Host selectively contributes to shaping intestinal microbiota of carnivorous and omnivorous fish

Running Title: Host influence on fish gut microbiota

Kristian Daly,1 Jennifer Kelly,1 Andrew W. Moran,1 Robert Bristow,1 Iain S. Young,1 Andrew R. Cossins,1 David Bravo,2 and Soraya P. Shirazi-Beechey1,\*

1Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK

2Pancosma SA, Voie-des-Traz 6, Le Grand-Saconnex, Geneva, Switzerland

Corresponding author: Soraya P. Shirazi-Beechey, Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK. Email: [spsb@liverpool.ac.uk](mailto:spsb@liverpool.ac.uk)

**Summary**

Fish production is increasingly important to global food security. A major factor in maintaining health, productivity and welfare of farmed fish is the establishment and promotion of a stable and beneficial intestinal microbiota. Understanding effects of factors such as host and environment on gut microbial community structure is essential for developing strategies for stimulating the establishment of a health-promoting gut-microbiota. We compared intestinal microbiota of common carp and rainbow trout, two fish with different dietary habits, sourced from various farm locations. There were distinct differences in gut microbiota of trout and carp intestine. The microbiota of carp was dominated by *Fusobacteriia* and *Gammaproteobacteria*, while the trout microbiota consisted predominantly of *Mollicutes* and *Betaproteobacteria*. The majority of bacterial sequences clustered into a relatively low number of operational taxonomic units (OTUs) revealing a comparatively simple microbiota, with *Cetobacterium*, *Aeromonas* and *Mycoplasma* being highly abundant. Within each species, fish from different facilities were found to have markedly similar predominant bacterial populations despite distinctly different rearing environments, demonstrating intra-species uniformity and significant influence of host selectivity. This study demonstrates that in fish the host species imparts substantial impact in shaping the community structure of the intestinal microbiota.

**Key Words:** carp; environment; host; intestine; microbiota; trout

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**Introduction**

Fish represent the most diverse taxonomic and ecological class of vertebrates, with approximately 28,000 species occupying an unparalleled number of habitats. This huge variation in species, host ecosystems, environmental factors, feeding habits, dietary constituents and functional and structural characteristics of the intestinal tract will undoubtedly impact on the resident gut microbiome. Indeed, it has been shown in many studies that fish harbor distinct and diverse gut microbial communities that are not simply a reflection of free-living microbes in the surrounding waters, but are shaped by a balance of host-derived and environmental pressures (Sullam et al., 2012).

Many aspects of host physiology including nutrition, stimulation of the immune system, protection against disease and gut-brain communication are all profoundly influenced by the microbiota of the gastrointestinal tract (Foster et al., 2016; Nayak, 2010; Pérez et al., 2010). A better understanding of the potential influence of host and environmental factors on gut microbial community structure is fundamental for developing strategies to rationally manipulate the gut microbiota in order to promote the establishment of a beneficial gut-microbiota ecosystem. This approach is particularly important in fish as the answers to what constitutes a ‘beneficial’ bacterial population and the factors affecting such populations are much less clear than for many terrestrial animals. Recent developments in next-generation sequencing have transformed the way in which microbial communities can be characterized, providing highly detailed analyses of diverse bacterial assemblages. Using this approach, the dynamics of bacterial communities can be assessed, allowing identification of predominant populations and determining how these may be influenced by various factors.

In this study, we used two species of commercially-important farmed fish, common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). By weight, common carp are the third most commercially reared fish with 4.2 million tonnes, representing a value of $5.9 billion, produced globally in 2014 (FAO, 2014). Rainbow trout are twelfth in the list of farmed species with over 800,000 tonnes produced in 2014, worth $3.9 billion (FAO, 2014). Common carp are an omnivorous species that will frequently consume plant matter, invertebrates and detritus. The gut lacks a defined stomach with only arbitrary demarcation between fore-, mid- and hindgut. Rainbow trout, however, are carnivorous, with their natural diet consisting mainly of insects, fish eggs, and smaller fish. They possess a true stomach, with sac-like protrusions known as pyloric ceca, which then leads into the intestine (Guillaume et al., 2001). There are also significant differences in the intestinal digestive and absorptive capacities of the two species (Polakof et al., 2012). Both carp and trout were sourced from different aquaculture facilities in the North West of England, with each facility characterized by distinct environmental factors such as water source, flow system, water treatment, pond design, and fish management. In each facility fish were fed commercial diets, containing appropriate sources and levels of protein, fat and carbohydrate suitable for carp or trout.

