposure to oxytetracycline (OTC). Distances were generated for the complete dataset including TA biofilter, tank biofilm, diet & NSC samples (A &C), and within the distal gut of Nile tilapia alone (B & D).

4.4.4. Microbial community composition and influence of oxytetracycline

The distribution of OTUs at phylum and genus level in the distal guts of Nile tilapia across time and treatment group is illustrated in Figure 4.5. The mean (+SD) abundance of the top bacterial phyla and genera (Table 4.3 (S1)) are available as supplementary information (section 4.12). The distal gut microbiome of fish in this study were colonised by OTUs assigned to 23 bacterial phyla and 470 bacterial genera, although 10 and 40 appeared to dominate these communities across treatment group and time, respectively (Table 4.3 (S1)). At day 0, the distal gut microbiome communities of pre-treated fish were dominated by Fusobacteria, followed by Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Bacteroidetes and Planctomycetes (Figure 4.5 A; Table 4.3 (S1)). At the genus level, *Cetobacterium* dominated the distal gut communities of fish, followed by *Aeromonas*, *Romboutsia* and *Clostridium_sensu_stricto_1* (Figure 4.5 B; Table 4.3 (S1)).

The composition of distal gut microbiome communities in fish was however recorded to shift throughout the 36-day trial period. This was largely associated with changes in the sequence abundance of Actinobacteria, Firmicutes, Fusobacteria and Proteobacteria, irrespective of OTC treatment (Figure 4.5 A). Overall Actinobacteria were found to decline in abundance within the distal guts of fish over time in this study (Figure 4.5 A). Within Actinobacteria, this decline was mainly associated with *Gordonia*, *Mycobacterium*, *Nocardia* and *Rhodococcus*, which were all found to have lower abundance in the distal guts of fish at day 22 compared with pre-treated fish on day 0 (Figure 4.5 B; Table 4.3 (S1)). Similarly, Firmicutes were also found to decline in representation within the distal guts of fish between days 0 and 22 (Figure 4.5 A). At the genus level, both *Clostridium_sensu_stricto_1* and *Romboutsia* decreased in abundance within the distal guts of fish between the start and end of the trial (Figure 4.5 B; Table 4.3 (S1)). The abundance of Fusobacteria and *Cetobacerium,* which dominated this phylum, followed similar patterns to aforementioned taxa and declined in abundance within the distal guts of fish at day 8 compared with day 0 (Figure 4.5; Table 4.3 (S1)). However, this group became enriched again within the distal guts of fish by day 15 (Figure 4.5; Table 4.3 (S1)). By day 22, temporal changes in Fusobacteria and *Cetobacterium* differed between treatment groups (Figure 4.5; Table 4.3 (S1)). Unlike other phyla, Proteobacteria was observed to increase in representation within the distal guts of fish from both treatment groups at day 8 compared with pre-treated fish (Figure 4.5 A). This was mainly attributed to *Plesiomonas*, which had higher sequence abundance at day 8 compared with day 0 (Figure 4.5 B). Likewise, *Plesiomonas* and *Pseudomonas* were also found in higher abundance within the distal guts of fish at day 22 compared with day 0 (Figure 4.5 B; Table 4.3 (S1)). However, this pattern was not universal across all Proteobacteria genera, as *Aeromonas*, *Aquicella*, *Crenobacter* and *Rhodobacter* were all present in lower abundances in the distal guts of fish at day 22 compared with day 0 (Figure 4.5 B; Table 4.3 (S1)).

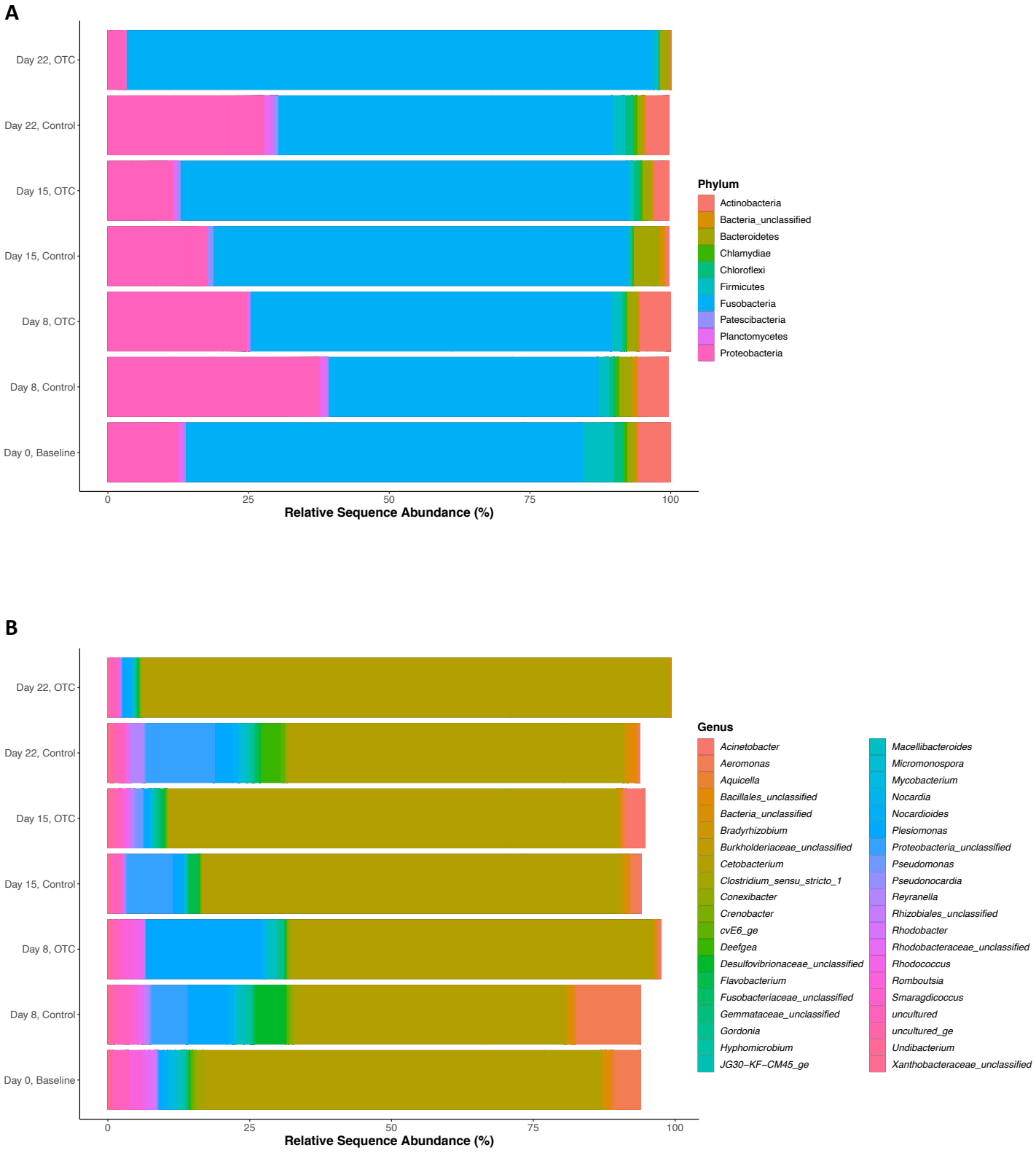

Figure 4.5. Mean relative sequence abundance (%) of the top 10 bacterial phyla (A) and top 40 bacterial genera (B) in the distal gut of control or oxytetracycline (OTC)-treated Nile tilapia before and after antibiotic treatment.

The distal gut microbiome community of Nile tilapia was also influenced by OTC treatment. In particular, the sequence abundance of Proteobacteria was decreased in OTC-treated fish compared with control fish at the end of antibiotic treatment (Figure 4.5 A). Furthermore, the proportion of this phylum continued to decrease in the gut microbiome of treated fish throughout the withdrawal period (Figure 4.5 A). The decline in Proteobacteria representation in response to OTC treatment was attributed to *Aeromonas, Bradyrhizobium, Hyphomicrobium, Reyranella* and *Rhodobacter*, which were depleted in the distal guts of treated fish compared with the control group at day 8 (Figure 4.5 B; Table 4.3 (S1)). However, not all Proteobacteria families responded to the OTC treatment in the same way, as *Aquicella*, *Plesiomonas* and *Undibacterium* had higher prevalence within OTC-treated fish compared with the control group at day 8 (Figure 4.5 B; Table 4.3 (S1)). The enrichment of *Plesiomonas* was found to be short-lived, as the abundance of this genus was reduced in the distal guts of treated fish compared with the control group following a 7-day withdrawal period at day 15 (Figure 4.5 B; Table 4.3 (S1)). Both *Acinetobacter* and *Pseudomonas* showed delayed enrichment in response to OTC following treatment, being detected more frequently in the distal guts of treated fish compared with the control group at day 15 (Figure 4.5 B; Table 4.3 (S1)). Similarly, *Hyphomicrobium* and *Reyranella* were also higher in abundance within treated fish compared with control fish at day 15 (Figure 4.5 B; Table 4.3 (S1)). However, by day 22, both *Hyphomicrobium* and *Reyranella* became undetectable in the distal guts of OTC-treated fish (Table 4.3 (S1)). This was similar across most Proteobacteria genera, as they were either undetectable or at a considerably lower prevalence within the distal gut microbiome communities of treated fish compared with the control group by the end of the trial (Figure 4.5 B; Table 4.3 (S1)).

Oxytetracycline also had an effect on other bacterial phyla within the distal gut microbiome of Nile tilapia (Figure 4.5 A; Table 4.3 (S1)). In particular, the Chlamydiae phylum was found to be present at a much lower sequence abundance within treated fish, compared with the control group at the end of antibiotic treatment (Table 4.3 (S1)). Similar patterns were also observed for Chloroflexi, Patescibacteria, Planctomycetes as well as most Firmicutes genera (Figure 4.5 A; Table 4.3 (S1)). However, within Firmicutes, *Romboutsia* was positively influenced by OTC treatment, becoming enriched in the distal guts of treated fish at day 8 (Figure 4.5 B). Likewise, whilst OTC was found to negatively influence the abundance of Bacteroidetes; *Macellibacteroides* also increased in abundance within the distal guts of treated fish at the end of antibiotic treatment (Figure 4.5 B). Furthermore, the abundance of this genus remained higher in OTC-treated fish compared with control fish by day 22 (Figure 4.5 B; Table 4.3 (S1)). Oxytetracycline treatment was also observed to shift Actinobacteria composition (Table 4.3 (S1)). However, similar to Proteobacteria, responses were not uniform across the entire Actinobacteria phylum. The sequence abundance of *Micromonospora*, *Mycobacterium*,

Nocardioides and *Pseudonocardia* were found to decline in the distal guts of treated fish at the end of antibiotic treatment, whereas *Conexibacter*, *Gordonia*, *Nocardia* and *Rhodococcus* read abundance became elevated at the same time point (Figure 4.5 B; Table 4.3 (S1)). Like Proteobacteria, most Actinobacteria genera became undetectable within the distal gut microbiome communities of treated fish by the end of the 14-day withdrawal period (Table 4.3 (S1)). Similar patterns of low abundance by day 22 was common across many of the genera detected, as the distal gut microbiome communities of treated fish showed low phylum and genus diversity by the end of the trial (Figure 4.5). This pattern followed that observed in the microbial diversity profiles of treated fish over time (Figure 4.3). This shift in microbiome communities was largely driven by OTUs assigned to Fusobacteria and *Cetobacterium* in particular (Figure 4.5). The OTUs assigned to *Cetobacterium* were observed in much higher sequence abundance in OTC-treated fish compared with the control group following antibiotic treatment (Figure 4.5 B). Furthermore, this genus increased steadily over time in the distal guts of treated fish throughout the withdrawal period, leading to the reduced representation of most other genera by day 22 (Figure 4.5 B).

Microbial communities associated with the feed pellets were less diverse than for fish guts, with a total of 229 bacterial genera detected within these samples. The vast majority of reads were assigned to *Lactobacillus*, *Moritella* and *Photobacterium* (Figure 4.6 A). Furthermore, the microbial community composition of the diet pellets remained relatively stable across treatment group and time (Figure 4.6 A). Likewise, similar compositions were also noted between the different NSC samples, which were primarily dominated by OTUs assigned to *Acidovorax*, *Acinetobacter*, *Flavobacterium*, *Polynucleobacter*, *Pseudomonas* and *Rhodoluna* (Figure 4.6 A). In contrast to feed pellets, the microbial communities from tank biofilms and the main biofilter unit were more diverse, with reads being assigned to a total of 437 bacterial genera. In addition, similar compositions were found between the microbial communities associated with the TA biofilter and the biofilms from different tanks, with no observable difference across treatment groups or time (Figure 4.6 B). In general, OTUs detected in these samples were primarily assigned to *11-24_ge*, *Deinococcus*, *Flavobacterium*, *Haliangium*, *Novosphingobium*, *Pedosphaeraceae_ge*, *RBG-13-54-9_ge*, *Rhizorhapis* and *Tepidisphaera* (Figure 4.6 B).

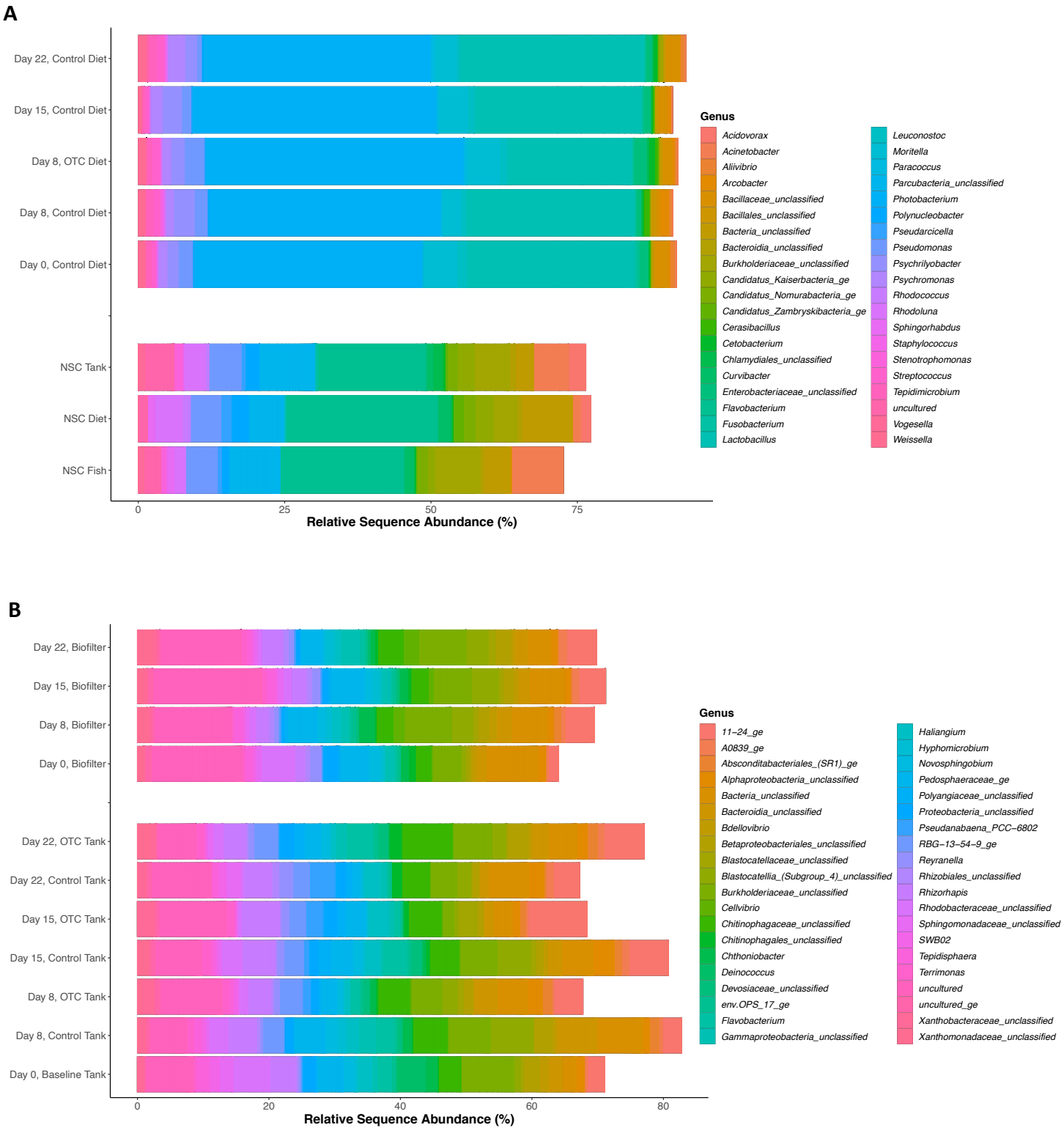

Figure 4.6. Relative sequence abundance (%) of the top 40 bacterial genera in NSC samples (A) as well as feed pellets (A), tank biofilms (B) and the main biofilter unit (B) across treatment and time.

Metastats and LEfSe analyses revealed that a number of OTUs were differentially abundant in the distal gut microbiome of fish in either control or OTC groups across time (Figure 4.7; Table 4.4 (S2)). Oxytetracycline exposure was found to have the greatest influence on Actinobacteria & Proteobacteria, as a number of OTUs assigned to these phyla were detected at significantly lower sequence abundances in distal guts of OTC-treated fish compared with the control group at day 8 (Figure 4.7). However, one Proteobacteria OTU classified as *Plesiomonas* was found to have significantly higher prevalence in OTC-treated fish compared with control fish by the end of antibiotic treatment (Figure 4.7; Table 4.4 (S2)). By day 15, most discriminatory OTUs were assigned to Actinobacteria or Proteobacteria, and found to be present at significantly higher sequence abundance within the distal guts of treated fish compared with the control group (Figure 4.7). A greater number of discriminatory OTUs were identified at day 22 compared with days 8 and 15 (Table 4.4 (S2)). Furthermore, the majority of discriminatory OTUs at day 22 were assigned to Proteobacteria, followed by Planctomycetes and Actinobacteria (Figure 4.7). Further analysis revealed that almost all of the differentially abundant OTUs detected at day 22, had significantly lower sequence abundance in the distal guts of fish treated with OTC compared with the control group (Figure 4.7). This followed findings in microbial diversity (Figure 4.3) and taxonomic profiles (Figure 4.5). Despite the decrease in prevalence for many OTUs at day 22, two OTUs were detected at higher sequence abundance in the distal guts of treated fish compared with control fish at the end of the trial (Figure 4.7). These were assigned to *Cetobacterium* and *Macellibacteroides*, respectively (Table 4.4 (S2)).

