Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout Oncorhynchus mykiss from both farm and aquarium settings

Journal:	*Applied Microbiology*
Manuscript ID	JAM-2016-1811.R1
Journal Name:	Journal of Applied Microbiology
Manuscript Type:	JAM - Original Article
Date Submitted by the Author:	25-Oct-2016
Complete List of Authors:	Lyons, Philip; University of Stirling, Institute of Aquaculture Turnbull, James; University of Stirling, Institute of Aquaculture Dawson, Karl; Alltech International, Nutrigenomics CRUMLISH, MARGARET; University of Stirling, Institute of Aquaculture
Key Words:	Intestinal microbiology, Aquaculture, Metagenomics, Metabolism, Diversity

Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout *Oncorhynchus mykiss* from both farm and aquarium settings

Philip P. Lyons[1]*, James F. Turnbull[1], Karl A. Dawson[2] and Mags Crumlish[1]

[1] *Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, United Kingdom*

[2] *Alltech Biotechnology Inc., 3031 Catnip Hill Pike, Nicholasville KY 40356, USA*

*Corresponding author: P Lyons, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK. E-mail: p.p.lyons@stir.ac.uk ; pptlyons@gmail.com

Running head: Phylogeny and function of trout gut microbiome

Abstract

Aims: This study focused on comparing the phylogenetic composition and functional potential of the intestinal microbiome of rainbow trout sourced from both farm and aquarium settings.

Methods and results: Samples of distal intestinal contents were collected from fish and subjected to high throughput 16S rRNA sequencing, to accurately determine the composition of the intestinal microbiome. The predominant phyla identified from both groups were Tenericutes, Firmicutes, Proteobacteria, Spirochaetae and Bacteroidetes. A novel metagenomic tool, PICRUSt, was used to determine the functional potential of the bacterial communities present in the rainbow trout intestine. Pathways concerning membrane transport activity were dominant in the intestinal microbiome of all fish samples. Furthermore, this analysis revealed that gene pathways relating to metabolism, and in particular amino acid and carbohydrate metabolism, were upregulated in the rainbow trout intestinal microbiome.

Conclusions: The results suggest that the structure of the intestinal microbiome in farmed rainbow trout may be similar regardless of where the fish are located and hence could be shaped by host factors. Differences were however noted in the microbial community membership within the intestine of both fish populations, suggesting that more sporadic taxa could be unique to each environment and may have the ability to colonize the rainbow trout GI tract. Finally, the functional analysis provides evidence that the microbiome of rainbow

trout contains genes that could contribute to the metabolism of dietary ingredients and therefore may actively influence the digestive process in these fish.

Significance and impact of the study: To better understand and exploit the intestinal microbiome and its impact on fish health, it is vital to determine its structure, diversity and potential functional capacity. This study improves our knowledge of these areas and suggests that the intestinal microbiome of rainbow trout may play an important role in the digestive physiology of these fish.

Keywords: intestinal microbiology, aquaculture, metagenomics, metabolism, diversity

Introduction

It is now well documented that animals harbour a vast number of microorganisms, collectively termed the microbiome, both on their body surfaces and particularly within their gastrointestinal (GI) tract. The most numerous of these microorganisms are the bacteria; however, yeasts, viruses, archaea and protozoans also inhabit these ecosystems. As molecular technologies develop and become more advanced, we are beginning to unravel the true diversity of these communities and the potential impact that they may have on host development, nutrition, disease resistance and immunity. Despite the widespread adoption of these technologies to study the microbiome of terrestrial animals, comparatively little is known of the intestinal microbiome of fish, and in particular economically important farmed fish species such as rainbow trout *Oncorhynchus mykiss*.