The aims of this study were to determine if there were differences in gut microbiota between carnivorous and omnivorous fish and, more pertinently, to further identify whether host innate selectivity or rearing environment is the foremost factor influencing intra-species bacterial community structure. We show here that gut microbial composition of common carp is significantly different to that of rainbow trout. We also demonstrate that host selectivity is a major influence on intra-species gut microbial community structure, resulting in uniformity of predominant bacterial populations despite differences in rearing conditions. Such insights into the selective pressures shaping fish gut microbiota are essential for understanding how microbial communities may be adapted to benefit the health and productivity of the host.

**Materials and Methods**

***Animals and collection of samples.*** Common carp (*Cyprinus carpio*) were sourced from 2 different aquaculture facilities located in the North West of England: University of Liverpool aquarium (LAQ); and Rodbaston aquaculture centre, Staffordshire (RB) (Fig. 1). LAQ uses a re-circulation/semi-flow through system with a continuous air pump. There is no UV or ozone treatment. Bagged coral stones are used to buffer the pH, with the filter containing filter socks, a waste collection vortex, a sand filter and bio-media balls. RB has a re-circulation system, containing 45,000 litres. Oxygen is pumped in with the inflow. All tanks are on automatic feeders. There is limited usage of cleaning products/disinfectants with 0.5–3.0 tonnes of fish in the system at all times. There is a large k1 moving filter bed and a drum filter, but no ozone or UV treatment. Carp from both farms were fed a commercial diet containing 33-36% protein, 7-11% fat and 45-47% carbohydrate.

Rainbow trout (*Oncorhynchus mykiss*) were also sourced from 2 different aquaculture facilities in the North West of England: Kilnsey fly fishery and trout farm, North Yorkshire (KF); and Chirk trout farm, Oswestry (CH) (Fig. 1). KF has a complete flow through system fed with water from a natural underground spring with fish reared through 10 raceways. The temperature never rises above 8°C and no disinfectants are used onsite. CH also uses a complete flow through system, fed with natural spring water from the River Ceiriog, maintained at 9-10°C. No initial filtration, UV or ozone treatments are used, however external parasites are controlled using chlorine. The ponds are natural earth-bottom pools that are lined with lime and contain between 2,000-6,000 fish. Trout from both farms were fed a commercial diet containing 41-44% protein, 27-30% fat and 15-17% carbohydrate. All commercial diets were irradiated to eliminate bacterial contamination.

After transportation of fish to the University of Liverpool aquarium, fish were sacrificed by concussion followed by pithing of brain according to UK Home Office Schedule 1 regulations. The average weight and length of carp was 405 g (range ± 184 g) and 22.6 cm (range ± 2.9 cm); whilst trout were 243 g (range ± 51 g) and 24.8 cm (range ± 2.0 cm). Immediately post-mortem, intestinal contents were removed, wrapped in aluminium foil and frozen in liquid nitrogen. All samples were subsequently stored at −80°C until use.

***Extraction of bacterial DNA from intestinal contents.*** Bacterial DNA was extracted from frozen intestinal contents using the ZR Fecal DNA MiniPrep kit (Zymo Research, California, USA) according to the manufacturer’s instructions. Purified DNA was quantified using PicoGreen Assay (Life Technologies Ltd, Paisley, UK) and integrity was assessed by agarose gel electrophoresis. We have observed that rapid freezing of samples in liquid nitrogen, followed by a single extraction procedure, is an effective method for preserving intact microbial DNA (Daly and Shirazi-Beechey, 2003). This approach also avoids repeated freeze-thawing of samples that may be deleterious to preservation of DNA from gram negative bacteria.