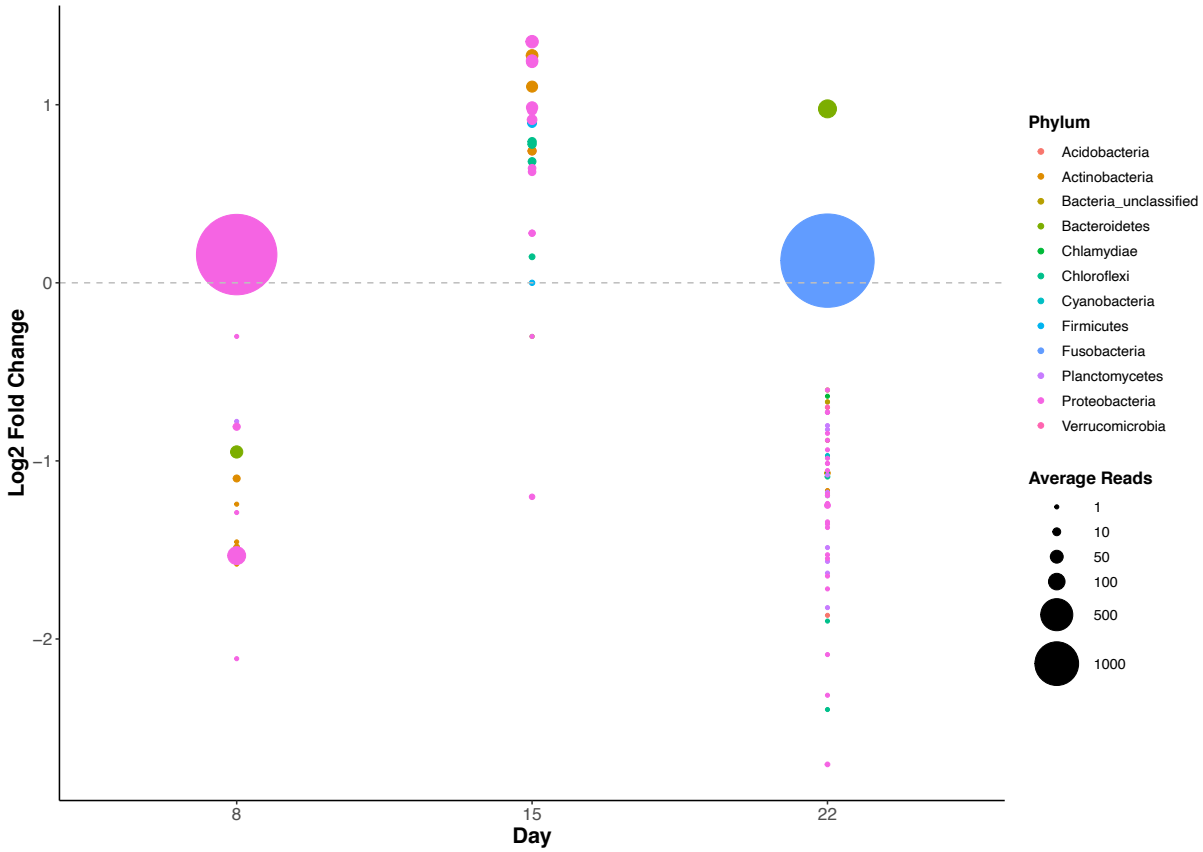

Figure 4.7. Plot of operational taxonomic units (OTU) that were significantly differentially abundant ($p < 0.05$) in the distal gut of Nile tilapia after treatment with oxytetracycline, compared with control fish. Effect size is represented as the log2 fold-change of each OTU observed in fish from the oxytetracycline diet treatment compared with fish fed the control diet. Each circle represents a single OTU and is coloured according to the phylum to which the OTU originates. Circle size is proportional to the mean read abundance of each OTU.

4.4.5. Antimicrobial resistance gene dynamics

Differences in the mean abundance of the AMR genes *intI1*, *tetA*, *tetM* and *tetX*, in the distal guts of Nile tilapia, were assessed between control and OTC-treated groups over time. All AMR genes were detected in pre-treated fish at day 0, as well as fish from both treatment groups (Figure 4.8). The mean abundance of the genes *intI1* and *tetA* remained constant in fish from both treatment groups over time (Figure 4.8 A & B). However, the mean abundance of *intI1* was frequently found at lower levels in the distal guts of OTC-treated fish compared with the control group (Figure 4.8 A). In contrast, the gene *tetA* was detected more frequently in the distal guts of OTC-treated fish compared with control fish following antibiotic treatment at day 8 (Figure 4.8 B). The mean abundance of *tetA* genes was however observed to decline in OTC-treated fish to levels below that of the control group by day 15 (Figure 4.8 B). By day 22, mean abundance levels of the *tetA* gene were comparable between both treatment groups (Figure 4.8 B). The mean abundance of the genes *tetM* and *tetX* were observed to vary over time in both treatment groups (Figure 4.8 C & D). Both genes were found in higher mean abundance in the distal guts of OTC-treated fish at day 15 compared with the control group (Figure 4.8 C & D). However, copies of both genes were detected less frequently in the distal guts of fish in the OTC group compared with the control group by the end of the trial (Figure 4.8 C & D). Despite these findings in AMR gene abundance, no significant differences were detected for any AMR gene between the control and treated groups at any time point ($p > 0.05$).

All AMR genes investigated were detected in the tank biofilms and TA biofilter in this study (Figure 4.9 B & C). However, the *intI1* and *tetA* genes were routinely detected in higher quantities compared with *tetM* and *tetX* (Figure 4.9 B & C). This followed similar findings in the distal guts of fish from both treatment groups (Figure 4.9 A). On further inspection, with the exception of OTC-treated fish at day 15, OTC exposure was associated with a higher prevalence of *tetA* genes within the distal gut and biofilm of treated fish and tanks compared with control groups, respectively (Figure 4.9 A & B). In contrast, the AMR gene dynamics within the TA biofilter unit was more resilient than that of the actual tanks, as the distribution of AMR genes was not found to fluctuate over time (Figure 4.9 C). Likewise, all AMR genes were also detected within most of the diet samples (Figure 4.9 D). In addition, with the exception of the control diet at day 8 and the diet given on day 22, similar AMR profiles were observed between the different diets given during the trial (Figure 4.9 D). More specifically, the *tetA* gene was not detected within the control diet at day 8, whereas the *tetX* gene dominated the AMR gene profile of the diet given on day 22 (Figure 4.9 D).

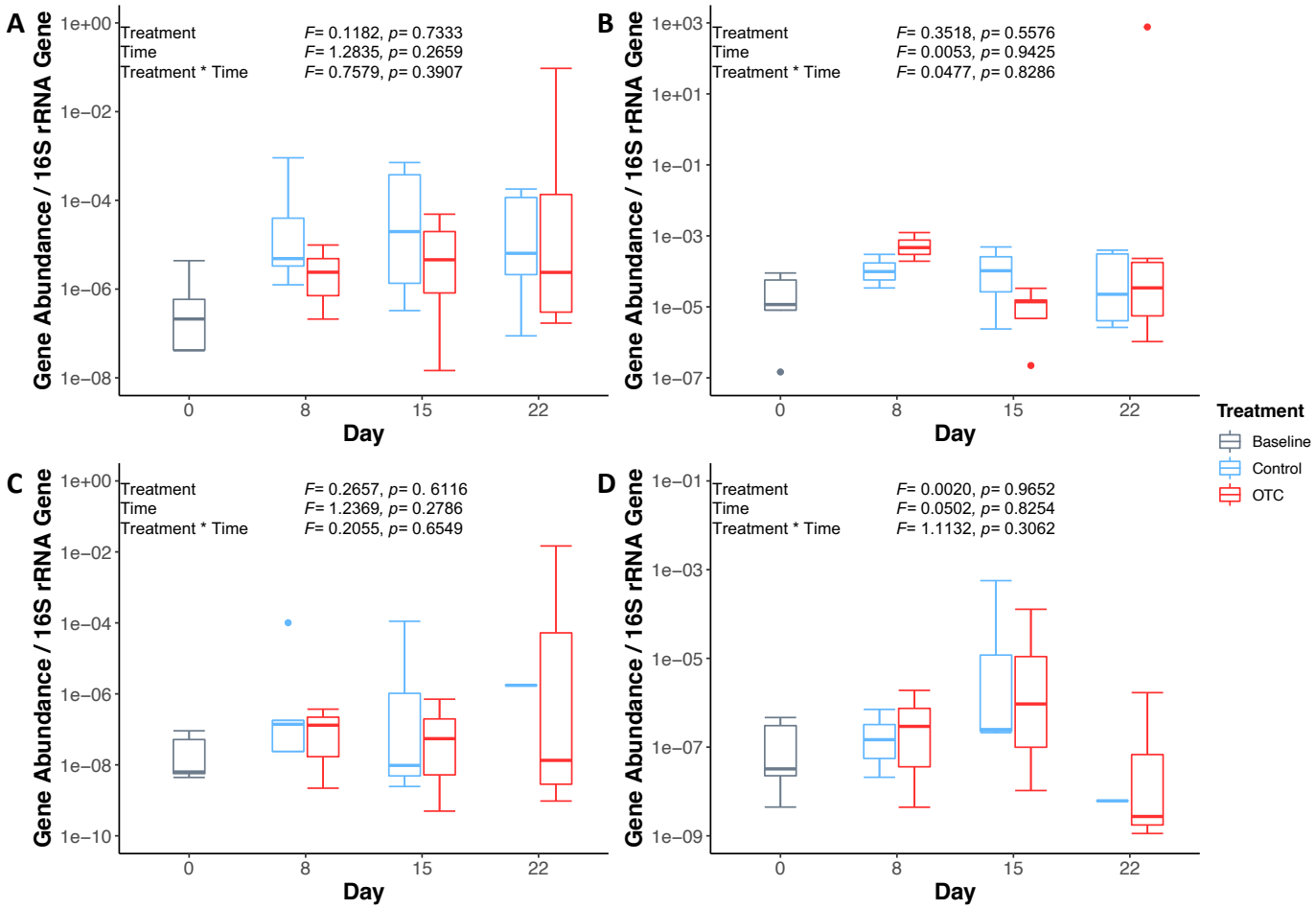

Figure 4.8. Box and whisker plot of absolute abundance of the AMR genes *intI1* (A), *tetA* (B), *tetM* (C) and *tetX* (D) in the distal guts of control and oxytetracycline (OTC)-treated Nile tilapia, before and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

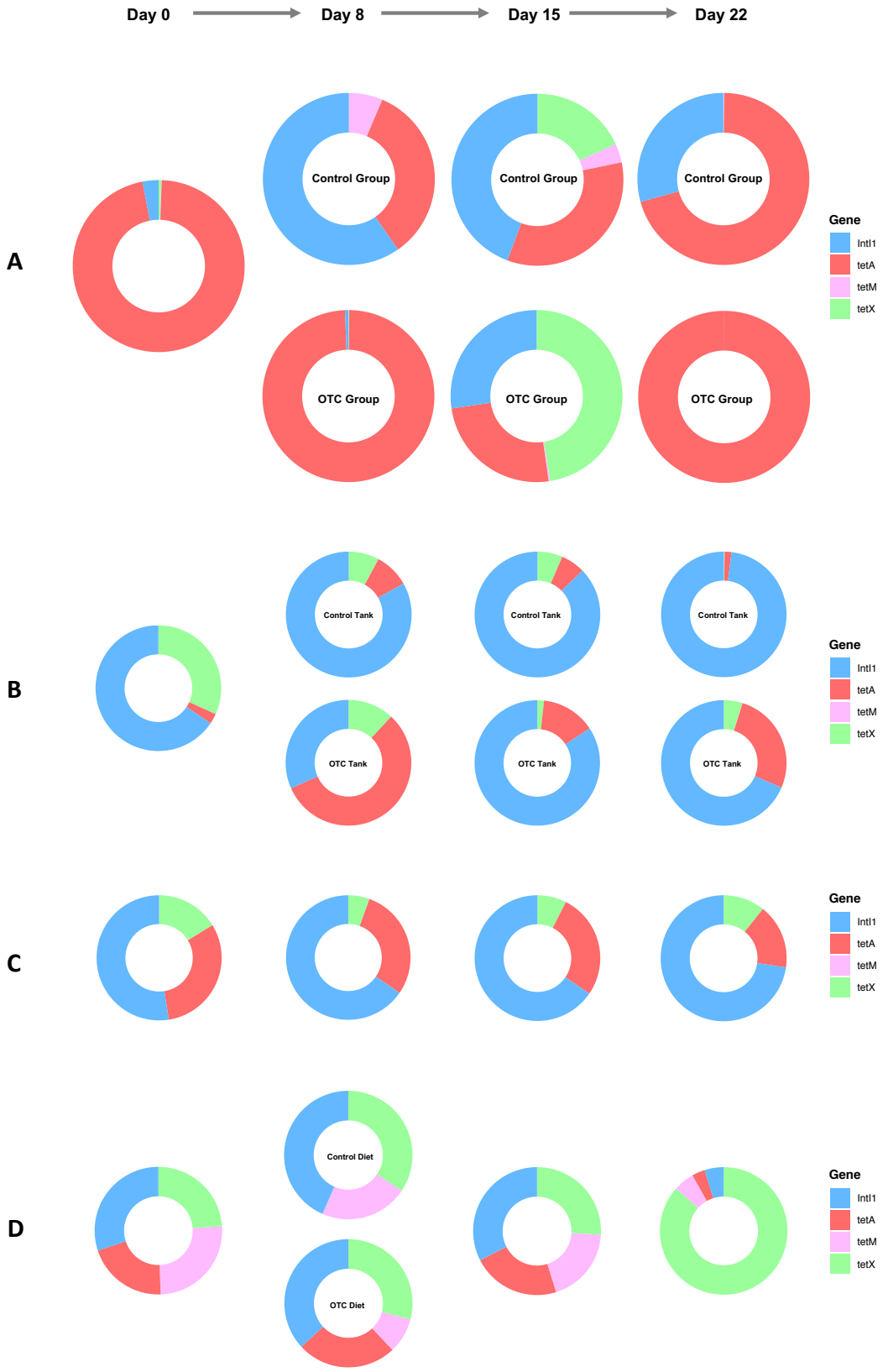

Figure 4.9. Distribution of *intI1*, *tetA*, *tetM* & *tetX* AMR genes in the distal guts of fish (A), tank biofilms (B), aquarium biofilter (C) and diets (D) before and after antibiotic treatment.

The AMR genes *intI1* and *tetM* were both positively correlated with microbiome community richness, diversity and evenness ($p < 0.01$) (Table 4.5). Whilst microbiome community richness and evenness were also positively associated with *tetX* gene abundance ($p < 0.05$), microbiome community diversity was only found to have weak correlations with this gene ($p > 0.05$) (Table 4.5). Furthermore, *tetA* abundance was not observed to have a strong correlation with any alpha diversity measure ($p > 0.05$) (Table 4.5).

Table 4.5. Associations between antimicrobial resistance (AMR) gene abundance and alpha diversity measures in the distal gut microbiome of Nile tilapia.

AMR Gene	Chao1 Richness		Inverse Simpson		Shannon Diversity Index	
	R	*p*	R	*p*	R	*p*
intI1	0.78	0.0000	0.58	0.0008	0.57	0.0011
tetA	0.21	0.2600	0.2	0.3000	0.24	0.2000
tetM	0.76	0.0001	0.69	0.0005	0.55	0.0092
tetX	0.5	0.0260	0.21	0.3700	0.45	0.0470

A correlation matrix of AMR abundance and microbial features showed varying degrees of correlations between these variables (Table 4.6 (S3)). A total of 109 OTUs were identified to be associated with AMR gene levels in the distal gut microbiome of Nile tilapia, where the magnitude of correlation ranged from -0.56 to 0.95. The strongest associations observed were positive associations with *intI1*, as 55 OTUs (50.46% of total OTUs) were found to have strong correlations ($R > 0.5$) with the abundance of this gene compared with any other AMR gene (Table 4.6 (S3)). In contrast, only five OTUs (4.58% of total OTUs) were found to be strongly correlated with the abundance of the *tetA* gene (Table 4.6 (S3)). At the phylum level, Actinobacteria, Planctomycetes and Proteobacteria were found to have the greatest influence on the abundance of AMR genes, as strong correlations between these genes and OTUs assigned to these phyla were repeatedly detected. Within the Actinobacteria phylum, 15, 14 and 12 OTUs were found to be positively associated with the abundance of *intI1*, *tetM* and *tetX* genes, respectively (Table 4.6 (S3)). These OTUs were assigned to several genera including *Gordonia, Mycobacterium, Nocardia, Nocardioides, Rhodococcus* and *Smaragdicoccus*. Furthermore, within *Smaragdicoccus*, OTU0063 was found to be positively associated with all three AMR genes (Table 4.6 (S3)). In regard to Planctomycetes, a total of eight, six and five OTUs were shown to have strong positive correlations with *intI1*, *tetM* and *tetX* gene abundance, respectively (Table 4.6 (S3)). Within this phylum, most OTUs were assigned to uncultured taxa. Additionally, similar to *Smaragdicoccus*, two Planctomycetes OTU's (OTU0165 and OTU0221) were also found to be associated with the *intI1*, *tetM* and *tetX* genes (Table 4.6 (S3)). A total of 23, 21 and 16 Proteobacteria OTUs were found to have positive correlations with the abundance of *intI1*, *tetM* and *tetX* genes, respectively (Table 4.6 (S3)). These positively correlated OTUs were assigned

to range of genera including *Aquicella*, *Edwardsiella*, *Hyphomicrobium*, *Plesiomonas* and *Reyranella*. Furthermore, among the five OTUs found to have a positive relationship with *tetA* gene abundance (Table 4.6 (S3)), four were classified as Proteobacteria, including OTU0004 which contributed the majority of reads assigned to *Plesiomonas*. Negative correlations were also observed between OTUs and AMR gene abundance (Table 4.6 (S3)). In particular, the abundance of OTU0001 assigned to Fusobacteria and *Cetobacterium*, which dominated the gut microbiome communities of fish, was found to have a strong negative correlation with *intI1* and *tetM* (Table 4.6 (S3)).

4.4.6. Host gene dynamics

The expression of genes related to immunity, digestion and gut functioning were tested as biomarkers to evaluate the influence of OTC exposure on gut health status in Nile tilapia (Figure 4.10). Results indicated that OTC only had minor effects on immune functioning within the distal gut tissue of Nile tilapia. Whilst at the end of antibiotic treatment, genes involved in bacterial recognition (*TLR21*, *NOD1* and *scarb1*), the NF-κB signalling pathway (*nkap*), cytokine expression (*TGF-β* and *IL-1β*) and innate (*TP4*) and adaptive defences (*sIgT*), all had higher mRNA expression within the distal gut tissue of OTC-treated fish compared with control fish, no significant difference ($p > 0.05$) was detected between treatment groups (Figure 4.10). Likewise, no significant difference ($p > 0.05$) was observed between treatment groups for any immune-related gene at day 15, although the expression of several genes remained elevated in the distal gut tissue of treated fish compared with control fish, including both cytokines as well as *nkap* and *TP4* (Figure 4.10). By day 22, all immune-related genes had lower expression levels in the distal gut tissue of OTC-treated fish compared with the control group (Figure 4.10). Furthermore, the expression profiles of several genes including *TLR21*, *TP4* and *sIgT* followed that of microbiome diversity, with mean mRNA expression levels decreasing within the distal gut tissue of OTC-treated fish throughout the withdrawal period (Figure 4.3 & Figure 4.10). However again, no significant differences ($p > 0.05$) were noted between OTC and control treatment groups for any immune-related genes at this time point. Similarly, with the exception of *TP4*, no significant difference ($p > 0.05$) was observed in the expression of immune genes over time in both treatment groups (Figure 4.10). The expression of both digestion (*slc2a6*) and gut barrier integrity (*atp1b1*) related genes within the distal guts of fish was not found to be significantly influenced by OTC treatment ($p > 0.05$) or time e.g. day 0, 8 etc. ($p > 0.05$). However, like most immune-related genes, both *slc2a6* and *atp1b1* genes were found to have increased expression at the end of OTC treatment (day 8) within the distal gut tissue of treated fish, reducing to a level below that of control fish at day 22 (Figure 4.10).