Rainbow trout are reared in a number of different aquaculture settings, such as earthen ponds, raceways, inshore tank systems and in freshwater/seawater cages. It has been hypothesized that these different farming environments can shape the composition of the gastrointestinal microbiome, with different taxa dominating according to the geographical location and environmental conditions of the farm in question (Cahill 1990, Spanggaard et al 2000, Nayak 2010). The ‘core’ microbiome concept proposes that individual hosts maintained under the same husbandry conditions, in the same environment and location will share similar microbial taxonomic compositions (Turnbaugh and Gordon 2009, Wong et al 2013). Novel nutritional strategies such as pro-, pre- and synbiotic feeds have been developed which aim to modulate the gut microbiota, especially in light of the industry’s commitment to reduce its use of both antibiotics, and fish meal/oil sourced from wild pelagic fisheries. Therefore, it is important to determine the potential existence of a core microbiome amongst rainbow trout, and whether such a core is shared even amongst fish reared in different geographical

locations and farming environments. This information could aid in refining nutritional strategies that aim to harness the potential of these communities, by improving our presently limited understanding of the normal or baseline composition of the rainbow trout intestinal microbiome.

More recent studies that have used high throughput sequencing technologies have shown that the fish intestine harbours a more complex and diverse microbiome than previously considered (Llewellyn et al 2014, 2015, Lowrey et al 2015, Ghanbari et al 2015). Some studies have shown that bacterial populations within teleost fish intestines can be altered in response to different dietary ingredients (Desai et al 2012, Carda-Dieguez et al 2014, Kormas et al 2014, Miyake et al 2015). Others have demonstrated that the core microbiome is resistant to changes in diet and rearing density, and that community profiles of individual fish, reared in the same aquaculture setting, can attain remarkable levels of uniformity (Wong et al 2013, Zarkasi et al 2014). However, it remains unclear whether the structure of the microbiome varies between individual fish of the same species reared in different farming environments, or whether these fish harbour a specialized microbiota independent of geographical location.

Furthermore, although a clearer picture has emerged of the extent of the microbial diversity within the rainbow trout intestinal microbiome, thus far no reports have been documented concerning the functional capability of these communities. Therefore, this study employed a novel but well validated computational approach, PICRUSt (Langille et al 2013) (http://picrust.github.io/picrust), to predict the potential functional capacity of the intestinal microbiome and to complement the phylogenetic data generated. PICRUSt uses an extended ancestral-state reconstruction algorithm to predict which gene families are present within 16S rRNA libraries, and then combines those gene families to estimate the composite metagenome. This approach has been used successfully to study the cecal microbiome of the farmed broiler chicken *Gallus gallus domesticus* (Corrigan et al 2015, Pourabedin and Zhao 2015, Shaufi et al 2015). A detailed knowledge of the phylogenetic profile and functional capacities of the intestinal microbiota is extremely important in order to aid our understanding of the role of these microorganisms in fish health and digestive physiology.

The aim of the present study was therefore to produce an in-depth taxonomic and functional characterization of the rainbow trout intestinal microbiome from individual fish maintained in separate rearing environments. It was hypothesized that the gut bacterial communities would

differ between the two farming locations due to the inherent differences in each system's environment. This research therefore would test whether the diversity and structure of these communities was affected by differences in rearing environment, in addition to elucidating fundamental information about their potential functional role within the intestinal ecosystem.

Materials and methods

Sample collection

A total of twelve rainbow trout were collected from a freshwater fish farm based on Loch Awe, Argyll, Scotland. Six fish were each randomly sampled from two separate pens, identified as A and B. The water temperature at the time of sampling was 9.4^{o}C. The fish from each pen originated from different egg sources, but were raised at the same hatchery. All individuals collected on the day of sampling were apparently healthy, that is, with no visual signs of disease or parasites on the skin or internal organs. All fish were fed the same commercial pelleted feed. The mean weight (± SD) of the fish from pen's A and B was 119 ± 24 g and 79 ± 10 g respectively at the time of sampling.

A further nine fish were collected from the Aquatic Research Facility (ARF) at the Institute of Aquaculture, University of Stirling, Scotland, UK. Three fish were sampled from each of three separate tanks of 100 L capacity. These tanks were maintained on a flow through system, with an ambient water temperature (11.8^{o}C), and a photoperiod of 12 h light 12 h dark. All of these fish originated from a local trout farm in Perthshire, UK. All were fed the same commercial pelleted feed. The mean weight (± SD) of these fish was 191 ± 45 g at the time of sampling. In addition, two samples of the pelleted feed and a single tank biofilm sample were taken to compare against the microbiome of the rainbow trout intestine.