***PCR amplification of bacterial 16S rRNA genes and Illumina MiSeq sequencing.*** Purified genomic DNA extracted from intestinal contents was used as template for PCR amplification of the hypervariable V4-region of bacterial 16S rRNA genes using custom primers (Caporaso et al., 2012), which comprised the universal forward and reverse bacterial primers 515f and 806r, and the required Illumina flowcell adaptor sequences (Caporaso et al., 2012). The reverse primer also contained a unique 12-base Golay barcode to allow multiplexing of numerous samples (Caporaso et al., 2012). To reduce PCR-associated bias and to increase DNA yield, the products of several amplification reactions for each sample were pooled. Each reaction mix contained 0.5 U of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Suffolk, UK), 0.5 *μ*M of each primer, 1 × Q5 Reaction Buffer (New England Biolabs) and 5 ng template DNA in a final volume of 25 *μ*l. PCR amplification was carried out using the following parameters: initial denaturation at 95°C for 1 min, 25 cycles of denaturation at 95°C for 10 s, annealing at 53°C for 20 s and extension at 72°C for 15 s, followed by a final extension step at 72°C for 2 min. Samples were then combined with loading buffer and subjected to agarose gel electrophoresis at 100 V in 1 × TAE buffer. Amplified product of the target length was visualized, excised and purified using the QIAquick Gel Extraction kit according to the manufacturer’s instructions (Qiagen). No-template controls were also generated for all samples. Purified amplicons were then quantified in duplicate using the Quant-it PicoGreen dsDNA kit (Life Technologies Ltd), combined in equimolar amounts and sequenced on the Illumina MiSeq sequencing platform at the Centre for Genomic Research (CGR) next-generation sequencing facility at the University of Liverpool, UK.

***Read filtering and paired-end assembly.*** Raw sequencing reads were filtered to remove low-quality reads: i) the removal of Illumina adaptor sequences using CutAdapt (version 1.2.1) (Martin, 2011); ii) the trimming of low-quality bases using Sickle (https://github.com/najoshi/sickle) (version 1.2), based on a sliding window of a defined size to remove read segments below a minimum phred quality value of 20, and iii) the removal of any trimmed reads below 10 bp in length. High-quality paired-end reads were then merged into overlapping sequences, and subsequently filtered for erroneous sequences using the USEARCH paired-read assembler and quality filtering commands utilizing a maximum expected error rate of 0.5 (Edgar, 2010). Assembled sequences were then filtered for any contaminating phiX sequence that may have been carried over from the sequencing process using BMtagger (Rotmistrovsky and Agarwala, 2011) and the NCBI reference sequence for *Enterobacteria* phage phiX174 (NCBI accession NC001422).

***Microbial profiling analysis.*** Unless otherwise stated, all analyses described in this section were carried out using the software package Quantitative Insights into Microbial Ecology (QIIME v1.8.0) (Caporaso et al., 2010b). Following quality filtering and assembly, a total of 7,699,382 reads were generated for 36 samples, with an average (±SD) of 213,872 ±42,695 reads per sample. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the UCLUST algorithm (Edgar, 2010) with a minimum cluster size of 2. Chimeric OTUs were identified using ChimeraSlayer (Haas et al., 2011) and subsequently removed. The most abundant sequence from remaining OTU clusters was selected and assigned taxonomic classification using the Ribosomal Database Project (RDP) (Cole et al., 2009) classifier tool with the most recent release of the Greengenes database (13.8) (DeSantis et al., 2006), using a confidence cut-off of 0.8. PyNAST (Caporaso et al., 2010a) was then used to generate alignments for all representative sequences, which were subsequently filtered and used to generate a phylogeny using the FastTree approach (Price et al., 2009). OTUs comprising < 0.005% relative abundance or taxonomically annotated as ‘Chloroplast’ or ‘Mitochondria’ were removed from the OTU table. Microbial richness and diversity (alpha diversity) was calculated using Shannon diversity index (Shannon and Weaver, 1971) and Simpson index (Simpson, 1949) metrics using a rarefaction depth of 89,000 reads. To determine the degree of similarity between samples (beta-diversity), weighted Unifrac distance matrices were calculated for all pair-wise sample comparisons, and used to generate non-metric multidimensional scaling (NMDS) ordination plots.