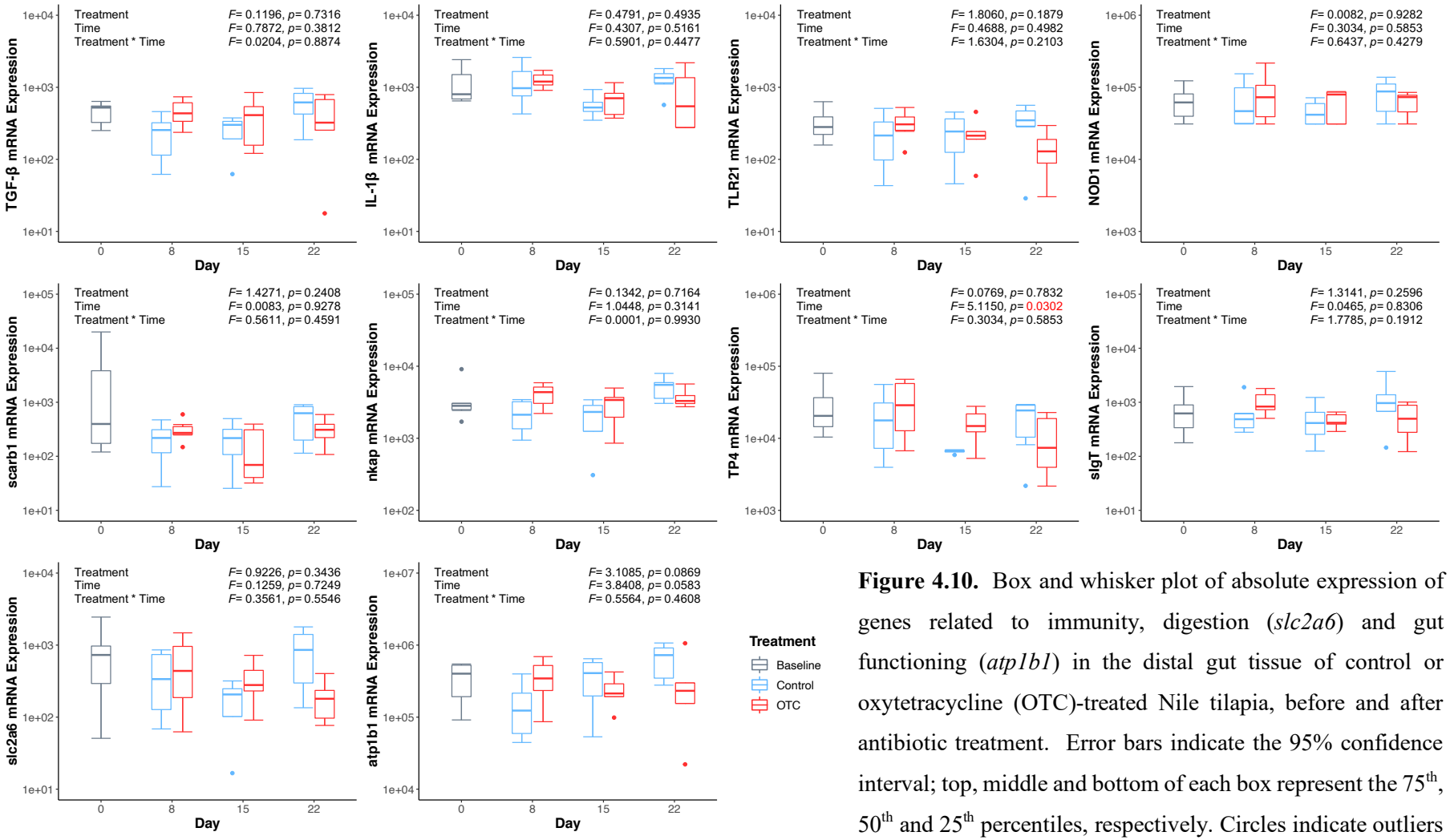

Figure 4.10. Box and whisker plot of absolute expression of genes related to immunity, digestion (*slc2a6*) and gut functioning (*atp1b1*) in the distal gut tissue of control or oxytetracycline (OTC)-treated Nile tilapia, before and after antibiotic treatment. Error bars indicate the 95% confidence interval; top, middle and bottom of each box represent the 75th, 50th and 25th percentiles, respectively. Circles indicate outliers from the dataset.

The expression of host genes related to immunity, digestion and gut functioning within the distal gut tissue of fish were not found to be significantly associated with any alpha diversity measure in this study ($p > 0.05$) (Table 4.7). Despite this, genes related to bacterial recognition (*TLR21*, *NOD1* and *scarb1*), intracellular signalling (*nkap*) and cell signalling (*TGF-β* and IL-1β) were found to have negative associations with microbiome community richness, diversity and evenness (Table 4.7). Furthermore, innate and adaptive defences were observed to have contrasting associations with gut microbiome diversity, as *TP4* and *sIgT* were found to have negative and positive correlations with all alpha diversity measures, respectively (Table 4.7).

Table 4.7. Associations between host gene expression levels and microbiome alpha diversity measures in the distal gut of Nile tilapia.

Host-related gene	Chao1 Richness		Inverse Simpson		Shannon Diversity Index	
	R	*p*	R	*p*	R	*p*
TGF-β	-0.028	0.89	-0.19	0.34	-0.25	0.2
IL-1β	-0.051	0.8	-0.076	0.7	-0.15	0.45
TLR21	0.056	0.78	-0.084	0.67	-0.071	0.72
NOD1	-0.16	0.42	-0.29	0.13	-0.21	0.28
scarb1	-0.12	0.55	-0.17	0.39	-0.21	0.29
nkap	-0.013	0.95	-0.17	0.4	-0.19	0.33
TP4	-0.27	0.17	-0.28	0.15	-0.21	0.29
sIgT	0.28	0.16	0.043	0.83	0.21	0.28
slc2a6	0.018	0.93	-0.087	0.66	-0.038	0.85
atp1b1	0.12	0.55	-0.033	0.87	-0.17	0.38

A total of 48 OTUs were identified to be associated with host-related gene expression in the distal guts of Nile tilapia, where the magnitude of correlation ranged from 0.5 to 0.95 (Table 4.8 (S4)). The greatest interactions between host and individual microbiome members were observed for genes involved in immune functioning compared with those for digestion or gut barrier integrity, as no strong associations were found between any OTU and *slc2a6* or *atp1b1* gene expression, respectively (Table 4.8 (S4)). The strongest interactions between the immune-related genes investigated and the distal gut microbiome community of Nile tilapia, was observed between five OTUs and *scarb1* gene expression with correlations of R = 0.68 to 0.95 (Table 4.8 (S4)). On further exploration, this gene was found to be strongly associated with Proteobacteria, as three out of four OTUs with strong correlations to *scarb1* gene expression were assigned to this phylum. However, the immune-related gene which had the most diverse interaction network was found to be *sIgT*, as 40 OTUs (83.33% of total OTUs) were observed to have positive associations with this gene (Table 4.8 (S4)). Furthermore, the gene expression of *sIgT* was found to be associated

with OTUs from eight bacterial phyla. This was followed by the *nkap* gene which had associations with 26 OTUs (54.16% of total OTUs), although these associations were not as strong compared with *sIgT*, as correlations ranged from R = 0.50 to 0.54 and R = 0.50 to 0.81 for *nkap* and *sIgT*, respectively (Table 4.8 (S4)). No strong associations were found between the distal gut microbiome and the genes *NOD1*, *TGF-β* or IL-1β (Table 4.8 (S4)). Likewise, no negative correlations were found between the microbiome community and any host-related gene expression (Table 4.8 (S4)).

At the phylum level, Proteobacteria was found to have the greatest influence over host gene expression within the distal guts of fish, followed by Firmicutes, Patescibacteria and Planctomycetes. Within the Proteobacteria phylum, OTUs were found more frequently to interact with the adaptive immune response compared with other host genes, as ten OTUs were found to have positive associations with *sIgT* gene expression (Table 4.8 (S4)). In comparison, six, three, two and two OTUs were found to have positive correlations with the genes *nkap*, *scarb1*, *TLR21* and *TP4*, respectively (Table 4.8 (S4)). Within this phylum, interactions were found between immune-related genes and *Acinetobacter* (*sIgT*), *Aeromonas* (*TP4*), *Crenobacter* (*sIgT* and *TLR21*), *Deefgea* (*nkap* and *sIgT*) and *Undibacterium* (*TP4*) (Table 4.8 (S4)). The Firmicutes phylum was found to be strongly associated with the gene expression of *sIgT* (R > 0.67) (Table 4.8 (S4)). Likewise, members of this phylum was also detected to have correlations with the gene expression of *nkap* (R = 0.52), although this was not as strong as for the *sIgT* gene (Table 4.8 (S4)). At the genus level, associations were found between the aforementioned genes and *Caldicellulosiruptor*, *Geobacillus*, *Pelosinus* and *Planifilum* (Table 4.8 (S4)). Similar patterns were also observed for the Patescibacteria phylum with ten OTUs observed to be positively correlated with *sIgT* gene expression (R > 0.69), as well as the expression of the *nkap* gene (R > 0.50) (Table 4.8 (S4)). These OTUs were predominantly classified as belonging to *Candidatus_Nomurabacteria_ge*. Following these same trends, the Planctomycetes were also found to have much greater interaction with the gene expression of *sIgT* than any other host-related gene investigated. Five OTUs were shown to have positive correlations with *sIgT* gene expression, whereas only two and one OTU were found to be associated with the genes *nkap* and *TLR21*, respectively (Table 4.8 (S4)). Within this phylum, OTUs were frequently classified as *Gemmata* and *Pirellula*.

4.5. Discussion

Findings from this study demonstrate that a single treatment with OTC at 100 mg kg bodyweight day^{-1}, was sufficient to induce a decline in the distal gut microbiome diversity of Nile tilapia within eight days. Furthermore, the microbial diversity within the distal guts of treated fish continued to decrease over time even after treatment had stopped, suggesting that this particular antibiotic can induce long-term disruption on the gut microbiome of this fish species. These findings follow that from other studies with Atlantic salmon (*Salmo salar*) and zebrafish, whose gut microbiomes have also been reported to reduce in microbial diversity in response to OTC (Navarrete et al., 2008; Zhou et al., 2018a, 2018b), as well as other antibiotic compounds e.g. sulfamethoxazole (Zhou et al., 2018a). As OTC has been reported to be administered on fish farms at similar doses (50 – 250 mg kg bodyweight day^{-1}) and using the same delivery mechanism (via feed) used in this study (Limbu et al., 2018), there is possibility that similar responses could take place in the distal guts of farmed Nile tilapia. This is concerning, given the fact that the microbiome plays a primary role in the protection of its host against the establishment of opportunistic pathogens, through direct colonisation resistance mechanisms (Van der Waaij et al., 1971). As such, any decline in the diversity of the gut microbiome through antibiotic treatment as evident in this study, may leave farmed fish susceptible to pathogen invasion and disease. This has already been demonstrated in zebrafish treated with low levels of OTC, as treated fish were found to have lower microbiome diversity and a higher mortality rate when challenged with *A. hydrophila*, compared with the control group (Zhou et al., 2018b). Therefore, if gut microbiome recovery is not supported following antibiotic treatment, antibiotic-induced disruptions in the gut microbiome diversity of treated fish could see fish farms stuck in a continuous cycle of antibiotic treatment, leading to poor colonisation resistance and disease outbreaks. However, as an experimental challenge or repeated antibiotic exposure were not investigated in this study, further research is required to explore these theories in more detail. Likewise, how best to support gut microbiome recovery in farmed fish following antibiotic treatment is an interesting question that remains to be fully understood.

Although no difference between treatment groups and time was observed in community structure based on the beta-diversity distances, OTC was observed to alter the distal gut microbiome community membership in Nile tilapia following an eight-day treatment, which persisted even after two weeks of withdrawal from the antibiotic. Indeed, in line with the observed alpha diversity of communities, most genera within the gut microbiome decreased in abundance within the guts of treated fish following OTC treatment at day 8, similar to what has been reported for this antibiotic in Atlantic salmon (Navarrete et al., 2008; Gupta et al., 2019). This was particularly evident in Gram-negative bacteria including those belonging to Chloroflexi and Planctomycetes,

as well as several Proteobacteria genera such as *Aeromonas* and *Hyphomicrobium*. A general decline in the Proteobacteria phylum was not surprising given that OTC is frequently used in the treatment of bacterial fish pathogens, many of whom belong to Proteobacteria (Smith et al., 1994; Serrano, 2005; Ibrahim Kholil et al., 2015). However, OTC was also shown to decrease the abundance of Gram-positive bacteria including the Firmicutes genera *Clostridium_sensu_stricto_1* and several Actinobacteria genera such as *Micromonospora*, and *Nocardioides*. These findings therefore demonstrate the diverse nature of organisms within the gut microbiome that can be unintentionally targeted by OTC during antibiotic treatment on the fish farm.

In this study OTC treatment was however found to enrich some bacterial groups, including *Plesiomonas* (Proteobacteria phylum) as well as *Nocardia* and *Rhodococcus* (Actinobacteria phylum). Bacterial members from the Actinobacteria and Proteobacteria phyla have previously been reported to increase in abundance within the fish gut microbiome, following treatment with OTC or other antibiotic compounds e.g. florfenicol (Tapia-Paniagua et al., 2015; Gupta et al., 2019). Bacteria can achieve resistance to antibiotic compounds through intracellular mechanisms encoded by antimicrobial resistance genes (ARG). In bacteria, ARGs can be acquired through the horizontal gene transfer (HGT) of mobile genetic elements (MGE) (Marano et al., 2019). One such gene associated with MGEs is the class 1 integron gene *intI1* (Subirats et al., 2018). In this study, the *intI1* gene was observed in high abundance across fish and environmental samples, indicating there was potentially a wide dissemination of ARGs between the bacteria in fish gut microbiome communities, as well as those associated with the environment e.g. tank biofilms, even prior to antibiotic treatment. Specific resistance to tetracycline compounds can be achieved through the production of efflux pumps, such as the pump encoded by the *tetA* gene, which are located on the inner membrane of the bacterial cell wall. Efflux pumps provide ribosomal protection by selectively transporting tetracycline compounds from the cytoplasm to the periplasmic space (Stavropoulos & Strathdee, 2000; Møller et al., 2016). In the present study, the *tetA* gene was detected in high abundance within the distal guts of fish and tank environments. Furthermore, this gene was highly correlated with OTU0004 assigned to *Plesiomonas*, as both were observed to increase in abundance within the distal guts of fish at the end of OTC treatment. These findings support previous studies which have detected the *tetA* gene in *Plesiomonas* species from aquatic environments as well as fish (Jun *et al.*, 2011; Adesiyan *et al.*, 2019), and demonstrate that antibiotic treatment may select for AMR within the gut microbiome of fish. Likewise, the strong correlation between the abundance of the *tetM* and *tetX* genes and several OTUs assigned to *Nocardia* and *Rhodococcus*, which had increased in abundance within the distal guts of treated fish on days 8 and 15, also support concerns surrounding antibiotic treatment and the selection of AMR populations. These findings were not surprising given that the first

tetracycline compounds originated from *Streptomyces aureofaciens*, another member of the Actinobacteria phylum (Grossman, 2016). As such, in addition to producing antimicrobial compounds, Actinobacteria members may also contain a range of mechanisms which aid in defending against their own antibiotics, as well as resistance to compounds excreted from similar organisms. In fact, the *tetM* gene, which encodes for a ribosomal protection protein, has previously been detected in several Actinobacteria genera (Fatahi-Bafghi, 2019). In farmed fish, the favourable selection of AMR within the commensal gut microbiome following antibiotic treatment, may mediate the transfer of ARGs to opportunistic pathogens in later disease outbreaks. Indeed, the HGT of vancomycin resistance has already been demonstrated between *Clostridium symbiosum* and the opportunistic pathogens *Enterococcus faecalis* and *Enterococcus faecium* in the guts of mice (Launay et al., 2006), thus similar processes could occur in the fish gut. This would be a concern for the aquaculture industry, as some fish farming countries e.g. the UK, only have a limited number of antibiotics prescribed for use in farmed fish (Serrano, 2005). As a result, the same antibiotic compound can be given more than once during the production cycle. However, if the bacterial pathogen being treated had already acquired ARGs to the specific antibiotic compound through HGT with the resistant microbiome community, this would ultimately limit the efficacy of this, and any future antibiotic treatment with the same compound, leading to significant economic losses on the farm.

Whilst this study found OTU0001 (*Cetobacterium*) to be negatively associated with several ARGs, *Cetobacterium* was also observed to display some resistance to the OTC treatment and increased in abundance within the distal guts of treated fish on day 8. Thus, the findings described previously do not support the observed resistance in some bacterial members within the distal gut microbiome of Nile tilapia in this study. These findings do however follow that described previously in zebrafish treated with OTC (Zhou et al., 2018a). Previous studies exploring tetracycline resistance in clinical and environmental isolates, have reported more than 50 tetracycline resistance genes (Wang et al., 2017) which confer resistance principally through the use of efflux pumps (e.g. *tetA*), ribosomal protection (e.g. *tetM*) and enzymatic modification (e.g. *tetX*). Therefore, it is entirely possible that the enrichment of *Cetobacterium* could have arisen through another gene and/or mechanism. Likewise, resistance of *Cetobacterium* may have resulted through the presence of outer membrane proteins (OMP), which are common in Gram-negative bacteria and play a critical role in antibiotic resistance through modulating cellular permeability (Lin et al., 2018; Choi & Lee, 2019). However, this would require further exploration as the current knowledge on OMPs in Fusobacteria members is scarce, with a primary focus being on *Fusobacterium* species which were not detected in fish in this study.

The enrichment of particular microbiome members may have also resulted from an increased availability of niches and/or adhesion sites, as a consequence of the decline in a number of bacterial genera following OTC treatment. Previously, reduced microbial diversity as evident in this study, has been suggested provide vacant niches for potential invaders (Mallon et al., 2015). However, given that ARGs were detected in the distal gut microbiome of pre-treated fish, the potential vacant niches that became available following OTC treatment, could have also allowed particular resistant members to proliferate. This is highly likely as the resistant commensal members e.g. *Cetobacterium*, were already established and accustomed to the intestinal environment of fish. Whilst the dominance of Fusobacteria and *Cetobacterium* in the distal guts of control and pre-treated fish demonstrate an importance of this bacterial group for host physiology, a further rise in their abundance may increase the potential for disease in farmed Nile tilapia. This increased risk of disease could occur through direct microbiome dysbiosis or the onset of virulence associated with changes in population dynamics.

Certain members within the microbiome e.g. keystone species, can play vital roles in how the overall community responds to disturbances, as changes in their abundance lead to considerable alterations in the membership and distribution of all other species (Foster et al., 2008). If *Cetobacterium* act as a keystone species within the distal guts of Nile tilapia, their continued proliferation within OTC-treated fish may positively influence the establishment of opportunistic pathogens, through the removal of other commensal members and reducing the competition for food and attachment sites. Likewise, further expansion of *Cetobacterium* may reduce the available attachment sites required by keystone species, thereby interrupting community networks which indirectly allows for pathogen invaders to colonise and establish. Furthermore, some species are also reported to alter their virulence in response to changes in co-operation (Rózsa et al., 2015). Therefore, the continued increase in abundance of *Cetobacterium* over long time frames, may further disrupt the gut microbiome of fish by reducing positive co-operation in the community network dynamics, and stimulating certain members to shift from a commensal/mutualistic state towards that of a pathogenic one. Finally, *Cetobacterium* may also shift from commensalism to pathogenic directly, as a result of changes in its population dynamics. A number of studies employing culture-independent techniques have detected the presence of opportunistic bacterial pathogens including members of the *Vibrio* and *Aeromonas* genera, as a component of the normal microbiome in fish (Reid et al., 2009; Al-Hisnawi et al., 2015). Similarly, *Aeromonas* as well as *Nocardia* which also contain important fish pathogens, were also detected in the distal guts of pre-treated fish in this study. The presence of these members could indicate that under stable and homeostatic conditions, the virulence of opportunistic pathogens in the microbiome is kept under control. However, in the event of microbiome dysbiosis, changes in population dynamics and inter-bacterial communication e.g. quorum sensing, result in a switch towards more aggressive

traits and the establishment of infection. This has already been demonstrated within the invertebrate *Drosophila* model (Kim et al., 2020), thus similar transitions could take place within the gut microbiome of fish. If this does occur, then the continued proliferation of *Cetobacterium* in the distal guts of fish treated with OTC, may cause members of this genus to transition from commensal to pathogenic behaviour, and increase the risk of future disease outbreaks in these animals.

Previous studies exploring the resident microbiome in fish have revealed a plethora of important functions served by this community which help in maintaining host physiology (Talwar et al., 2018). In this study, whilst a considerable shift in the distal gut microbiome community was observed in treated fish following antibiotic treatment and the withdrawal period, differences in the mRNA expression level of host genes related to immunity, digestion or gut functioning between treatment groups were minor. One factor to consider is the length of time following the withdrawal of OTC, as in this study, the final sampling point occurred after two-weeks following the termination of OTC treatment. However, in other vertebrate species such as farmed chicken (*Gallus gallus*) and humans, recovery times of microbiome communities following antibiotic treatment can vary considerably, lasting many years in some cases (Jakobsson et al., 2010; Videnska et al., 2013). Therefore, given the functional importance of the gut microbiome; if the changes in the microbiome community of treated fish in this study persist long-term, it is possible that more significant differences in physiological processes within the distal gut could be observed between control and treated fish following a longer time frame. On the other hand, small differences in distal gut physiology between treatment groups irrespective of microbial community differences, could also be attributed to host-microbiome evolution history. More specifically, it is possible that due to the complexity and importance of the microbial-mediated functions provided by the gut microbiome, instead of selecting for individual OTUs, the fish host may select for functional assemblages e.g. proteolytic groups, during microbiome establishment or recovery following a disturbance. Under the former scenario, if an OTU is lost through a disturbance event e.g. antibiotic treatment and not available for re-colonisation, the absence of this specific OTU following recovery could result in a loss of a particular host-function provided by that OTU, which would likely impact on host fitness. However, under the latter scenario, the loss of specific OTUs following a disturbance would not be as detrimental, as the host would select for OTUs within the environment which provide a similar function, thereby maintaining optimal physiological status. If the fish host does select for function rather than OTU, then this would undoubtably allow for better resilience to microbiome changes as a result of antibiotic treatment, with minimal consequences on host physiology and health. Currently, the majority of microbiome meta-analysis in fish species are conducted by 16S rRNA metabarcoding techniques, therefore only predicted functional capacity can be inferred which are not as accurate as other

methods. Future studies combining 16S rRNA surveys with other "omics" approaches that investigate host/microbial transcriptomes or their functional metabolites, such as transcriptomics or metabolomics, respectively, would therefore be vital to explore this theory further.

Despite the minor changes in gene expression within the distal gut tissue between OTC and control fish, findings from this study demonstrate the existence of host-microbiome interactions within Nile tilapia. This was particularly evident in the expression of several immune-related genes, which were found to have strong correlations with the abundance of a variety of microbiome members. For example, present results demonstrated an interaction between the microbiome and innate immunity within the distal gut of Nile tilapia, as the gene expression of *scarb1* which is vital for phagocytosis (Fink et al., 2015), was strongly associated with several Proteobacteria OTUs. Likewise, the gene expression of *TP4* which encodes for the antimicrobial peptide moronecidin (Peng et al., 2012), was also found to be strongly associated with OTU0005 assigned to *Aeromonas*. These results agree with previous reports where *Aeromonas* species have been found to modulate the activity of other innate immune responses including serum amyloid A (Rawls et al., 2004). Furthermore, as both *TP4* gene expression and the proportion of *Aeromonas* were found to decrease within the distal gut of treated fish over time, results from this study demonstrate that OTC-induced reductions in the microbiome diversity and loss of particular bacterial taxa, can lead to a decrease in innate immune mechanisms within the distal guts of Nile tilapia. This is concerning as the gut has been demonstrated to be a primary site of entry for fish bacterial pathogens (Ringø et al., 2010). Likewise, the innate immune response of fish is considered to be vital for defending against invading bacterial pathogens, due to the limitations in the adaptive immune response such as the slow proliferation and memory of lymphocytes (Kordon et al., 2018). As a result, any impairment in the innate immune system may have consequences on the gut health and subsequent disease resilience in farmed individuals of this fish species.

In the present study, the microbiome was also found to interact with the adaptive immune response within the distal gut of Nile tilapia, as the gene expression of *sIgT*, which encodes for secretory IgT was strongly associated with a diverse assemblage of bacterial genera including *Deefgea* and *Flavobacterium*. This correlation partly explains the observed decrease in *sIgT* gene expression within the distal guts of treated fish by day 22, as these bacterial groups also became underrepresented at the same timepoint. The highly diverse interaction between the *sIgT* gene and certain gut microbiome members, supports previous findings which demonstrate the role played by *sIgT* in microbiome homeostasis (Salinas et al., 2018). As *sIgT* is thought to coat commensal bacteria to aid in immune exclusion and to regulate the microbiome community (Kelly & Salinas, 2017), the reduction in *sIgT* gene expression within the distal gut tissue of treated fish by day 22,

could be indicative of poor immune tolerance and host-microbiome dysbiosis in Nile tilapia following OTC treatment. However, further investigation using histological or immunohistochemistry approaches, combined with exploring the expression of a wider panel of genes related to intestinal integrity and health, would be required to support this theory. Despite this, these findings raise concerns for the long-term impacts of antibiotic treatment in fish and their gut health.

4.6. Conclusions

Findings from this study provide evidence that a single high dose OTC treatment can disturb the gut health of Nile tilapia, through changes in the microbiome community and minor disturbances in distal gut physiology. Oxytetracycline treatment reduced the gut microbiome diversity of treated fish within eight days, which was accompanied by alterations in the microbiome community membership. Furthermore, the decline in microbial diversity and shift in the distal gut microbiome community, continued in the OTC-treated fish even after the antibiotic treatment was terminated. An increase in the prevalence of several bacterial genera was however evident within the distal guts of treated fish following antibiotic treatment, which was correlated with the abundance of several ARGs. The consequences of these effects are unknown, however the findings demonstrate that antibiotic treatment can induce selection pressures which favour the resistant communities already present within the gut microbiome of fish. The present study also supports previous findings of host-microbiome interactions in other fish species, as shifts in the microbiome community were strongly associated with changes in the expression of immune-related genes within the distal gut of Nile tilapia. However, despite the observed host-microbiome interactions, OTC treatment was not found to have a significant effect on the expression of genes related to immunity, metabolism and gut integrity within the distal gut tissue of fish. Therefore, further work is required to clarify the long-term consequences of antibiotic-induced changes in the gut microbiome on the gut health in this fish species.

4.7. Acknowledgments

This study was funded by the Fisheries Society of the British Isles as part of their PhD Studentship program. The authors would like to thank aquarium staff for their technical assistance during the feeding trial. Thanks, are also given to Dr. J. Taggart and Mrs. J. Ireland for their expertise and guidance during the molecular phase of this research as well as Miss. C. Broughton, Mr. F. Legario and Mr. A. Ahmed for their help during sample processing. We would also like to thank Prof. J. Turnbull for his help during data analysis.

4.8. Author Contributions

CJP and MC developed the study concept and design. CJP collected stool samples from fish and performed all laboratory work. CJP analysed all data with input from SM and MC.

4.9. Conflict of interest

The authors declare that they have no competing interests.

4.10. Ethical Approval

All work carried out was approved by the Animal Welfare and Ethical Review Body (AWERB) at UoS (AWERB (18 19) 151 New ASPA) and followed guidelines set out by the UK Home Office Animals (Scientific Procedures) Act 1986.

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4.12. Supplementary Information

Table 4.3 (S1). Mean (± SD) abundance of top bacterial phyla and genera in the distal gut of control or oxytetracycline (OTC)-treated Nile tilapia, before and after antibiotic treatment.

Phylum	Genus	Day 0	Day 8		Day 15		Day 22	
		Baseline	Control	OTC	Control	OTC	Control	OTC
Actinobacteria	**All Genera (%)**	5.78 ± 6.24	5.57 ± 6.48	5.55 ± 9.24	0.76 ± 0.83	2.89 ± 4.39	4.19 ± 8.66	0.14 ± 0.17
	Conexibacter (%)	0.22 ± 0.33	0.24 ± 0.27	0.35 ± 0.60	0.00 ± 0.00	0.06 ± 0.13	0.23 ± 0.39	0.00 ± 0.00
	Gordonia (%)	0.17 ± 0.24	0.07 ± 0.10	1.22 ± 2.12	0.00 ± 0.01	0.20 ± 0.39	0.01 ± 0.02	0.00 ± 0.00
	Micromonospora (%)	0.02 ± 0.02	0.10 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	1.16 ± 2.84	0.00 ± 0.00
	Mycobacterium (%)	0.89 ± 1.14	0.43 ± 0.57	0.38 ± 0.67	0.04 ± 0.06	0.17 ± 0.26	0.16 ± 0.33	0.00 ± 0.01
	Nocardia (%)	0.61 ± 1.22	0.00 ± 0.00	0.25 ± 0.43	0.00 ± 0.00	0.04 ± 0.09	0.10 ± 0.25	0.00 ± 0.00
	Nocardioides (%)	0.74 ± 0.93	1.02 ± 1.42	0.27 ± 0.45	0.01 ± 0.01	0.26 ± 0.46	1.06 ± 2.24	0.06 ± 0.07
	Pseudonocardia (%)	0.16 ± 0.19	0.30 ± 0.38	0.06 ± 0.08	0.00 ± 0.01	0.19 ± 0.40	0.23 ± 0.47	0.00 ± 0.00
	Rhodococcus (%)	0.53 ± 1.18	0.09 ± 0.17	1.50 ± 2.61	0.01 ± 0.01	0.05 ± 0.07	0.01 ± 0.01	0.00 ± 0.00
	Smaragdicoccus (%)	0.02 ± 0.03	0.69 ± 0.97	0.68 ± 1.18	0.00 ± 0.00	0.50 ± 1.00	0.03 ± 0.05	0.00 ± 0.00
Bacteria_unclassified	**All Genera (%)**	0.48 ± 0.77	0.87 ± 1.24	0.26 ± 0.46	1.11 ± 2.11	0.34 ± 0.52	0.40 ± 0.53	0.02 ± 0.03
	Bacteria_unclassified (%)	0.48 ± 0.77	0.87 ± 1.24	26 ± 0.46	1.11 ± 2.11	0.34 ± 0.52	0.40 ± 0.53	0.02 ± 0.03
Bacteroidetes	**All Genera (%)**	1.58 ± 1.24	2.39 ± 1.74	1.95 ± 1.13	4.47 ± 4.86	1.51 ± 2.07	1.00 ± 1.96	1.84 ± 2.67
	Flavobacterium (%)	0.00 ± 0.00	0.08 ± 0.10	0.08 ± 0.15	1.96 ± 3.87	0.74 ± 1.57	0.83 ± 1.78	0.00 ± 0.00
	Macellibacteroides (%)	0.84 ± 0.78	1.27 ± 1.02	1.61 ± 0.86	0.40 ± 0.65	0.38 ± 0.39	0.03 ± 0.04	0.67 ± 0.78
Chlamydiae	**All Genera (%)**	0.63 ± 0.97	0.81 ± 0.82	0.38 ± 0.66	0.33 ± 0.66	0.48 ± 0.79	0.84 ± 1.50	0.01 ± 0.01
	cvE6_ge (%)	0.35 ± 0.54	0.37 ± 0.34	0.33 ± 0.57	0.03 ± 0.06	0.28 ± 0.39	0.44 ± 0.80	0.00 ± 0.00
Chloroflexi	**All Genera (%)**	1.62 ± 1.76	1.02 ± 1.40	0.49 ± 0.85	0.01 ± 0.01	1.06 ± 1.80	1.39 ± 2.22	0.00 ± 0.00
	JG30-KF-CM45_ge (%)	0.33 ± 0.51	0.21 ± 0.38	0.09 ± 0.16	0.00 ± 0.00	0.15 ± 0.27	0.26 ± 0.41	0.00 ± 0.00
Firmicutes	**All Genera (%)**	5.53 ± 8.07	1.83 ± 20.02	1.88 ± 1.49	0.78 ± 0.18	1.00 ± 1.12	2.26 ± 3.69	1.07 ± 1.03
	Bacillales_unclassified (%)	0.94 ± 1.56	0.39 ± 0.49	0.04 ± 0.07	0.06 ± 0.12	0.15 ± 0.22	1.26 ± 2.82	0.07 ± 0.10
	Clostridium_sensu_stricto_1 (%)	1.32 ± 2.35	0.17 ± 0.33	0.01 ± 0.01	0.02 ± 0.03	0.07 ± 0.10	0.06 ± 0.10	0.10 ± 0.17
	Romboutsia (%)	2.32 ± 2.82	0.87 ± 1.18	1.63 ± 1.67	0.43 ± 0.41	0.53 ± 0.92	0.25 ± 0.24	0.85 ± 1.00
Fusobacteria	**All Genera (%)**	70.58 ± 28.91	47.98 ± 32.05	64.15 ± 22.24	73.58 ± 30.96	79.57 ± 21.57	59.44 ± 43.18	93.66 ± 4.19
	Cetobacterium (%)	70.07 ± 29.78	47.78 ± 31.94	64.00 ± 22.18	73.53 ± 30.92	79.26 ± 21.71	59.20 ± 43.06	93.08 ± 4.19
	Fusobacteriaceae_unclassified (%)	0.51 ± 1.03	0.20 ± 0.12	0.15 ± 0.23	0.05 ± 0.10	0.32 ± 0.68	0.24 ± 0.34	0.54 ± 0.60

Table 4.3 (S1). *Continued.*

Phylum	Genus	Day 0	Day 8		Day 15		Day 22	
		Baseline	Control	OTC	Control	OTC	Control	OTC
Patescibacteria	**All Genera (%)**	0.10 ± 0.12	0.16 ± 0.20	0.08 ± 0.13	1.05 ± 2.10	0.53 ± 1.07	0.73 ± 1.58	0.00 ± 0.00
Planctomycetes	**All Genera (%)**	1.18 ± 1.04	1.32 ± 1.70	0.56 ± 0.96	0.06 ± 0.09	0.80 ± 1.51	1.67 ± 2.22	0.05 ± 0.09
	Gemmataceae_unclassified (%)	0.22 ± 0.26	0.19 ± 0.36	0.01 ± 0.02	0.00 ± 0.00	0.23 ± 0.52	0.27 ± 0.39	0.00 ± 0.00
Proteobacteria	**All Genera (%)**	12.46 ± 12.88	37.59 ± 20.50	24.61 ± 10.59	17.52 ± 21.68	11.47 ± 11.14	27.70 ± 33.76	3.21 ± 2.61
	Acinetobacter (%)	0.08 ± 0.10	0.01 ± 0.01	0.01 ± 0.02	0.58 ± 0.69	3.35 ± 4.89	0.29 ± 0.61	0.05 ± 0.10
	Aeromonas (%)	4.67 ± 8.42	11.52 ± 18.53	0.67 ± 0.84	1.45 ± 1.48	0.49 ± 0.69	0.14 ± 0.17	0.00 ± 0.00
	Aquicella (%)	0.35 ± 0.33	0.04 ± 0.05	0.22 ± 0.38	0.00 ± 0.01	0.22 ± 0.27	0.27 ± 0.67	0.00 ± 0.00
	Bradyrhizobium (%)	0.26 ± 0.27	0.21 ± 0.26	0.06 ± 0.10	0.03 ± 0.06	0.05 ± 0.05	0.33 ± 0.53	0.00 ± 0.00
	Burkholderiaceae_unclassified (%)	0.14 ± 0.23	0.27 ± 0.31	0.18 ± 0.28	0.87 ± 1.42	0.27 ± 0.41	0.22 ± 0.32	0.10 ± 0.15
	Crenobacter (%)	0.40 ± 0.41	0.51 ± 0.73	0.05 ± 0.02	0.00 ± 0.01	0.02 ± 0.03	0.34 ± 0.54	0.21 ± 0.31
	Deefgea (%)	0.20 ± 0.34	0.12 ± 0.11	0.03 ± 0.06	0.27 ± 0.45	0.04 ± 0.06	3.67 ± 8.15	0.13 ± 0.26
	Desulfovibrionaceae_unclassified (%)	0.00 ± 0.00	5.59 ± 11.06	0.00 ± 0.00	0.09 ± 0.11	0.01 ± 0.01	0.05 ± 0.12	0.00 ± 0.00
	Hyphomicrobium (%)	0.32 ± 0.26	1.17 ± 1.56	0.13 ± 0.16	0.00 ± 0.00	0.35 ± 0.56	0.81 ± 1.11	0.00 ± 0.00
	Plesiomonas (%)	0.90 ± 0.69	7.11 ± 7.34	20.49 ± 7.59	2.24 ± 1.95	0.84 ± 1.49	3.20 ± 6.44	1.79 ± 1.84
	Proteobacteria_unclassified (%)	0.03 ± 0.04	6.41 ± 12.80	0.00 ± 0.00	8.03 ± 16.05	0.29 ± 0.64	12.11 ± 28.97	0.02 ± 0.03
	Pseudomonas (%)	0.01 ± 0.02	0.00 ± 0.01	0.01 ± 0.01	0.41 ± 0.57	1.25 ± 2.52	0.09 ± 0.19	0.03 ± 0.05
	Reyranella (%)	0.11 ± 0.14	0.57 ± 0.69	0.01 ± 0.02	0.00 ± 0.00	0.59 ± 1.24	2.18 ± 3.19	0.00 ± 0.00
	Rhizobiales_unclassified (%)	0.37 ± 0.44	0.36 ± 0.52	0.10 ± 0.15	0.06 ± 0.11	0.11 ± 0.15	0.43 ± 0.74	0.00 ± 0.01
	Rhodobacter (%)	0.71 ± 1.32	0.19 ± 0.39	0.15 ± 0.26	0.00 ± 0.00	0.02 ± 0.02	0.05 ± 0.12	0.00 ± 0.00
	Rhodobacteraceae_unclassified (%)	1.02 ± 1.48	0.70 ± 1.18	0.37 ± 0.64	0.00 ± 0.01	0.53 ± 0.66	0.36 ± 0.61	0.00 ± 0.00
	Undibacterium (%)	0.16 ± 0.12	0.26 ± 0.20	0.55 ± 0.57	0.44 ± 0.40	0.42 ± 0.42	0.12 ± 0.16	0.24 ± 0.11
	Xanthobacteraceae_unclassified (%)	0.30 ± 0.27	0.32 ± 0.38	0.17 ± 0.28	0.03 ± 0.06	0.29 ± 0.65	0.64 ± 1.01	0.00 ± 0.00
Uncultured	*Uncultured* (%)	2.66 ± 2.52	2.66 ± 1.91	1.15 ± 1.65	1.74 ± 2.70	1.12 ± 1.92	1.89 ± 2.85	1.20 ± 2.06
	uncultured_ge (%)	0.46 ± 0.80	0.49 ± 0.54	0.20 ± 0.35	0.13 ± 0.18	0.43 ± 0.82	0.31 ± 0.57	0.03 ± 0.06

Table 4.4 (S2). Operational taxonomic units (OTU) identified as discriminatory according to oxytetracycline exposure by Metastats and LEfSe algorithms in Mothur.