***Statistical analysis.*** To test for differences in intra-species microbial diversity/richness between different aquaculture facilities, non-parametric two-sample t tests were used with Monte Carlo permutations to calculate the *p* value (999 permutations). The significance and strength of a range of sample groupings (i.e. location, individual) was determined by comparing beta-diversity matrices of weighted Unifrac distances and Bray-Curtis dissimilarity measures using a range of non-parametric tests including ADONIS and ANOSIM (Anderson et al., 2006). The ADONIS test also revealed the amount of sample variance (effect size) attributable to each grouping category. Differentially abundant OTUs were identified using the nonparametric Kruskal-Wallis method with *p*-values corrected for multiple testing using family wise error rate (FWER). Features were considered significant if *p* values were < 0.05. Plots were generated using the software packages R, version 3.1.1 (Grimonprez et al., 2014), QIIME, versions 1.8 and 1.9 (Caporaso et al., 2010b) and Phyloseq (McMurdie and Holmes, 2013).

***Quantitative real-time PCR analysis of microbial abundance.*** Quantification of 16S rRNA gene copy number in DNA extracted from fish intestinal contents was determined by qPCR using a Rotorgene 3000 (Qiagen) and SYBR Green JumpStart Taq ReadyMix for qPCR (Sigma Aldrich Co. Ltd). Total 16S rRNA gene copy number was determined in each sample, as a measurement of total bacterial numbers (Barman et al., 2008), by amplification of bacterial DNA with universal 16S rRNA gene primers, in comparison to standard curves constructed from a reference plasmid containing the 16S rRNA gene from *Lactobacillus* *amylovorus* (Daly et al., 2016). PCR cycling was performed as follows: initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 63°C for 15 s, 72°C for 30 s. Assays were performed in triplicate and 16S rRNA gene copy number was calculated using RG-3000 quantification software.

***Accession number(s).*** Illumina sequencing data has been deposited in the European Nucleotide Archive (ENA) under project accession number PRJEB18770.

***Use of animals.*** National/institutional guidelines for the care and use of animals were followed, fish were sacrificed according to UK Home Office Schedule 1 regulations and all experiments were approved by the University of Liverpool Ethics Committee.

**Results**

***Characterization of common carp and rainbow trout intestinal microbiota***

DNA was extracted from the intestinal contents of 2 sets of 9 common carp (*Cyprinus carpio*) and 2 sets of 9 rainbow trout (*Oncorhynchus mykiss*) each set sourced from different aquaculture facilities (Fig. 1). DNA sequencing of amplicons of the hypervariable V4-region of bacterial 16S rRNA genes was used to profile the community structure and diversity of intestinal microbiota across the different farms.

Following removal of chimeric and contaminating sequences, the average number of assembled sequence reads per sample was over 200,000 for carp and over 220,000 for trout, each with a mean read length of 250 bp. Employing a sequence dissimilarity threshold of 3% to classify operational taxonomic units (OTUs) and a relative abundance threshold of 0.005% (to remove low abundance OTUs), we identified 117 and 135 distinct OTUs residing in the intestinal tracts of carp and trout respectively, of which 100 OTUs were observed in both species. However, the respective compositions of the intestinal microbiota differed significantly between carp and trout, demonstrating strong host selectivity between these species. NMDS analysis of Bray-Curtis matrices clearly demonstrated complete and significant separation of intestinal bacterial communities in carp from those observed in trout (ADONIS R2 = 0.689; ANOSIM R = 0.873; *p* < 0.05; weighted Unifrac) (Fig. 2).

***Host influence on intra-species microbial composition and diversity***

Classification of OTUs showed the microbiota of carp to be almost exclusively dominated by *Fusobacteriia* and *Gammaproteobacteria*, with trout dominated by *Mollicutes* and *Betaproteobacteria*. Taxonomic classification of OTUs comprising > 0.5% relative abundance identified 6 families in carp encompassing 4 bacterial classes: *Fusobacteriia, Gammaproteobacteria, Betaproteobacteria* and *Bacilli*; and 7 families in trout within 6 bacterial classes: *Mollicutes, Betaproteobacteria, Gammaproteobacteria, Bacilli, Clostridia* and *Bacteroidia*.

Although a small number of low abundance microbial OTUs differed between fish from different facilities, the overall composition of the intestinal microbiome, for both carp and trout, were significantly associated with host. For both species, there was no significant difference in intra-species alpha diversity, using Shannon and Simpson indices, indicating a similar level of microbial diversity between locations. Furthermore, NMDS analysis of Bray-Curtis matrices clearly shows overlapping grouping within both carp and trout populations demonstrating a substantial level of intra-species similarity regardless of source location (ADONIS R2 = 0.292 [carp], 0.092 [trout]; ANOSIM R = 0.301[carp], 0.028 [trout]; weighted Unifrac) (Fig. 2).

For both fish species, the intestinal microbiota was dominated by a limited number of highly abundant OTUs. For carp, only 6 different OTUs in LAQ and 7 OTUs in farm RB had a relative abundance > 0.5%, representing 95% and 97% of the total microbiota respectively (Table 1). Four of these abundant OTUs were found in fish from both locations. The dominant OTU in carp showed 100% sequence identity to *Cetobacterium somerae*, a member of the *Fusobacteriia*. This OTU comprised 51% and 68% of the total microbiota in LAQ and RB carp respectively (Fig. 3). The second most abundant OTU in these carp showed 100% sequence identity to *Aeromonas* sp., a member of the *Gammaproteobacteria*, with a mean relative abundance of 33% and 19% of total respectively (Fig. 3).

In trout, the number of OTUs with a relative abundance of > 0.5% was 7 in farm KF and 4 in farm CH. These abundant OTUs accounted for 83% of the total microbiota in fish from each respective farm (Table 2). Two of these abundant OTUs were found in trout from both locations. The most abundant OTU in trout from either farm was related to *Mycoplasma* sp.(95% sequence identity), a member of the *Mollicutes* class, with mean relative abundances of 55% and 74% in KF and CH trout, respectively (Fig. 3).

Quantitative PCR analysis of total bacterial abundance showed similar densities of bacteria in these fish irrespective of source location, ranging from 108-109 bacteria/g intestinal contents (Fig. 4).

**Discussion**

The gastrointestinal microbiota is influenced by external factors such as diet, habitat, disease, and internal host-derived influences such as immune status, physicochemical conditions and gut anatomy (Goldsmith and Sartor, 2014). This balance between host and environment defines both the community structure and activity of the microbiome (Sullam et al., 2012; Wong and Rawls, 2012).

The composition of gut microbiota has been studied in several fish species within the same habitat suggesting that microbiota may be influenced by host associated factors (Larsen et al., 2014; Li et al., 2014; Li et al., 2012; Liu et al., 2016; Wong et al., 2013). Although these studies provide valuable information on microbiota composition of particular fish species, the potential impacts of confounding environmental pressures, if any, on host influence have not been fully evaluated.

In this study, we have investigated the impact of host and environment upon intra-species microbial variation within two different commercially-important and diet-selective farmed fish, the omnivorous common carp (*Cyprinus carpio*) and the carnivorous rainbow trout (*Oncorhynchus mykiss*). Using DNA extracted from the intestinal contents of 4 sets of fish, sourced from 4 different aquaculture facilities, and Illumina sequencing of 16S rRNA gene amplicons, we characterized the composition of gut microbiota.