Day	Phylotype			*p* value	
	OTU	Phylum	Genus	Metastats	LEfSe
8	OTU0140	Actinobacteria	*Kineosporiaceae_unclassified*	0.040	-
	OTU0237	Actinobacteria	*uncultured_ge*	0.027	-
	OTU0321	Actinobacteria	*Iamia*	0.049	-
	OTU0359	Actinobacteria	*Microtrichales_unclassified*	0.028	-
	OTU0802	Actinobacteria	*Microtrichales_unclassified*	0.046	-
	OTU0008	Bacteroidetes	*uncultured*	0.019	-
	OTU0308	Planctomycetes	*uncultured*	0.041	-
	OTU0004	Proteobacteria	*Plesiomonas*	0.007	0.022
	OTU0006	Proteobacteria	*Aeromonas*	0.029	-
	OTU0059	Proteobacteria	*Reyranella*	0.027	-
	OTU0163	Proteobacteria	*Deefgea*	0.032	-
	OTU0005	Proteobacteria	*Pseudomonas*	-	0.030
	OTU0017	Proteobacteria	*Pedomicrobium*	-	0.008
	OTU0155	Proteobacteria	*uncultured*	-	0.042
	OTU0208	Proteobacteria	*Reyranella*	-	0.044
15	OTU0065	Acidobacteria	*DS-100_ge*	0.039	-
	OTU0010	Actinobacteria	*Nocardioides*	0.044	-
	OTU0053	Actinobacteria	*Mycobacterium*	0.037	-
	OTU0609	Actinobacteria	*Corynebacterium*	0.026	-
	OTU0062	Chloroflexi	*Flavobacterium*	0.031	-
	OTU0219	Chloroflexi	*RBG-13-54-9_ge*	0.022	-
	OTU0434	Chloroflexi	*KD4-96_ge*	0.046	-
	OTU0945	Chloroflexi	*S085_ge*	0.034	-
	OTU0750	Firmicutes	*JG30-KF-CM45_ge*	0.028	-
	OTU2501	Firmicutes	*Lactobacillales_unclassified*	0.024	-
	OTU0018	Proteobacteria	*Bacillales_unclassified*	0.028	-
	OTU0024	Proteobacteria	*Desulfovibrionaceae_unclassified*	0.030	-
	OTU0028	Bacteroidetes	*Hyphomicrobium*	-	0.010
	OTU0044	Proteobacteria	*Aquicella*	0.013	-
	OTU0051	Proteobacteria	*Rhodobacteraceae_unclassified*	0.006	-
	OTU0098	Proteobacteria	*Rhizobiales_unclassified*	0.014	-
	OTU0113	Proteobacteria	*Methyloligellaceae_unclassified*	0.048	-
	OTU0116	Proteobacteria	*Rhizobiales_unclassified*	0.004	-
	OTU0136	Proteobacteria	*Sphingomonadaceae_unclassified*	-	0.032
	OTU0169	Proteobacteria	*Pseudorhodoplanes*	0.020	-
	OTU0187	Proteobacteria	*Stenotrophomonas*	0.028	-
	OTU0196	Proteobacteria	*Rhodobacteraceae_unclassified*	0.016	-
	OTU0246	Proteobacteria	*Alphaproteobacteria_unclassified*	0.037	-

Table 4.4 (S2). *Continued.*

Day	Phylotype			*p* value	
	OTU	Phylum	Genus	Metastats	LEfSe
22	OTU0065	Acidobacteria	*DS-100_ge*	0.028	-
	OTU0063	Actinobacteria	*Smaragdicoccus*	0.014	-
	OTU0217	Actinobacteria	*uncultured_ge*	0.042	-
	OTU0582	Actinobacteria	*Corynebacterium_1*	0.038	-
	OTU0685	Actinobacteria	*Nocardioides*	0.044	-
	OTU1031	Actinobacteria	*Mycobacterium*	0.024	-
	OTU0132	Bacteria_unclassified	*Bacteria_unclassified*	0.033	-
	OTU0505	Bacteria_unclassified	*Bacteria_unclassified*	0.029	-
	OTU0782	Bacteria_unclassified	*Bacteria_unclassified*	0.030	-
	OTU1130	Bacteria_unclassified	*Bacteria_unclassified*	0.030	-
	OTU0020	Bacteroidetes	*Macellibacteroides*	0.024	-
	OTU0435	Chlamydiae	*Chlamydiales_unclassified*	0.013	-
	OTU0544	Chlamydiae	*Chlamydia*	0.017	-
	OTU1093	Chlamydiae	*Parachlamydiaceae_unclassified*	0.040	-
	OTU0012	Chloroflexi	*uncultured*	0.029	-
	OTU0058	Chloroflexi	*JG30-KF-CM45_ge*	0.021	-
	OTU0219	Chloroflexi	*KD4-96_ge*	0.044	-
	OTU0976	Cyanobacteria	*Obscuribacterales_ge*	0.021	-
	OTU0002	Fusobacteria	*Cetobacterium*	0.004	-
	OTU0073	Planctomycetes	*Planctopirus*	0.018	-
	OTU0080	Planctomycetes	*uncultured*	0.006	-
	OTU0121	Planctomycetes	*Gemmataceae_unclassified*	0.027	-
	OTU0221	Planctomycetes	*uncultured*	0.004	-
	OTU0238	Planctomycetes	*uncultured*	0.035	-
	OTU0262	Planctomycetes	*Gemmata*	0.011	-
	OTU0478	Planctomycetes	*Pirellulaceae_unclassified*	0.011	-
	OTU0574	Planctomycetes	*uncultured*	0.003	-
	OTU0588	Planctomycetes	*Gemmata*	0.002	-
	OTU0626	Planctomycetes	*uncultured*	0.007	-
	OTU0005	Proteobacteria	*Aeromonas*	0.006	-
	OTU0006	Proteobacteria	*Reyranella*	0.013	-
	OTU0024	Proteobacteria	*Hyphomicrobium*	0.005	-
	OTU0034	Proteobacteria	*Novosphingobium*	0.045	-
	OTU0036	Proteobacteria	*Bradyrhizobium*	0.045	-
	OTU0046	Proteobacteria	*Proteobacteria_unclassified*	0.013	-
	OTU0048	Proteobacteria	*Rhodobacteraceae_unclassified*	0.043	-
	OTU0051	Proteobacteria	*Rhodobacteraceae_unclassified*	0.047	-
	OTU0116	Proteobacteria	*Rhizobiales_unclassified*	0.019	-
	OTU0150	Proteobacteria	*uncultured*	0.049	-
	OTU0189	Proteobacteria	*Xanthobacteraceae_unclassified*	0.009	-
	OTU0201	Proteobacteria	*Hyphomicrobiaceae_unclassified*	0.036	-
	OTU0204	Proteobacteria	*Hyphomicrobium*	0.042	-
	OTU0206	Proteobacteria	*Enterobacteriaceae_unclassified*	0.046	-
	OTU0229	Proteobacteria	*uncultured*	0.041	-
	OTU0258	Proteobacteria	*Arenimonas*	0.048	-
	OTU0271	Proteobacteria	*Deltaproteobacteria_unclassified*	0.011	-
	OTU0314	Proteobacteria	*Xanthobacteraceae_unclassified*	0.009	-
	OTU0324	Proteobacteria	*Reyranella*	0.001	-
	OTU0332	Proteobacteria	*Pedomicrobium*	0.022	-
	OTU0334	Proteobacteria	*Phreatobacter*	0.033	-
	OTU0412	Proteobacteria	*Gammaproteobacteria_unclassified*	0.019	-
	OTU0503	Proteobacteria	*Pedomicrobium*	0.030	-
	OTU0594	Proteobacteria	*Rickettsiaceae_unclassified*	0.010	-
	OTU0679	Proteobacteria	*Dechloromonas*	0.038	-
	OTU1564	Proteobacteria	*Deltaproteobacteria_unclassified*	0.030	-
	OTU0425	Verrucomicrobia	*Verrucomicrobiaceae_unclassified*	0.012	-

Table 4.6 (S3). Correlation of antimicrobial resistance (AMR) gene abundance and the distal gut microbiome in Nile tilapia. Correlations were determined using Pearson's correlation algorithm.

OTU	Phylum	Genus	AMR Gene	Pearson's Correlation	
				R	*p* value
OTU0065	Acidobacteria	*DS-100_ge*	*intI1*	0.63	1
OTU1605	Acidobacteria	*Subgroup_10*	*tetA*	0.91	0.0062
OTU0362	Actinobacteria	*67-14_ge*	*tetM*	0.57	1
OTU0473	Actinobacteria	*Acidimicrobiia_unclassified*	*tetM*	0.87	0.0837
OTU0473	Actinobacteria	*Acidimicrobiia_unclassified*	*tetX*	0.65	1
OTU1182	Actinobacteria	*Actinobacteria_unclassified*	*intI1*	0.95	<.0001
OTU0050	Actinobacteria	*Conexibacter*	*tetM*	0.86	0.1671
OTU0050	Actinobacteria	*Conexibacter*	*tetX*	0.59	1
OTU0284	Actinobacteria	*Crossiella*	*intI1*	0.75	1
OTU0253	Actinobacteria	*Fodinicola*	*tetM*	0.78	1
OTU0253	Actinobacteria	*Fodinicola*	*tetX*	0.54	1
OTU0291	Actinobacteria	*Gaiella*	*tetM*	0.59	1
OTU0902	Actinobacteria	*Gaiella*	*tetM*	0.87	0.0837
OTU0902	Actinobacteria	*Gaiella*	*tetX*	0.65	1
OTU0850	Actinobacteria	*Gaiellales_unclassified*	*intI1*	0.95	<.0001
OTU0476	Actinobacteria	*Galbitalea*	*intI1*	0.69	1
OTU0476	Actinobacteria	*Galbitalea*	*tetM*	0.60	1
OTU0104	Actinobacteria	*Gordonia*	*tetM*	0.87	0.1284
OTU0104	Actinobacteria	*Gordonia*	*tetX*	0.70	1
OTU0321	Actinobacteria	*Iamia*	*intI1*	0.76	1
OTU0492	Actinobacteria	*Microbacteriaceae_unclassified*	*intI1*	0.95	<.0001
OTU0622	Actinobacteria	*Microtrichaceae_unclassified*	*tetM*	0.87	0.0837
OTU0622	Actinobacteria	*Microtrichaceae_unclassified*	*tetX*	0.65	1
OTU0802	Actinobacteria	*Microtrichales_unclassified*	*intI1*	0.95	<.0001
OTU0912	Actinobacteria	*Mycobacterium*	*tetM*	0.88	0.0478
OTU0912	Actinobacteria	*Mycobacterium*	*tetX*	0.66	1
OTU0350	Actinobacteria	*Nocardia*	*tetM*	0.87	0.0865
OTU0350	Actinobacteria	*Nocardia*	*tetX*	0.65	1
OTU0526	Actinobacteria	*Nocardioides*	*intI1*	0.95	<.0001
OTU0684	Actinobacteria	*PeM15_ge*	*intI1*	0.53	1
OTU0114	Actinobacteria	*Pseudonocardia*	*intI1*	0.77	1
OTU1390	Actinobacteria	*Quadrisphaera*	*intI1*	0.95	<.0001
OTU0064	Actinobacteria	*Rhodococcus*	*tetM*	0.71	1
OTU0064	Actinobacteria	*Rhodococcus*	*tetX*	0.50	1
OTU0063	Actinobacteria	*Smaragdicoccus*	*intI1*	0.66	1
OTU0063	Actinobacteria	*Smaragdicoccus*	*tetM*	0.81	1
OTU0063	Actinobacteria	*Smaragdicoccus*	*tetX*	0.57	1
OTU0702	Actinobacteria	*uncultured*	*intI1*	0.95	<.0001
OTU0327	Actinobacteria	*uncultured*	*tetM*	0.85	0.3315
OTU0327	Actinobacteria	*uncultured*	*tetX*	0.60	1
OTU0237	Actinobacteria	*uncultured_ge*	*intI1*	0.71	1
OTU0257	Actinobacteria	*uncultured_ge*	*intI1*	0.57	1
OTU0217	Actinobacteria	*uncultured_ge*	*tetX*	0.57	1
OTU0153	Bacteria_unclassified	*Bacteria_unclassified*	*intI1*	0.95	<.0001
OTU0158	Bacteria_unclassified	*Bacteria_unclassified*	*tetM*	0.56	1
OTU0311	Bacteria_unclassified	*Bacteria_unclassified*	*tetM*	0.90	0.0171
OTU0311	Bacteria_unclassified	*Bacteria_unclassified*	*tetX*	0.67	1
OTU0007	Bacteroidetes	*Flavobacterium*	*tetM*	0.92	0.0022
OTU0007	Bacteroidetes	*Flavobacterium*	*tetX*	0.68	1

Table 4.6 (S3). *Continued.*

OTU	Phylum	Genus	AMR Gene	Pearson's Correlation	
				R	*p* value
OTU0157	Chlamydiae	*Candidatus_Fritschea*	*intI1*	0.66	1
OTU0435	Chlamydiae	*Chlamydiales_unclassified*	*intI1*	0.77	1
OTU0031	Chlamydiae	*cvE6_ge*	*tetM*	0.69	1
OTU0562	Chlamydiae	*Parachlamydiaceae_unclassified*	*intI1*	0.95	<.0001
OTU0296	Chlamydiae	*Simkaniaceae_unclassified*	*tetM*	0.87	0.0837
OTU0809	Chlamydiae	*Simkaniaceae_unclassified*	*tetM*	0.87	0.0837
OTU0296	Chlamydiae	*Simkaniaceae_unclassified*	*tetX*	0.65	1
OTU0809	Chlamydiae	*Simkaniaceae_unclassified*	*tetX*	0.65	1
OTU0062	Chloroflexi	*RBG-13-54-9_ge*	*tetM*	0.55	1
OTU0372	Dadabacteria	*Dadabacteriales_ge*	*intI1*	0.94	0.0002
OTU0035	Firmicutes	*Bacillales_unclassified*	*tetM*	0.81	1
OTU0035	Firmicutes	*Bacillales_unclassified*	*tetX*	0.58	1
OTU0416	Firmicutes	*Tumebacillus*	*tetM*	0.78	1
OTU0416	Firmicutes	*Tumebacillus*	*tetX*	0.55	1
OTU0001	Fusobacteria	*Cetobacterium*	*intI1*	-0.56	1
OTU0001	Fusobacteria	*Cetobacterium*	*tetM*	-0.63	1
OTU1395	Fusobacteria	*Fusobacterium*	*tetX*	0.56	1
OTU1312	Patescibacteria	*Saccharimonadales_ge*	*intI1*	0.95	<.0001
OTU1522	Patescibacteria	*Saccharimonadales_ge*	*tetM*	0.87	0.0837
OTU1522	Patescibacteria	*Saccharimonadales_ge*	*tetX*	0.65	1
OTU0262	Planctomycetes	*Gemmata*	*intI1*	0.95	<.0001
OTU0138	Planctomycetes	*Pirellula*	*intI1*	0.95	<.0001
OTU0509	Planctomycetes	*Planctomycetales_unclassified*	*intI1*	0.94	0.0003
OTU0073	Planctomycetes	*Planctopirus*	*intI1*	0.94	0.0004
OTU1464	Planctomycetes	*Singulisphaera*	*intI1*	0.95	<.0001
OTU0165	Planctomycetes	*uncultured*	*intI1*	0.68	1
OTU0221	Planctomycetes	*uncultured*	*intI1*	0.76	1
OTU0383	Planctomycetes	*uncultured*	*intI1*	0.95	<.0001
OTU0091	Planctomycetes	*uncultured*	*tetM*	0.78	1
OTU0126	Planctomycetes	*uncultured*	*tetM*	0.92	0.0041
OTU0147	Planctomycetes	*uncultured*	*tetM*	0.52	1
OTU0165	Planctomycetes	*uncultured*	*tetM*	0.80	1
OTU0221	Planctomycetes	*uncultured*	*tetM*	0.65	1
OTU0452	Planctomycetes	*uncultured*	*tetM*	0.89	0.0267
OTU0091	Planctomycetes	*uncultured*	*tetX*	0.65	1
OTU0126	Planctomycetes	*uncultured*	*tetX*	0.68	1
OTU0165	Planctomycetes	*uncultured*	*tetX*	0.57	1
OTU0221	Planctomycetes	*uncultured*	*tetX*	0.53	1
OTU0452	Planctomycetes	*uncultured*	*tetX*	0.66	1
OTU0564	Proteobacteria	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	*intI1*	0.94	0.0005
OTU0250	Proteobacteria	*Alphaproteobacteria_unclassified*	*intI1*	0.82	1
OTU0044	Proteobacteria	*Aquicella*	*tetM*	0.52	1
OTU0456	Proteobacteria	*Bosea*	*intI1*	0.95	0.0001
OTU0322	Proteobacteria	*Burkholderiaceae_unclassified*	*intI1*	0.77	1
OTU0081	Proteobacteria	*Burkholderiaceae_unclassified*	*tetM*	0.79	1
OTU0081	Proteobacteria	*Burkholderiaceae_unclassified*	*tetX*	0.71	1
OTU0128	Proteobacteria	*Coxiella*	*intI1*	0.95	0.0001
OTU0043	Proteobacteria	*Crenobacter*	*intI1*	0.80	1
OTU0018	Proteobacteria	*Desulfovibrionaceae_unclassified*	*intI1*	0.53	1
OTU1016	Proteobacteria	*Devosia*	*tetM*	0.87	0.0837
OTU1016	Proteobacteria	*Devosia*	*tetX*	0.65	1
OTU0590	Proteobacteria	*Duganella*	*tetM*	0.89	0.0354
OTU0590	Proteobacteria	*Duganella*	*tetX*	0.65	1
OTU0335	Proteobacteria	*Edwardsiella*	*tetM*	0.88	0.0781
OTU0335	Proteobacteria	*Edwardsiella*	*tetX*	0.65	1
OTU0139	Proteobacteria	*Enhydrobacter*	*tetA*	0.57	1
OTU1190	Proteobacteria	*Haematobacter*	*tetM*	0.87	0.0837
OTU1190	Proteobacteria	*Haematobacter*	*tetX*	0.65	1

Table 4.6 (S3). *Continued.*

OTU	Phylum	Genus	AMR Gene	Pearson's Correlation	
				R	*p* value
OTU1046	Proteobacteria	*Hyphomicrobiaceae_unclassified*	*tetM*	0.91	0.0098
OTU1046	Proteobacteria	*Hyphomicrobiaceae_unclassified*	*tetX*	0.66	1
OTU0024	Proteobacteria	*Hyphomicrobium*	*intI1*	0.77	1
OTU0094	Proteobacteria	*Hyphomicrobium*	*intI1*	0.65	1
OTU0151	Proteobacteria	*Hyphomicrobium*	*intI1*	0.95	<.0001
OTU0535	Proteobacteria	*Legionella*	*intI1*	0.87	0.1226
OTU1420	Proteobacteria	*Legionella*	*tetM*	0.89	0.0368
OTU1420	Proteobacteria	*Legionella*	*tetX*	0.66	1
OTU0835	Proteobacteria	*Methylobacterium*	*tetA*	0.91	0.0062
OTU0445	Proteobacteria	*Micavibrionales_unclassified*	*tetM*	0.87	0.0837
OTU0445	Proteobacteria	*Micavibrionales_unclassified*	*tetX*	0.65	1
OTU0155	Proteobacteria	*Pedomicrobium*	*intI1*	0.87	0.1354
OTU0587	Proteobacteria	*Pedomicrobium*	*tetA*	0.93	0.0013
OTU0503	Proteobacteria	*Pedomicrobium*	*tetM*	0.87	0.0837
OTU0503	Proteobacteria	*Pedomicrobium*	*tetX*	0.65	1
OTU0334	Proteobacteria	*Phreatobacter*	*intI1*	0.95	<.0001
OTU0607	Proteobacteria	*Phreatobacter*	*intI1*	0.92	0.0029
OTU0004	Proteobacteria	*Plesiomonas*	*tetA*	0.80	1
OTU0004	Proteobacteria	*Plesiomonas*	*tetM*	0.83	0.7202
OTU0089	Proteobacteria	*Polynucleobacter*	*tetM*	0.87	0.0837
OTU0089	Proteobacteria	*Polynucleobacter*	*tetX*	0.65	1
OTU0003	Proteobacteria	*Proteobacteria_unclassified*	*intI1*	0.95	<.0001
OTU0274	Proteobacteria	*Proteobacteria_unclassified*	*intI1*	0.95	<.0001
OTU0006	Proteobacteria	*Reyranella*	*intI1*	0.92	0.0024
OTU0067	Proteobacteria	*Reyranella*	*intI1*	0.83	0.8527
OTU0077	Proteobacteria	*Reyranella*	*intI1*	0.95	<.0001
OTU0324	Proteobacteria	*Reyranella*	*tetM*	0.86	0.1792
OTU0324	Proteobacteria	*Reyranella*	*tetX*	0.62	1
OTU0178	Proteobacteria	*Rhizobiaceae_unclassified*	*tetM*	0.54	1
OTU0697	Proteobacteria	*Rhizobiales_unclassified*	*intI1*	0.72	1
OTU0433	Proteobacteria	*Rhizobiales_unclassified*	*tetM*	0.88	0.0492
OTU0433	Proteobacteria	*Rhizobiales_unclassified*	*tetX*	0.66	1
OTU0042	Proteobacteria	*Rhodobacteraceae_unclassified*	*tetM*	0.88	0.0767
OTU0042	Proteobacteria	*Rhodobacteraceae_unclassified*	*tetX*	0.65	1
OTU0150	Proteobacteria	*uncultured*	*intI1*	0.54	1
OTU1644	Proteobacteria	*uncultured*	*intI1*	0.95	<.0001
OTU0660	Proteobacteria	*uncultured*	*tetM*	0.73	1
OTU0660	Proteobacteria	*uncultured*	*tetX*	0.51	1
OTU0451	Proteobacteria	*Unknown_Family_ge*	*tetM*	0.50	1
OTU0367	Proteobacteria	*Vibrionaceae_unclassified*	*tetM*	0.87	0.0837
OTU0367	Proteobacteria	*Vibrionaceae_unclassified*	*tetX*	0.65	1
OTU0022	Proteobacteria	*Xanthobacteraceae_unclassified*	*intI1*	0.54	1
OTU0022	Proteobacteria	*Xanthobacteraceae_unclassified*	*tetM*	0.72	1
OTU0214	Proteobacteria	*Xanthobacteraceae_unclassified*	*tetM*	0.87	0.0837
OTU0214	Proteobacteria	*Xanthobacteraceae_unclassified*	*tetX*	0.65	1
OTU0549	Tenericutes	*Mycoplasma*	*intI1*	0.95	<.0001
OTU0425	Verrucomicrobia	*Verrucomicrobiaceae_unclassified*	*intI1*	0.89	0.0366
OTU0289	Verrucomicrobia	*Verrucomicrobiales_unclassified*	*tetM*	0.87	0.0837
OTU0289	Verrucomicrobia	*Verrucomicrobiales_unclassified*	*tetX*	0.65	1

Table 4.8 (S4). Correlation of host-related gene expression and the distal gut microbiome in Nile tilapia. Correlations were determined using Pearson's correlation algorithm.

OTU	Phylum	Genus	Host Related Gene	Pearson's Correlation	
				R	*p* value
OTU1012	Actinobacteria	*Fodinicola*	*nkap*	0.52	1
OTU1012	Actinobacteria	*Fodinicola*	*sIgT*	0.77	0.1447
OTU0634	Actinobacteria	*Thermoleophilia_unclassified*	*scarb1*	0.68	1
OTU0227	Bacteria_unclassified	*Bacteria_unclassified*	*sIgT*	0.72	1
OTU0285	Bacteria_unclassified	*Bacteria_unclassified*	*sIgT*	0.58	1
OTU0948	Bacteroidetes	*Flavobacterium*	*nkap*	0.52	1
OTU0948	Bacteroidetes	*Flavobacterium*	*sIgT*	0.77	0.1447
OTU0544	Chlamydiae	*Chlamydia*	*sIgT*	0.59	1
OTU1574	Chlamydiae	*Chlamydiales_unclassified*	*nkap*	0.52	1
OTU1574	Chlamydiae	*Chlamydiales_unclassified*	*sIgT*	0.77	0.1447
OTU1871	Chloroflexi	*vadinBA26_ge*	*nkap*	0.52	1
OTU1871	Chloroflexi	*vadinBA26_ge*	*sIgT*	0.77	0.1447
OTU1208	Firmicutes	*Caldicellulosiruptor*	*nkap*	0.52	1
OTU1674	Firmicutes	*Caldicellulosiruptor*	*nkap*	0.52	1
OTU1208	Firmicutes	*Caldicellulosiruptor*	*sIgT*	0.77	0.1447
OTU1674	Firmicutes	*Caldicellulosiruptor*	*sIgT*	0.77	0.1447
OTU0463	Firmicutes	*Geobacillus*	*sIgT*	0.67	1
OTU0160	Firmicutes	*Pelosinus*	*nkap*	0.52	1
OTU0160	Firmicutes	*Pelosinus*	*sIgT*	0.77	0.1447
OTU1761	Firmicutes	*Planifilum*	*nkap*	0.52	1
OTU1761	Firmicutes	*Planifilum*	*sIgT*	0.77	0.1447
OTU1252	Patescibacteria	*Absconditabacteriales_(SR1)_ge*	*nkap*	0.52	1
OTU1252	Patescibacteria	*Absconditabacteriales_(SR1)_ge*	*sIgT*	0.77	0.1447
OTU1067	Patescibacteria	*Candidatus_Falkowbacteria_ge*	*nkap*	0.51	1
OTU1067	Patescibacteria	*Candidatus_Falkowbacteria_ge*	*sIgT*	0.76	0.2012
OTU0354	Patescibacteria	*Candidatus_Kaiserbacteria_ge*	*nkap*	0.50	1
OTU0354	Patescibacteria	*Candidatus_Kaiserbacteria_ge*	*sIgT*	0.76	0.2569
OTU0823	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*nkap*	0.54	1
OTU0892	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*nkap*	0.52	1
OTU1735	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*nkap*	0.52	1
OTU1897	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*nkap*	0.52	1
OTU0405	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*sIgT*	0.70	1
OTU0823	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*sIgT*	0.77	0.1881
OTU0892	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*sIgT*	0.77	0.1447
OTU1735	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*sIgT*	0.77	0.1447
OTU1897	Patescibacteria	*Candidatus_Nomurabacteria_ge*	*sIgT*	0.77	0.1447
OTU1356	Patescibacteria	*Parcubacteria_ge*	*nkap*	0.52	1
OTU1356	Patescibacteria	*Parcubacteria_ge*	*sIgT*	0.77	0.1629
OTU0304	Patescibacteria	*Parcubacteria_unclassified*	*nkap*	0.52	1
OTU0234	Patescibacteria	*Parcubacteria_unclassified*	*sIgT*	0.69	1
OTU0304	Patescibacteria	*Parcubacteria_unclassified*	*sIgT*	0.77	0.1447
OTU0927	Patescibacteria	*Parcubacteria_unclassified*	*sIgT*	0.72	1
OTU1251	Patescibacteria	*Saccharimonadales_ge*	*nkap*	0.51	1
OTU1251	Patescibacteria	*Saccharimonadales_ge*	*sIgT*	0.77	0.1835

Table 4.8 (S4). *Continued.*

OTU	Phylum	Genus	Host Related Gene	Pearson's Correlation	
				R	*p* value
OTU1113	Planctomycetes	*Gemmata*	*nkap*	0.52	1
OTU0262	Planctomycetes	*Gemmata*	*sIgT*	0.50	1
OTU1113	Planctomycetes	*Gemmata*	*sIgT*	0.77	0.1788
OTU0818	Planctomycetes	*Gemmataceae_unclassified*	*sIgT*	0.69	1
OTU0722	Planctomycetes	*Pir4_lineage*	*TLR21*	0.50	1
OTU0984	Planctomycetes	*Pirellula*	*nkap*	0.51	1
OTU0984	Planctomycetes	*Pirellula*	*sIgT*	0.76	0.2163
OTU0857	Planctomycetes	*uncultured*	*sIgT*	0.50	1
OTU1477	Proteobacteria	*Achromobacter*	*nkap*	0.52	1
OTU1477	Proteobacteria	*Achromobacter*	*sIgT*	0.77	0.1447
OTU0168	Proteobacteria	*Acinetobacter*	*sIgT*	0.65	1
OTU0005	Proteobacteria	*Aeromonas*	*TP4*	0.51	1
OTU0417	Proteobacteria	*Amb-16S-1323_ge*	*scarb1*	0.94	<.0001
OTU0043	Proteobacteria	*Crenobacter*	*sIgT*	0.81	0.0176
OTU0043	Proteobacteria	*Crenobacter*	*TLR21*	0.54	1
OTU0137	Proteobacteria	*Curvibacter*	*nkap*	0.51	1
OTU0137	Proteobacteria	*Curvibacter*	*sIgT*	0.76	0.3076
OTU0017	Proteobacteria	*Deefgea*	*nkap*	0.53	1
OTU0017	Proteobacteria	*Deefgea*	*sIgT*	0.78	0.1062
OTU0888	Proteobacteria	*Deltaproteobacteria_unclassified*	*TLR21*	0.50	1
OTU0835	Proteobacteria	*Methylobacterium*	*sIgT*	0.70	1
OTU0431	Proteobacteria	*Nordella*	*sIgT*	0.76	0.2297
OTU1175	Proteobacteria	*Rhodoferax*	*nkap*	0.53	1
OTU1175	Proteobacteria	*Rhodoferax*	*sIgT*	0.78	0.1066
OTU1575	Proteobacteria	*Silanimonas*	*nkap*	0.52	1
OTU1575	Proteobacteria	*Silanimonas*	*sIgT*	0.77	0.1447
OTU1051	Proteobacteria	*Sphingomonas*	*scarb1*	0.95	<.0001
OTU1692	Proteobacteria	*uncultured*	*nkap*	0.52	1
OTU1692	Proteobacteria	*uncultured*	*sIgT*	0.77	0.1447
OTU0037	Proteobacteria	*Undibacterium*	*TP4*	0.60	1
OTU0911	Proteobacteria	*Unknown_Family_ge*	*scarb1*	0.95	<.0001

CHAPTER 5. General Discussion

5.1. Context & Aims

Disease management is among the top priorities for the aquaculture industry as the sector continues its growth in production to support global food security. As such, there has been a considerable rise in the number of studies investigating the influence of antibiotic compounds on the gut microbiome of fish and host-microbiome interactions. However to date, much of our understanding on the effects of antimicrobial compounds on the fish gut microbiome, derives from studies using non-farmed fish species or non-licensed antimicrobial compounds (Brugman et al., 2009; Narrowe et al., 2015; Carlson et al., 2017; He et al., 2017; López Nadal et al., 2018; Zhou et al., 2018a, 2018b). As a result, this PhD thesis therefore aimed to use next generation sequencing and quantitative-PCR methods to explore (i) the distal gut microbiome community dynamics of two farmed fish species in response to a commercially licensed antibiotic treatment, and (ii) how these changes influence host-microbiome interactions and gut health.

In line with the 3Rs framework (Bara & Joffe, 2014), the studies presented in this thesis were designed to use the minimal number of animals required to still provide robust and reproducible data. This is currently a challenge in many microbiome studies due to the high level of inter-individual variability in the gut microbiome community diversity and composition, as demonstrated in a number of terrestrial vertebrate and fish species (Mansfield et al., 2010; Kim et al., 2011; Fjellheim et al., 2012; Stanley et al., 2013; Webster et al., 2018). On a more technical side, protocols which generate 16S rRNA libraries by standardising total genomic DNA (tgDNA) material may influence this variability in the microbiome community, as tgDNA from animal samples will contain varying levels of host and microbial DNA (Feehery et al., 2013). Therefore, before addressing the primary aims of this thesis, chapter 2 set out to determine whether standardising bacterial DNA (bDNA) concentration in 16S rRNA libraries, could improve the characterisation of the distal gut microbiome by reducing the inter-individual variability between samples. Findings from this study were then used to inform the experimental design in the remaining chapters of this thesis. In chapter 3, we set out to explore the short-term longitudinal changes in the microbiome community and expression of key inflammatory cytokine genes within the distal gut of rainbow trout (*Oncorhynchus mykiss*), a cold, freshwater and carnivorous species, in response to and following oxytetracycline (OTC) treatment. This is of particular importance for the rainbow trout sector, which dominates the UK's freshwater aquaculture production (FAO, 2017), as this sector relies on antibiotic treatment for disease management due to limited alternative disease control strategies e.g. vaccines, being available (Brudeseth et al., 2013).

Likewise, OTC was used as the model antibiotic, as this compound is extensively used across the aquaculture industry due to having broad-spectrum activity against both Gram-positive (GPB) and Gram-negative (GNB) bacteria (Leal et al., 2019; Yang et al., 2019). Following the results from the rainbow trout study, additional research was performed as described in chapter 4, to investigate the effect of OTC on the gut health of Nile tilapia (*Oreochromis niloticus*), a globally important tropical, freshwater and omnivorous species (Eknath et al., 1998; Lèveque, 2002; FAO, 2018). In this study, we addressed this aim by profiling the dynamic changes in the microbiome community, in addition to antimicrobial resistance gene (ARG) abundance and the expression of host-related genes within the distal guts of treated fish over time following antibiotic treatment.

5.2. Conclusions

5.2.1. Standardising bacterial DNA does not reduce the inter-individual variability in the fish gut microbiome

In chapter 2, both the tgDNA and bDNA yield recovered from the distal gut digesta was found to vary between individual rainbow trout. This was likely associated with DNA extraction bias between the different samples in this study, as differences in the physiochemical nature of the gut digesta has been reported to affect the recovery of bDNA (McOrist et al., 2002; Thomas et al., 2015). Likewise, the configuration of the microbiome communities present within these samples, which can vary in their microbial load, bacterial distribution and bacterial cell wall properties (Wesolowska-Andersen et al., 2014), may have also influenced the yield of bDNA recovered. In this study, no clear trend was observed between the levels of tgDNA and bDNA recovered from the distal gut digesta samples. However, the samples with the highest recovery of tgDNA were overall found to contain lower yields of bDNA, indicating the some of the variation observed in bDNA recovery may be from the co-extraction of contaminating host genomic material. This was expected as most DNA extraction methods, including that used in this study, are not selective and will extract all genomic material including the host DNA present within a sample (El Bali et al., 2014).

Despite the considerable differences measured in the bDNA concentration between the samples in chapter 2, titrating the bDNA template in 16S rRNA libraries, did not reduce the level of inter-individual variability observed in the diversity or composition of the distal gut microbiome in rainbow trout. In fact, analysis of the beta-diversity between samples revealed that microbiome communities clustered according to fish, with no significant change in beta-diversity distances between fish following titration of bDNA. The results from this study therefore suggested that the inter-individual variability in the distal gut microbiome community of rainbow trout may be more influenced by biological factors, rather than differences in genomic template material. This

follows the current understanding that the fish gut microbiome community can be influenced by endogenous host factors. Such factors include host genetic components and immune status, as these have previously been demonstrated to shape the gut microbiome in several carp species and rainbow trout, respectively (Li et al., 2012; Kelly et al., 2017). These host-associated components could well have varied in fish in the present study, as whilst fish were sourced from one supplier, this farm was rearing multiple stocks of fish from different sources at the time of collection. Thus, fish used in this study may have originated from different genetic populations, resulting in both genetic and phenotypic e.g. immune system variation. Likewise, when the alpha diversity of individual microbiome communities was explored, sample-specific responses to the bDNA titration process were observed which increased the variability in the distal gut microbiome diversity between fish. These sample-specific responses may be due to the method of titration, as the 16S rRNA gene copy number was used as a marker for bDNA concentration. Recent published literature has highlighted a huge diversity in the copies of this gene between the genomes of different bacterial species (Louca et al., 2018). Therefore, titrating bDNA according to 16S rRNA concentration could favour bacteria with a particular range of 16S rRNA genes within their genome. As individual fish can already vary in their microbiome community due to factors described above amongst others, this method of genomic template standardisation may further increase this inter-individual variability through artificial selection and enrichment of particular taxa. Further work is therefore required to explore how inter-individual variability can be reduced in gut microbiome studies, perhaps through biological pathways or better experimental design as describe in section 5.3. Nonetheless, this study demonstrated the potential of titrating bDNA in fish gut microbiome research through (i) increased sequencing yield and (ii) reduced influence of foreign microbial DNA contamination, which could improve the characterisation of the fish gut microbiome. As a result, this approach was used for the remaining studies in this thesis.

5.2.2. A core gut microbiome community exists within different farmed fish species, but the composition is host-specific

The results presented in this thesis demonstrated the existence of a core gut microbiome community within fish, as members from the Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria phyla, were detected in the distal gut microbiomes of fish from both species in this PhD study. This is in agreement with previous studies investigating the gut microbiome communities of Nile tilapia (Souza et al., 2020) and rainbow trout (Michl et al., 2017). Bacteroidetes, Firmicutes and Proteobacteria have also been found to dominate the gut microbiomes of animals from other vertebrate lineages including the classes amphibia, aves, reptilia and mammalia (Hird et al., 2015; Rosshart et al., 2017; Zhang et al., 2019; Zhou et al., 2020). These patterns thus suggest a core gut microbiome community across all vertebrate animals. The presence of a core gut microbiome community in fish was first proposed by

Roeselers et al., (2011), who reported similar microbiome community memberships between laboratory-reared and wild zebrafish (*Danio rerio*) individuals. However, since then, numerous studies have reported the aforementioned phyla to represent more than 90% of the gut microbiome community in individuals from across the teleost lineage, thereby strengthening this hypothesis (Talwar et al., 2018). The common occurrence of these phyla across fish species in this study indicated a strong functional importance within the fish host, which agrees with other reports highlighting their role in the health and physiology of fish (Kihara & Sakata, 1997, 2002; Kanther et al., 2011; Li et al., 2013; Nie et al., 2017; Tan et al., 2019).

Both fish species investigated in this study also displayed low phylogenetic diversity in that their distal gut microbiomes were often dominated by one particular genus, which represented >70% of the total sequence abundance in pre-treated fish. The low phylogenetic diversity could be a concern for aquaculture, as diverse microbiome communities are thought to be more resilient to environmental stressors (Lozupone et al., 2012), thus the gut microbiome of these farmed fish species may be more susceptible to alterations through husbandry practices. Despite the detection of a core set of microbial phyla in the distal gut microbiome of both fish species, species-specific compositions were noted between Nile tilapia and rainbow trout in this study. In chapter 3, the distal gut of rainbow trout was found to be dominated by *Mycoplasma*, and follows previous findings for this species (Brown et al., 2019; Mora-Sánchez et al., 2020). Despite the dominance of *Mycoplasma* within the guts of many salmonid species, the function of this taxa within the fish host is still not fully understood, however a symbiotic relationship between *Mycoplasma* and the salmonid host has recently been proposed (Cheaib et al., 2020). In comparison, the distal gut microbiome community of Nile tilapia in chapter 4 was dominated by *Cetobacterium*, which again were in agreement with previous investigations with this and other freshwater fish species, where it is thought to play a role in vitamin production for the fish host (Ramírez et al., 2018; Chang et al., 2019; Suphoronski et al., 2019).

Differences in the dominant bacterial taxa between fish species may have resulted due to variances in the environmental conditions at the aquaria sites used in these chapters, such as water temperature or the surrounding water microbiome, as these have been previously reported to steer the gut microbiome community in fish (Zarkasi et al., 2014; Giatsis et al., 2015). However, on closer inspection, it was found that *Mycoplasma* and *Cetobacterium* were not present in high sequence abundance (<1%) within the tank biofilms at respective aquaria sites, therefore this theory seems unlikely. Instead, disparities in each species' biology may have resulted in the different distal gut microbiome community structures observed between fish species. For example, in the wild, Nile tilapia and rainbow trout display different feeding habits at distinct trophic levels, which have both been suggested to exert strong selective pressures on the gut

microbiome community (Desai et al., 2012; Sullam et al., 2012; Ingerslev et al., 2014). However, in the studies described in this thesis, the source and content of food items was controlled by giving a commercial diet to fish. Whilst the commercial diets did differ between each study, when the diet-associated microbiome communities were profiled, the two dominating genera within the distal guts of fish were only detected in low sequence abundance (<1%) within the respective diets. Therefore, further research would be required to better understand the bacterial sources of the distal gut microbiome in fish from these studies. One possibility for the difference in microbiome composition would be host-specific and genetic-based selection, as demonstrated by Li et al., (2012). This is likely, as host genetic components can modulate the gut morphology and physiology of fish, which can interact to shape the microbiome community (Li et al., 2014). Thus, amongst other host factors, differences in the anatomical structure and physical environment of the gut between the fish species investigated, may have in turn contributed to the differences in the microbiome community. Indeed, Nile tilapia and rainbow trout display distinct gut morphologies to suit the requirements of their respective feeding habits and food types, thus they may comprise different attachment sites for different commensal bacteria. In general, carnivorous species such as rainbow trout display a short, simple intestinal tract, whereas omnivorous species e.g. Nile tilapia have a longer, more complex intestinal tract due to the additional digestion requirements of the complex plant components within their diet (Kramer & Bryant, 1995; German & Horn, 2006; Çalta, 2016).

Across the fish species used in this PhD study, a number of genera which comprise economically important bacterial pathogens, were consistently detected within the distal guts of fish. For example, *Aeromonas* was detected in the distal gut of both rainbow trout and Nile tilapia individuals in chapters 3 & 4. This genus encompasses a wide range of fish pathogens, including *Aeromonas salmonicida* and *Aeromonas hydrophila*, which are the aetiological agents of furunculosis and motile *Aeromonas* septicaemia (MAS), respectively in farmed fish (Austin & Austin, 2012). Likewise, the *Nocardia* genus which contain the aetiological agents of fish nocardiosis (Chen et al., 2019a), was detected in the distal gut microbiome community of Nile tilapia in chapter 4. The presence of these genera, amongst others within the distal guts of apparently healthy and pre-treated fish, support the working hypothesis that potentially pathogenic bacteria may form part of the natural gut microbiome community in these particular fish species. This follows previous culture-dependant and culture-independent analysis which revealed that many opportunistic bacterial pathogens frequently occur as components of the normal microbiome in fish, along with non-pathogenic and commensal bacteria. For example, *Vibrio anguillarum*, which is one of the aetiological agents of vibriosis in fish (Mohamad et al., 2019), has been detected in the gut microbiome of healthy hatchery-reared Atlantic cod (*Gadus morhua*) larvae (Reid et al., 2009). Likewise, *Aeromonas sobria* another causative agent of MAS,

has been identified as a resident community member in the gut microbiome of farmed brown trout (*Salmo trutta*) (Al-Hisnawi et al., 2015). Similar findings have also been reported in other vertebrate animals, such as the detection of the chicken pathogen *Campylobacter jejuni* in the faecal material of apparently healthy individuals at commercial broiler farms (Kaakoush et al., 2014).