We found significant differences between carp and trout gut microbiota, two species with contrasting dietary requirements, habitats, lifestyle and intestinal tract anatomy. It is notable that for both fish species, relatively few OTUs comprised the majority of the microbiota. Indeed, despite there being over 100 distinct OTUs observed in each species, only 7 OTUs individually represented > 0.5% of the carp total community in farms LAQ and RB, while only 7 and 4 OTUs comprised > 0.5% of the trout microbiota in farms KF and CH respectively. For both carp and trout, those OTUs with an abundance > 0.5% described 83-97% of the entire microbiota. This demonstrates a much simpler microbial community in fish in comparison to terrestrial mammalian species; the latter typically possess a far greater diversity of intestinal bacterial species (Daly et al., 2016; Daly et al., 2001; Lagier et al., 2016; Myer et al., 2015). Furthermore, the density of bacteria within the intestine of these fish (108-109 bacteria/g intestinal contents) is up to 2-3 orders of magnitude lower than that reported for many terrestrial mammals (1011 bacteria/g intestinal contents) (Barman et al., 2008; Mao et al., 2015; Metzler-Zebeli et al., 2010).

Most notably, by characterizing the gut microbiota of trout and carp sourced from different aquaculture facilities, we found that microbial composition and diversity within each species was predominantly influenced by host and not by rearing conditions. In carp, there was a dominance of *Fusobacteriia* and *Gammaproteobacteria*, with the microbiota in carp from both LAQ and RB farms being dominated by *Cetobacterium somerae*. This bacterium is a prominent member of the carp gastrointestinal tract community, having the ability to ferment carbohydrates (Finegold et al., 2003) and synthesise vitamin B12 (Tsuchiya et al., 2008). An OTU relating to multiple *Aeromonas* sp. was also a major component of the intestinal microbiota in carp from both locations. Although *Aeromonas* sp. are generally regarded as a pathogenic, they are a common intestinal inhabitant of fish, and several species are reported to produce extracellular enzymes involved in carbohydrate and protein fermentation (Jiang et al., 2011; Ray et al., 2012; Sugita et al., 1995).

A *Mycoplasma* sp. was the most abundant OTU in trout obtained from farms KF and CH. It has been shown previously that this species is a dominant member of the intestinal microbiota of rainbow trout (Lowrey et al., 2015) and of wild and farmed Atlantic salmon (Holben et al., 2002), suggesting conservation of host selection for this bacterial population within salmonids. However, the reasons why this bacterium is so abundant in the intestinal tract of these fish and the role it might play are unclear.

Most notably, we show that different farmed populations of both trout and carp contain highly uniform intra-species microbial communities, irrespective of rearing conditions. This highlights the pressures imposed by the host in shaping the gut microbiota that are manifest in terms of global community structure, microbial diversity, population abundance and predominance of shared OTUs. These distinct and characteristic intestinal microbiotas have evolved in response to long term dietary adaptation and ecological influences in both carnivorous and omnivorous fish.

The data presented here provide the basis for identification of beneficial members of the gut microbiota and the role they play in promoting fish health and growth in an era of increasing demand for fish production as a sustainable food source.

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**FIGURE LEGENDS**

**Fig. 1.** Location of aquaculture facilities used to source common carp and rainbow trout in the North West of England (Red: common carp; Blue: rainbow trout). LAQ: University of Liverpool aquarium; RB: Rodbaston aquaculture centre; KF: Kilnsey fly fishery and trout farm; CH: Chirk trout farm.

**Fig. 2.** Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity measures for common carp and rainbow trout gut microbiota calculated and plotted according to species and source location. Confidence ellipses represent the co-variance matrices. Each individual symbol represents one individual fish.

**Fig. 3.** Relative contributions (expressed as mean % of total sequence reads) of identified bacterial families to the composition of common carp and rainbow trout gut microbiota.

**Fig. 4.** Quantitative real-time PCR determination of total 16S rRNA gene copy number (as a measure of total bacterial numbers) in common carp and rainbow trout intestinal contents.