Under homeostatic conditions, it is possible that pathogenic communities are kept under control by commensal members within the gut microbiome community through direct commensal-pathogen interactions. Such interactions include the production of toxic secondary metabolites e.g. antimicrobials by commensal bacteria, in response to microbial competition with opportunistic pathogens or other commensal bacteria within the microbiome. In fish, several antimicrobial compounds have been isolated and characterised from members of the gut microbiome community, including 1-hydroxyphenazine from *Pseudomonas monteilii* within the guts of grass carp (*Ctenopharyngodon idella*), as well as several bacteriocin-like compounds from various *Lactobacillus* species in the guts of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) (Ghanbari et al., 2013; Qi et al., 2020). Furthermore, members of the commensal community can also compete with the residing opportunistic pathogens for the available bindings sites and nutrients within the gut (Buffie & Pamer, 2013). However, in the event of dysbiosis when these commensal-pathogen interactions become disturbed, the new conditions may permit the opportunistic bacteria to establish an infection. This has been previously shown in farmed Ayu (*Plecoglossus altivelis*) during an experimental *V. anguillarum* infection, whereby diseased individuals displayed disturbed microbiome communities, with less co-operative and complex interspecies interactions (Nie et al., 2017). It is accepted that the analysis presented in this thesis only went to genus level, therefore we were unable to report on whether particular bacterial pathogens were present in the distal gut microbiome community of fish. In future work studying the gut microbiome of fish, it would be advantageous to employ the use of shotgun metagenomics, as this would allow complete 16S rRNA genes to be annotated and subsequent classification down to species or even strain level (Mas-Lloret *et al.*, 2020). Nonetheless, the detection of particular genera which contain opportunistic pathogen members, raises concerns for the aquaculture industry, as common husbandry practices may alter the microbiome community network, increasing the likelihood of disease outbreaks within the production system.

5.2.3. The gut microbiome community of fish can shift throughout time

Results from this study highlight the level of temporal variability that can exist within the microbiome community of fish, as the distal gut microbiome was observed to vary across time in the control groups of the studies presented in chapters 3 & 4. Temporal variability can arise through the types of samples collected in fish gut microbiome studies, as the digesta-associated

microbial community, which was profiled in both studies, has been described to be more transient than those associated with mucosal surfaces (Gajardo et al., 2016). Furthermore, this community has been suggested to fall under great influence from external environmental conditions (Tarnecki et al., 2017). In chapter 3, further inspection of environmental conditions at the aquaria site revealed tank water temperature to decline from 4°C at day 0, to 1.6°C by day 22 of the study. Thus, findings from this study indicate that temperature can influence the distal gut microbiome community of rainbow trout, which is in agreement with a previous study in Atlantic salmon (*Salmo salar*) (Zarkasi et al., 2014). Temperature-induced shifts in the microbiome community are likely associated with the ectothermic nature of fish and the inability to regulate their internal body temperature (Coutant, 1976). All bacteria have a thermal range for microbial growth, which is related to the thermodynamics of metabolism, and the effect of temperature on enzyme activity or microbial-mediated reactions within bacterial cells (Harrison et al., 2015; Großkopf & Soyer, 2016). As the environmental temperature changes, temperatures may fall outside the optimal threshold of particular bacterial species, severely impacting their microbial growth. This ultimately leads to a competitive advantage for other bacterial species which have optimal growth at the new temperatures to outcompete existing bacteria, thus causing a shift in the microbial community of the respective environment. In line with this theory, it is possible that the changes in the tank water temperature could have played some role in the temporal variability observed in the distal gut microbiomes of control fish in chapter 3, as the internal gut temperature of fish likely reflected that of the tank water. This is supported by the previous work from Naviner et al., (2006), who reported an increase in Firmicutes abundance within the gut microbiomes of farmed rainbow trout reared at colder temperatures, as similar findings were observed within the distal guts of control fish between days 0 and 22 in this study. Likewise, in another study exploring the gut microbiome of rainbow trout, a negative relationship was observed between Firmicutes abundance and rearing temperature, with lower abundances of lactic acid bacteria e.g. *Lactobacillus* species, in the guts of fish reared at higher temperatures (Huyben et al., 2018). With this in mind, future studies would greatly benefit from more control in environmental conditions, to limit the influence of external variables on the fish microbiome community and improve the clarity of trends between biological treatment groups.

The longitudinal sampling approach taken in chapter 3 resulted in stocking densities reducing from n=15 fish tank^{-1} at day 0, to n=5 fish tank^{-1} by day 15. As stocking density has previously been reported to alter the gut microbiome communities of other fish species such as blunt snout bream (*Megalobrama amblycephala*) and gilthead sea bream (*Sparus aurata*) (Du et al., 2019; Parma et al., 2020), it is therefore possible that changes in stocking density may also partly explain the temporal changes observed in the distal gut microbiome of rainbow trout in this PhD study. Stocking density is a source of stress (Parma et al., 2020), which can potentially stimulate changes

in the gut microbiome community of fish through a variety of mechanisms. Firstly, stress has been demonstrated to induce the release of glucocorticoids e.g. cortisol, which play a critical role in mediating the physiological adaptations of fish in response to stressful stimuli (Sánchez-Vázquez et al., 2019). Such physiological changes may affect the microbiome community indirectly, as many of these systems e.g. the immune system, have been demonstrated to regulate the gut microbiome of fish under normal homeostasis (Kelly & Salinas, 2017). Secondly, stress-induced cortisol release has also been shown to directly influence both the diversity and composition of the gut microbiome in Atlantic salmon (Uren Webster et al., 2020). As low stocking densities have previously been shown to induce cortisol release in rainbow trout (North et al., 2006), it is therefore possible that reducing stocking densities throughout the trial in chapter 3 may have been associated with increasing levels of cortisol within the distal guts of fish, thus stimulating the observed changes in the abundance of particular taxa. Whilst Uren Webster et al., (2020) were unable to determine exactly how cortisol may affect different taxa within the microbiome community, they proposed several hypotheses which may have played a role in this chapter, such as the potential inhibitory mechanisms of cortisol through direct toxicity and disruption to metabolism and ion-regulation within bacteria cells. Finally, stress has also been associated with the cessation of feeding in fish (Leal et al., 2011). Feeding behaviour is an essential regulator of the gut microbiome, as it provides an important source of bacteria for colonisation as well as nutritional substrates for use by the existing members within the microbiome community (Smith et al., 2015; Egerton et al., 2018). Therefore, if fish in this study underwent periods of stress-induced fasting in response to changes in stocking density, the lack of food intake may have removed an essential source of bacteria or nutrition for the existing microbiome community, thereby inducing shifts in the distal gut microbiome of fish through changes in the community dynamics and the complex microbial food web. This would support findings from previous studies which have demonstrated that starvation or fasting can induce shifts in the gut microbiome of grass carp and Asian seabass (*Lates calcarifer*) (Xia et al., 2014; Tran et al., 2018). Whilst the feeding response of individual fish was unable to be monitored in chapter 3, these theories highlight the advantages of utilising larger stocking densities of fish in future longitudinal gut microbiome studies, to minimise the aforementioned effects of stocking density changes in fish and their gut microbiome communities.

Temporal variability was also noted in the distal gut microbiome community of Nile tilapia in chapter 4. However, unlike the study conducted in chapter 3 which relied on a flow-through system and group stocking in tanks, fish in chapter 4 were stocked into individual tanks that were operated on a recirculatory system whereby tank conditions e.g. water temperature were tightly controlled. With this in mind, it is unlikely that the temporal shifts in the distal gut microbiome community of fish in chapter 4 were related to changes in the tank water temperature or stocking

density. The study conducted in chapter 4 did however utilise a mixed sex population of fish and on numerous occasions throughout the study, female fish from both treatment groups were observed to spawn. Due to the mouthbrooding nature of Nile tilapia (Barreto et al., 2003), this meant that spawning events in this study were accompanied by a period of fasting in some fish which lasted at least 1.5 days. Therefore, the lack of feeding in some fish prior to each sampling point may have influenced the temporal variability observed in the distal gut microbiome, due to the effects of starvation described previously. Whilst the effects of spawning and starvation on distal gut microbiome communities were not investigated in more detail in this study, the challenges encountered do highlight the benefits of using monosex male populations of Nile tilapia in gut microbiome studies, to remove the potential bias induced by spawning and the associated lack of feeding.

5.2.4. Treatment with a licensed antibiotic compound can alter the gut microbiome diversity of farmed fish species

The effect of a single antibiotic compound was not similar between the cold-water and tropical fish species investigated in this study, as the distal gut microbiome communities of rainbow trout and Nile tilapia displayed contrasting longitudinal responses in diversity following treatment with OTC. More specifically, the distal gut of rainbow trout was found to increase in operational taxonomic unit (OTU) richness over time following OTC treatment, whereas a decrease in the community richness was found in the distal gut microbiome of Nile tilapia following antibiotic treatment. Whilst there should be caution when comparing data from different studies due to the effects of technical bias (Antosca et al., 2020), these findings reflect those of other studies. For example, low level concentrations of OTC at 420ng L^{-1} have been reported to reduce the gut microbiome community richness in zebrafish (Zhou et al., 2018a), however the same concentration of OTC was found to have the opposite effect on the gut microbiome in Nile tilapia (Limbu et al., 2018). Whilst evidence from the study by Limbu et al., (2018) contradicts findings presented in this thesis, this disparity may be related to the different concentrations of OTC used in the respective studies as described below. Nonetheless, these findings are important to the aquaculture industry which produces a vast diversity of fish species, as they demonstrate that antibiotic treatment guidelines should be species specific and a “one size fits all” approach should not be adopted across the industry. Therefore, to better inform these guidelines, further investigation should be conducted to better understand the gut microbiome response of individual fish species to OTC and other antibiotic compounds.

The contrasting effects of OTC on the distal gut microbiome communities of Nile tilapia and rainbow trout could be intensity-dependant, as both studies investigated different concentrations of the antibiotic compound. The study presented in chapter 3 used a subtherapeutic level of OTC

at 35 mg kg bodyweight^{-1}, leading to increases in the alpha diversity of the distal gut microbiome in rainbow trout over time. However, the OTC treatment at 100 mg kg bodyweight^{-1} used in chapter 4 was associated with reduced diversity within the distal guts of Nile tilapia over the same time frame. These findings offer support to the intermediate disturbance hypothesis (Connell, 1978). In this hypothesis; community diversity often increases within an ecosystem experiencing intermediate disturbance intensity, as both the colonisers and competitors can co-exist. However, diversity often declines during periods of low or high intensity, as only the competitors or colonisers can exist, respectively. Thus in these studies, the subtherapeutic concentration of OTC in chapter 3 may have reflected an intermediate disturbance on the distal gut microbiome of rainbow trout, whereas the high concentration of OTC used in chapter 4, induced a high disturbance intensity on the distal gut microbiome of Nile tilapia. Although limited information is available on the effect of OTC on the gut microbiome community in fish, the metabolic profile of the human gut microbiome has been demonstrated to have a dose dependent response to tetracycline antibiotics (Keerthisinghe et al., 2019). Despite the gut microbiome community not being profiled in the study by Keerthisinghe et al., (2019), it is likely that these changes in bacterial metabolism were related to dose-dependant changes in the gut microbiome diversity and membership, thus similar responses could occur in fish.

As described previously, whilst the distal guts of Nile tilapia and rainbow trout displayed similar core microbiome community membership, their compositions were markedly different. Thus, the effect of OTC on the distal gut microbiome communities of fish in this PhD study may have been community dependant, as different bacterial groups may respond differently to OTC treatment. In fact, GPB and GNB have been reported to display different patterns of antibiotic sensitivity, as a result of differences in their cell envelope structure (Figure 5.1). For example, the direct exposure of the peptidoglycan layer of GPB to the external environment, makes this group of bacteria particularly sensitive to compounds which target the cell wall e.g. beta-lactam antibiotics (Davis, 2018). In comparison, the cells of GNB have an additional protective layer in the form of an outer lipopolysaccharide membrane, which is mosaiced with porins and other outer membrane proteins making the cells highly impermeable (Miller & Salama, 2018). This outer membrane protects the cells from toxic compounds within the external environment, and therefore provides additional protection from antibiotic compounds. As a result, GNB are generally considered to have a higher resistance to antibiotics compared with GPB (Danilova et al., 2020). However, given that the distal guts of both fish species in this PhD study were dominated mostly by GNB, this hypothesis does not fully support the observed differences in microbiome diversity in this thesis. Despite this, the above hypothesis does further highlight the need to explore the gut microbiome responses of different farmed fish species to antibiotic compounds, as certain fish species that have higher proportions of GPB in their gut microbiome e.g. herbivorous fish (Das

et al., 2014), may be more susceptible to microbiome community disturbances through antibiotic treatment.

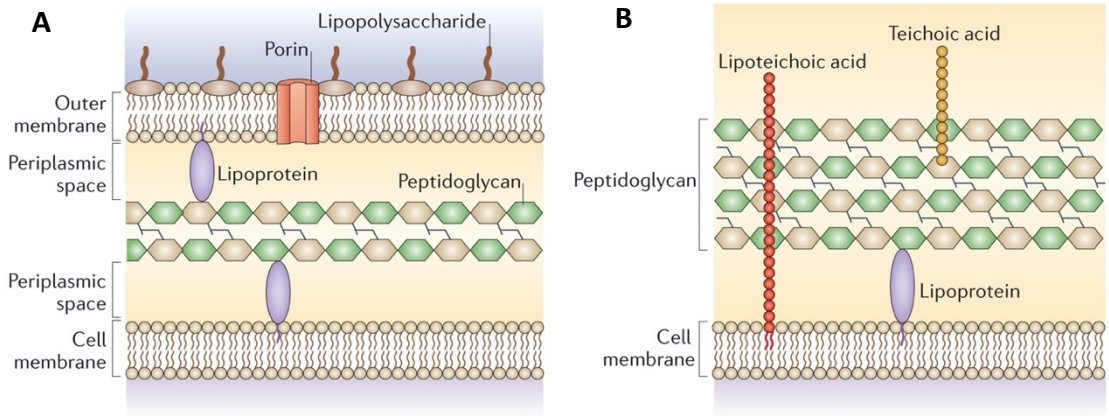

Figure 5.1. The cell envelope of Gram-negative (A) and Gram-positive (B) bacteria. Taken from Brown et al., (2015).

5.2.5. Treatment with a licensed antibiotic compound can induce shifts in the gut microbiome composition of farmed fish species

Findings from this thesis also demonstrated that OTC treatment can disturb the distal gut microbiome composition of two farmed fish species. The distal gut microbiome communities of both fish species were observed to undergo successional changes following antibiotic treatment and throughout the withdrawal period, although the patterns of succession differed between Nile tilapia and rainbow trout. In chapter 3, OTC was associated with the suppression of *Mycoplasma* within the distal guts of rainbow trout, accompanied by an enrichment in Proteobacteria members including *Aeromonas*, *Deefgea* and *Pseudomonas*. However, following antibiotic treatment, the distal gut microbiome of treated fish was found to undergo successional changes leading to communities which were more diverse compared with pre-treated fish or the control group. In contrast, antibiotic-induced restructuring of the distal gut microbiome in Nile tilapia in chapter 4, lead to a community with much lower diversity compared with pre-treated or control fish by the end of the study period. This was mainly associated with *Cetobacterium*, which had higher representation within the distal guts of treated fish compared with the control group by the end of the antibiotic treatment. Furthermore, this genus steadily increased in sequence abundance throughout the withdrawal period, accompanied by a decline in the abundances of other core bacterial taxa including those belonging to Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Collectively, these findings are in agreement with previous studies that showed successional changes in the gut microbiome of 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).

Bacterial succession in the distal gut microbiome of treated fish in this study, may have been associated with alterations in niche availability as a direct result of antibiotic-induced changes in bacterial membership. For example, the decline in dominant taxa e.g. *Mycoplasma* during the OTC treatment in the rainbow trout study, may have increased the availability of attachment sites or niches for occupation by other communities e.g. Proteobacteria members, which survived through resistance mechanisms. This trend is supported by a general consensus that members of the Proteobacteria phylum display fast growing, r-strategist life-histories (Brzeszcz et al., 2016). In biofilms and other microbial ecosystems, r-strategist bacteria are usually the first to colonise newly exposed surfaces, as they exhibit characteristics that allow them to thrive in unstable or unpredictable environments (Verschuere et al., 1997; Hoppert & König, 2006). This life history trait would also fit with the community changes that occurred following withdrawal of OTC in rainbow trout, as a number of Proteobacteria genera became outcompeted at day 10 by taxa from other bacterial phyla. This could have occurred through natural bacterial succession processes, as the build-up of toxic compounds and resource limitation which occurs during the rapid growth of r-strategists, enabled slow-growing competitive bacteria displaying K- life strategies e.g. Firmicutes, to outcompete and replace early successional groups (Ringelberg et al., 2008; Rui et al., 2009). On the other hand, bacterial succession in the microbiome may have resulted indirectly through antibiotic-induced morphological changes within the distal gut environment of treated fish. Such changes, including the disruption of the lamina propria and mucosal folds, can modify the attachment sites on mucosal surfaces for the commensal bacteria and therefore stimulate changes in the microbiome community (Ringø & Gatesoupe, 1998). This theory seems likely given that OTC has been shown to alter the intestinal villi width and muscularis thickness in Nile tilapia (Limbu et al., 2018), thus similar changes could have occurred in the distal guts of Nile tilapia as well as in rainbow trout in this PhD study.

Findings presented in this thesis highlight the need to further investigate how the gut microbiome community recovers following antibiotic treatment, and how this can be managed successfully within the farming system to benefit the health and production of farmed fish. In chapter 3, treated rainbow trout were found to utilise the resident bacterial community associated with the feed pellets, as *Chloroplast_ge* (Cyanobacteria phylum) which dominated this community became established in the distal gut microbiome of treated fish at the conclusion of the experiment. This finding supports existing evidence that demonstrates the role of the diet in manipulating the gut microbiome community of fish (Kashinskaya et al., 2018; Antonopoulou et al., 2019). Establishment of the diet-associated Cyanobacteria communities within the distal guts of OTC-treated fish could have resulted from deterministic processes, and as such were selected for by the fish host during recovery to replace certain lost communities and their associated functions. As the Cyanobacteria phylum has previously been reported to have high abundance in the guts of

other freshwater fish species such as silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*) (Liu et al., 2016), members of this particular phylum may serve an important role within the fish host. Therefore, selection of this phylum during gut microbiome recovery in rainbow trout following antibiotic treatment could be likely. However, the establishment of Cyanobacteria may have also occurred through ecological and stochastic processes. In this scenario, the diet-associated microbial communities randomly colonised the distal guts of treated fish due to the increased availability of unoccupied niches/adhesion sites following OTC treatment. This is possible as Cyanobacteria could survive the harsh conditions within the gut environment, as evident in the detection of this phylum within the gut microbiome of several fish species including channel catfish (*Ictalurus punctatus*), gizzard shard (*Dorosoma cepedianum*), grass carp, and freshwater carp (*Labeo rohita*) (Ye et al., 2014; Burgos et al., 2018; Tyagi et al., 2019). These communities could then have become established during the microbiome recovery period, as a result of poor direct colonisation resistance and antibiotic-induced disturbances in the co-operative interaction pathways between the commensal bacteria of the gut microbiome. 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. Disruptions in colonisation resistance may have also been exacerbated by suboptimal environmental conditions. The immune system and microbiome can interact to provide an integrated defence system (Kitano & Oda, 2006). Therefore, along with disturbances in direct colonisation resistance mechanisms, the reduced immunological activity of fish resulting from the low tank water temperatures observed in this study (see section 5.2.7.), may have allowed Cyanobacteria to establish in the distal gut microbiome of treated fish through disturbances in indirect colonisation resistance pathways. Ecological processes could have also been in play in chapter 4 during gut microbiome recovery in Nile tilapia following antibiotic treatment. However, instead of utilising the environmental sources of bacteria, the disturbed microbiome communities within the distal guts of treated Nile tilapia recovered using the remaining endogenous populations that had not been completely wiped out during antibiotic treatment. As *Cetobacterium* already dominated the microbiome community within the distal guts of pre-treated fish, OTC-induced disruptions in ecological interactions could have allowed this genus to overpopulate where it made up > 90% of the total sequences from the distal gut digesta of treated fish by the end of the study.

Together, findings from these studies provide important evidence which could better inform antibiotic management practices within aquaculture. The establishment of the diet-associated bacterial communities following antibiotic treatment as observed in chapter 3, could be beneficial during the rearing of fish larvae and juveniles. Throughout the early stages of production, disease outbreaks and subsequent antibiotic use can be high. This is likely associated with the fact that

the immune response of fish at early developmental stages is immature and thus not fully functional (Faruk & Anka, 2017). However, if the diet can be used as a platform for tailoring gut microbiome recovery following antibiotic treatment, aquafeed could be formulated with a bespoke community of desired bacteria. This community could then colonise the fish gut and have potential benefits during later stages of production. For example, this route may allow for the manipulation of the gut microbiome towards a "healthy" community, or one that has the desired functional potential e.g. growth or immune promotion (Jurado et al., 2018; Chen et al., 2019b). However, before this can be investigated, further exploration is required in the hunt for what a "healthy" microbiome may look like in fish, and how this varies across the many fish species currently farmed within the industry. In addition, this form of intervention may only be valuable during the early stages of production, as deterministic and host-selection processes are strongest in larvae/juvenile fish (Yan et al., 2016; Li et al., 2017). In the later stages of development, drift and stochastic processes have a greater influence on the microbiome community landscape. As a result, similar patterns of gut microbiome recovery as reported in chapter 4 may be observed in adult farmed fish following antibiotic treatment. If the random colonisation and establishment of bacteria does occur during microbiome recovery, farmers would need to ensure that the microbiome communities of both the fish and wider production environment e.g. diet and surrounding water, were not already compromised prior to antibiotic treatment. This would be imperative as otherwise, gut microbiome recovery following antibiotic treatment could lead to the proliferation and dominance of "bad" bacteria, such as opportunistic pathogens or those which do not promote beneficial microbiome functioning. Establishment of these communities would have detrimental consequences for the future health and production of the farmed fish. Nonetheless, temporary fortification of the gut microbiome after a disturbance e.g. antibiotic treatment, through specially formulated aquafeed in adult fish, may still be beneficial in order to reduce the niche availability for potential invading pathogens.

5.2.6. Antibiotic treatment can promote antimicrobial resistance within the fish gut microbiome

In this PhD study, a number of Proteobacteria genera were found to be associated with OTC and increased in sequence abundance within the distal guts of Nile tilapia and rainbow trout fish, either during or at the end of antibiotic treatment. This was in agreement with previous studies in fish which also reported increases in Proteobacteria following antibiotic treatment with OTC (Navarrete et al., 2008; Tapia-Paniagua et al., 2015). It was hypothesised that the increase in abundance for some of these taxa may be related to the presence of ARGs, as genes encoding for tetracycline resistance have been reported in several Proteobacteria members isolated from fish or aquaculture environments (Agersø et al., 2007; Dubert et al., 2016). This hypothesis was tested in chapter 4 whereby the abundance of four ARGs; three of which encode for tetracycline resistance, were quantified in the distal gut digesta of Nile tilapia before and after antibiotic

treatment. In this study, the efflux pump encoded by the *tetA* gene was found to be highly correlated with *Plesiomonas*, as both increased in abundance within the guts of treated fish following OTC treatment. In addition, the genes which encoded for a ribosomal protection protein (*tetM*) and enzymatic modification of tetracycline (*tetX*), were also found to be strongly associated with Actinobacteria members including *Nocardia*, as they all increased in abundance at some point following OTC treatment. Together, these results demonstrated that antibiotic treatment with OTC at 100 mg kg bodyweight^{-1} can induce selective pressures in the distal gut microbiome of fish in favour of resistant populations. This can potentially be applied to farmed fish in production systems, as OTC is used at similar doses within the industry (Limbu et al., 2018). This would be in agreement with other production sectors where antibiotic use has been associated with changes in ARG abundance, such as in broiler chickens (*Gallus gallus*), feedlot cattle (*Bos taurus*) and domestic pigs (*Sus scrofa*) (Looft et al., 2012; Xiong et al., 2018; Holman et al., 2019).

The evidence in this study raises concerns for the aquaculture industry, as farmed fish can receive multiple antibiotic treatments throughout the production cycle. Therefore, the selection of antimicrobial resistance (AMR) within the gut microbiome from previous antibiotic exposures, may reduce the effectiveness of future treatments with the same compound. Findings from this study also raise another important issue that needs to be addressed under the One Health paradigm (White & Hughes, 2019). The establishment of resistant communities within the gut microbiome of farmed fish is a potential public health challenge, as it may allow the transfer of AMR or resistant populations to human-associated microbiome communities, through the ingestion of contaminated fish products. This threat is a real possibility as evident by the previous recovery of resistant human pathogens including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates from farmed Atlantic salmon and catfish (*Pangasius hypophthalmus*) food products imported into Switzerland (Boss et al., 2016). Likewise, Rezai et al., (2018) recently isolated a number of isolates belonging to the food-borne pathogens *Listeria monocytogenes* and *Listeria ivanovii* from farmed and retail rainbow trout products in Western Iran. Among those isolates recovered, a high percentage were found to be resistant to tetracycline, enrofloxacin and ciprofloxacin antibiotic compounds. Whilst the promotion of AMR in the gut microbiome of farmed fish would not be an immediate concern for people who eat processed products such as fillets, this would be a concern for people in countries which consume whole fish such Bangladesh, Cambodia and several Southern Mediterranean countries (Martinsdóttir et al., 2008; Thilsted, 2012).

In the same study presented in chapter 4, all four ARGs were observed in the distal gut digesta of fish prior to OTC treatment. Given that these fish had never previously received any antibiotic

treatment before this study, detection of these genes in the distal gut microbiome was alarming, as this finding highlighted the potential role played by the gut microbiome as an AMR reservoir. In fish, the larval gut is thought to be sterile upon hatching, and can subsequently be colonised by the microbial communities present within the surrounding environment (Wang et al., 2018). Thus, it is possible that bacterial communities within the recirculation system of the aquaria site, already possessed these particular ARGs through a history of antibiotic treatment. Furthermore, these communities were able to colonise the immature guts of Nile tilapia during development of the gut microbiome. This theory is likely as the distal guts of fish and tank biofilms in this study shared several OTUs in common. In addition, all four ARGs investigated in this study were also detected in the tank biofilms and main biofilter unit within the aquarium. Likewise, the reproductive physiology of this fish species may have also aided in the transmission of AMR in the developing gut microbiome of individuals in this study. Nile tilapia are one of the many cichlid species which display mouthbrooding behaviour. Following spawning, female individuals will incubate fertilised eggs in their mouths until the larvae are released at swim-up fry stage (Barreto et al., 2003). As the egg-associated microbiome community is also thought to facilitate the initial colonisation of the gut microbiome in fish (Egerton et al., 2018), members with tetracycline ARGs within the maternal buccal cavity microbiome may have transferred onto the egg surface, where they subsequently were able to colonise the distal guts in developing fish. This would be in agreement with the transfer of ARGs through the vertical transmission of maternal microbiome communities to offspring, that has been reported for other vertebrate animals such as humans (de Vries et al., 2011). However, at the time of writing this thesis, the vertical transmission of gut microbiome communities in fish remains to be explored. Despite this, findings from this study highlight the need to further investigate gut microbiome colonisation and establishment in farmed fish species. Particularly if this process is accompanied by the development of AMR from the surrounding production environment, as a result of previous antibiotic exposure history.

5.2.7. Antibiotic treatment has the potential to disturb fish gut health through changes in host-microbiome interactions

It is known that the gut microbiome plays a vital role in the health and physiology of the fish host through various microbial-mediated functions. Due to the considerable changes in the distal gut microbiome community of rainbow trout following OTC treatment in chapter 3, it was hypothesised that such antibiotic-induced changes may lead to alterations in gut health through disruptions in host-microbiome interactions. Findings from this chapter demonstrated that OTC did not have an effect on the expression of key inflammatory cytokine genes (*IL-1β* and *TGF-β*) within the distal gut tissue of rainbow trout. However, both cytokine genes investigated were found to decline in expression within the distal guts of fish from both treatment groups between days 0 and 22. It was postulated that findings may be associated with tank conditions at the aquaria

site, as the tank water temperature was observed to decline between days 0 and 22 (see section 5.2.3). This hypothesis is supported by previous studies in rainbow trout or rainbow trout cell lines, which collectively found that both pro-inflammatory and anti-inflammatory cytokine gene expression can be suppressed at water temperatures below 5°C (Zou et al., 2000; Raida & Buchmann, 2007). As a result, further investigation would be required in rainbow trout reared at optimal water temperatures, to gain a better understanding of the implications of antibiotic-induced changes in the gut microbiome on host-microbiome interactions and host physiology.

We therefore sought to investigate the effect of OTC on host-microbiome interactions further in chapter 4 using Nile tilapia, which were reared close to water temperatures considered optimal for this species' growth and physiology (El-Sayed & Kawanna, 2008). Due to previous evidence demonstrating that the gut microbiome supports other aspects of fish gut health including acquisition of dietary nutrients and modulating gut morphology (Wu et al., 2015; Asaduzzaman et al., 2018), in this study we quantified the absolute expression of host genes related to immunity, as well as digestion and gut barrier integrity within the distal gut tissue of fish. Despite observing changes in the gut microbiome community of Nile tilapia following antibiotic treatment, OTC was not found to significantly affect the gene expression of *slc2a6*, which encoded for a glucose transporter protein, within the distal gut tissue of treated fish. In addition, the gene expression of *slc2a6* was not found to be correlated with the sequence abundance of any OTU within the distal gut microbiome of Nile tilapia. These results would indicate that the high concentration of OTC was not sufficient to disrupt glucose metabolism within the distal guts of treated fish, either directly or through changes in host-microbiome interactions. Similar findings were also found for gut integrity, as the expression of the gene *atp1b1* which encoded for a Na/K-transporting ATPase subunit and essential for osmoregulation, was not found to be significantly different between OTC and control groups. These findings contradict that found previously for Nile tilapia in which OTC at low and aquaculture relevant concentrations, were reported to disturb gut integrity and glucose metabolism in treated fish, potentially through changes in the gut microbiome (Limbu et al., 2018). Whilst the full genome of Nile tilapia has been published (Conte et al., 2017), the genome has not been well characterised and thus much of the genomic sequence data available for designing primers is unconfirmed. As a result, although we had initially selected a large panel of target genes to monitor the changes in the distal gut integrity and digestion/metabolism of fish over time, the final panel comprised of one gene for each pathway following the primer design and optimisation process. It is therefore likely that monitoring the expression of more genes e.g. through transcriptomic approaches, which can be used to monitor global gene expression of the host and microbiome, would have generated a better picture of how OTC may disrupt gut health, through changes in host-microbiome interactions involved in the gut integrity and metabolism of

the fish host. However, as highlighted by this study, there is an urgent need to better characterise the genome of Nile tilapia for such molecular-based studies before they have any real value.

Similar findings were also found for distal gut immunity in Nile tilapia in chapter 4, as changes in the expression profiles of immune-related genes between treatment groups were not found to be significant. However, the expression of several immune genes within the distal gut was highly correlated with the sequence abundance of particular OTUs, indicating a strong host-microbiome interaction in the localised immune status of Nile tilapia. These findings support those of other reports, which demonstrate a strong communication pathway between the resident gut microbiome community and fish host immune system (Galindo-Villegas et al., 2012; Chi et al., 2014; Tan et al., 2019). In this study, the *sIgT* gene was found to be under considerable influence by the gut microbiome community, as the expression of this gene within the distal gut tissue of fish was highly correlated with the abundance of 40 OTUs spanning eight bacterial phyla. This finding is in agreement with previous reports which suggest that this particular immunoglobulin serves a vital role in regulating the fish gut microbiome community (Salinas et al., 2018). In addition to *sIgT*, the gene expression of *nkap*, *scarb1*, *TLR21* and *TP4,* were also found to have strong associations with the abundance of numerous OTUs within the distal guts of Nile tilapia. These findings thus demonstrated that within the distal gut of Nile tilapia, the microbiome can influence pathways which span the entirety of the immune system process e.g. from perception to cell signalling, and finally induction of innate/adaptive defences. These associations can partly explain the subtle but suppressed expression in these genes within the distal gut tissue of treated Nile tilapia by day 22 in this study, as many of these OTUs were also found to decline in sequencing abundance by the end of the experiment. These findings therefore have implications for the aquaculture industry, as they indicate that antibiotic-induced changes in the distal gut microbiome may not only impair disease resilience of farmed fish through reduced direct colonisation resistance, but potentially also alter the indirect colonisation resistance of the fish host through microbial-mediated changes in immune functioning. As we were unable to perform a disease challenge in this study, this theory would need to be explored further to better understand the consequences of antibiotic treatment on disease susceptibility through changes in host (immune)-microbiome interactions. Nonetheless, certain gut bacterial taxa such as *Bacillus* species have already been shown to improve disease resilience through promotion and thus interactions with the immune response in grouper (*Epinephelus coioides*) and Rohu (*Labeo rohita*) (Sun et al., 2010; Mukherjee et al., 2019). Thus, it is highly likely that antibiotic-induced disruptions in particular bacterial communities will affect the disease resilience of farmed fish, through the loss or change in their interactions with the host's immune system.

5.3. Future Work

Findings reported in this thesis highlight the importance of utilising individual rather than pooled samples in fish gut microbiome studies, as chapters 2 and 3 revealed a considerable amount of inter-individual variability in the distal gut microbiome community and diversity of rainbow trout. Similar findings were also found for Nile tilapia in chapter 4, and support previous studies which have found considerable variation between individuals in the gut microbiomes of farmed Atlantic cod (Fjellheim et al., 2012), wild Atlantic salmon (Webster et al., 2018) and farmed rainbow trout (Mansfield et al., 2010). At the time of conducting the studies presented in chapters 2 - 4, little information was available on the minimal sample size required for microbiome studies, thus a sample size of n=6 was used in these chapters. This followed international recommendations for RNA-seq experiments (Schurch et al., 2016) which use similar molecular methods, and met conditions for the 3Rs framework in animal research (Bara & Joffe, 2014). However, the small sample size accompanied by individual variability in the microbiome community composition and diversity, made finding clear patterns between treatment groups difficult. Future gut microbiome research would therefore benefit from a better understanding of the minimal sample size and statistical power requirements for these types of studies. Moreover, improvements in the experimental design, particularly larger sample sizes, would help to identify distinct groups within the population and improve the clarity of observable trends between treatment groups.

It would also be beneficial for future gut microbiome research if other metadata was collected in addition to gut microbiome profiles. For example, capturing information on the sex of individual fish used in studies would be valuable, as sex differences in the gut microbiome have been reported in other vertebrate animals such as humans and mice (Dominianni et al., 2015; Org et al., 2016). Furthermore, there is likely to be personality-driven differences in the gut microbiome communities of fish, as the gut microbiome has been shown to influence behaviour in zebrafish through the gut-brain axis (Borrelli et al., 2016; Davis et al., 2016). Differences in these biological/behavioural factors may contribute to the level of inter-individual variability observed in fish gut microbiome studies. Thus, capturing this increased level of detail may allow for individuals in treatment groups to be further grouped based on these differences e.g. male/female. This may ultimately help to improve the transparency of gut microbiome differences between treatment groups, as the gut microbiome communities from different subgroups may respond differently to the applied stressor in the respective study e.g. antibiotic treatment.

Current gut microbiome research in fish is often performed using terminal sampling of intestinal or digesta material, which prevent the repeated sampling of individuals over time. This sampling approach can challenge the ethics of experimental design in longitudinal microbiome studies, as it requires a higher number of animals to be used which does not support a key objective of the

3Rs framework (Bara & Joffe, 2014). In addition, this sampling approach can also hamper the outputs of such microbiome studies (e.g. chapters 3 and 4), as the differences observed in the community diversity and composition of animals which are thought to be associated with time, may in fact be due to differences between the individuals sampled at each time point. Whilst research efforts have been on-going to investigate the viability of non-invasive sampling approaches in other vertebrate groups, such as faecal collection or cloacal swabs in birds and amphibians (Berlow et al., 2020; Zhou et al., 2020), the drive to investigate the use of non-invasive alternatives in fish gut microbiome research has been slow. However, the development of robust non-lethal sampling techniques, which permit the use of repeated sampling of individuals throughout time would greatly benefit the fish gut microbiome research field. For example, non-invasive sampling could be of value in studies investigating antibiotic treatment, as it would allow for the gut microbiome community of individual fish to be monitored throughout time in response to a respective antibiotic compound. This would permit the exploration of how antibiotic treatments at early stages in the production cycle e.g. larvae/juvenile rearing, impact on the gut microbiome and physiology of individual fish in the later, grow-out stages of production. This knowledge would be beneficial to the aquaculture industry, as the standard production cycle of farmed fish is short, e.g. around 24 months for Atlantic salmon (Thorland et al., 2020). Therefore, if early life antibiotic-induced changes in the gut microbiome led to long-term (e.g. several months) disruption in the gut health and physiology of farmed fish, this would ultimately be detrimental to production.

Findings presented in this thesis provide further evidence to support the ongoing efforts to reduce antibiotic use in aquaculture. We have shown that not only can antibiotic treatment at industry-relevant doses induce changes in the distal gut microbiome of farmed fish species (chapter 3 & 4), but it can also promote AMR (chapter 4), and has the potential to affect localised host physiology through changes in host-microbiome interactions (chapter 4). Such disruptions could potentially lead to reduced disease resilience in farmed fish, as observed in fish species such as zebrafish (Zhou et al., 2018b), which would have economical and welfare implications for production. However, recent research efforts have shown promise for the aquaculture industry in the use of probiotic or prebiotic therapies to alleviate some of the downstream consequences of antibiotic-disturbed microbiome communities. For example, black molly (*Poecilia sphenops*) fish given the probiotic bacteria *Phaeobacter inhibens* and *Bacillus pumilus* following streptomycin treatment, have improved resistance against *V. anguillarum* compared with those fish only given the antibiotic (Schmidt et al., 2017). In their study, authors attributed this improved disease resilience to changes in the direct colonisation resistance potential of probiotic-manipulated microbiome communities, and the probiotic bacteria's competition with the pathogen. In addition, early reports of a "combo" therapy strategy whereby antibiotics and

prebiotics are given simultaneously, have been shown to mitigate against some of the detrimental effects of antibiotics on the gut microbiome community *in vitro*. In a recent study by Johnson et al., (2015), supplementation with pectin and inulin reduced the negative effects of ampicillin on the taxonomic composition of a batch culture originating from healthy human faecal samples. As dietary fibres e.g. inulin provide an energy source and metabolic substrate for commensal bacteria (Nazzaro et al., 2012), it is likely that prebiotic administration may improve the resistance of the resident gut microbiome community during antibiotic treatment. At the time of writing this thesis, the use of prebiotic compounds to moderate the antibiotic-induced changes on the gut microbiome, has not been investigated in any vertebrate animal *in vivo*. Therefore, it would be of benefit to the aquaculture industry to explore this theory further in fish.

Whilst the molecular methods employed in chapter 4 were able to detect several host-microbiome interactions within Nile tilapia, this approach did not detect any significant antibiotic-induced disruptions in the expression of genes involved in the gut physiology of treated fish. These findings contradict existing evidence for this species and antibiotic (Limbu et al., 2018). However, currently there is a growing trend to employ a multi- "omics" approach in fish microbiome research, to investigate host-microbiome interactions and microbiome-derived changes in host physiology. For example, transcriptomics was recently coupled with 16S rRNA sequencing, to profile the changes in the skin microbiome and host response of rainbow trout during a parasitic infection by *Ichthyophthirius multifillis* (Zhang et al., 2018). Likewise, metabolomics or the study of metabolites and other biomolecules, can permit the profiling of the functional status of the host-microbiome "supraorganism". This emerging and powerful tool has been applied alongside 16S rRNA sequencing, to investigate changes in the gut microbiome and metabolic profile of zebrafish in response to microplastic ingestion (Qiao et al., 2019). By applying these tools in future fish gut microbiome studies, the information collected will greatly improve our understanding of how the gut microbiome of farmed fish responds to husbandry practices e.g. antibiotic treatment, and how the fish host may respond to the changes in the gut microbiome community. Thus, findings from studies which employ these combined "omics" style approaches will help to make better informed recommendations for aquaculture practices in the future.

5.4. References

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