All fish were sacrificed with a lethal dose of the anaesthetic benzocaine (Sigma Aldrich®, Poole, UK) and swabbed with 100% ethanol before dissection of the ventral surface. The tissues surrounding the visceral fat were aseptically removed and the distal intestine identified. The distal gut contents (~150 mg) were removed by gently massaging the tissue with a sterile forceps and were placed into sterile 2 ml capped microtubes (Alpha laboratories®, Eastleigh, UK) containing 1 ml of buffer ASL (Qiagen, Hilden, Germany). The tissue was then incised and washed with a sterile 0.85% (w/v) salt solution, and the intestinal mucous was carefully removed from the gut wall. This material was placed into the same tube as the gut contents. All tubes were immediately placed on dry ice after sampling, before being transferred to the laboratory for subsequent same-day DNA extraction.

DNA extraction

A total of 150 mg of intestinal content material from each individual fish suspended in 1 ml of buffer ASL (Qiagen), was processed for DNA extraction. The extractions were performed on the same day as sampling to ensure optimal sample integrity. A sample of 1 ml buffer ASL was processed as a negative control. Samples were firstly disrupted using a Mini- Bead-Beater 16 (Biospec Products Inc.), incorporating sterile zirconia beads at maximum speed for four separate cycles of 35 s each. Samples were allowed to settle, and total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with the following modifications to the manufacturer's protocol : 150 mg starting material in 1 ml buffer ASL; suspension heated at 95^{o}C for 10 min to improve lysis of Gram positive bacteria; 0.5 Inhibitex tablet per sample in 700 µl supernatant ; final sample elution volume of 50 µl. Intestinal content samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions. The QIAamp kit is specifically designed to remove these inhibitors and final purified eluates are enriched for microbial community DNA. After extraction, the DNA concentration of all samples was determined both spectrophotometrically (NanoDrop 1000®, Thermo Scientific Ltd., DE, USA) and fluorometrically (Qubit®, Life Technologies Ltd., Paisley, UK) to ensure optimal DNA purity, and samples were stored at -20^{o}C for downstream processing.

16S rRNA PCR and Illumina sequencing

A PCR was firstly carried out using universal eubacterial primers 27F AGAGTTTGATCMTGGCTAG and 1492R TACGGYTACCTTGTTACGACTT (Weisburg et al 1991) that target the full length bacterial 16S rRNA gene sequence, to confirm the presence of ample microbial community DNA and to rule out the presence of any potential inhibitory compounds. The extraction from the sample containing buffer ASL only was included in this PCR as a negative control. The PCR conditions for this confirmatory reaction were as follows: denaturation at 95^{o}C for 5 min, followed by 30 cycles of denaturation at 94^{o}C for 2 min, annealing at 50^{o}C for 1 min and elongation at 72^{o}C for 2 min, before final elongation of 72^{o}C for 10 min. Products were then visualized on a 1.5% (w/v) agarose gel, run at 100V for approximately 75 min. The presence of a single strong PCR product of ~1500bp was considered to be indicative of the presence of microbial community DNA. Illumina libraries were prepared following the method described by Caporaso et al (2012) using the NEXTflex 16S Amplicon-Seq kit (Bioo Scientific, Austin USA). A total of 50 ng of template DNA was used for each individual sample and the V4 hypervariable region of the

bacterial 16S rRNA gene (length 292bp) was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (GATC Biotech AG, Konstanz, Germany). The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; followed by a final extension step at 72°C for 5 min. All samples were amplified in triplicate and all products purified using Agencourt AMpure XP beads (Beckman Coulter (UK) Ltd.).

The products of the first PCR served as a template for a second PCR with the same conditions as the first, however the number of cycles was reduced to eight, and Illumina sequencing adapters were added to the primers in the reaction mix. Following amplification, PCR products were purified using Agencourt AMpure XP (Beckman Coulter (UK) Ltd.) with a modified 1:1 volume of PCR product to AMpure XP beads. Purified amplicons were quantified with Qubit®, pooled in equal concentration and the final quality of the pooled library was validated using an Agilent Bioanalyzer 2100® (Agilent Technologies, Waldbronn, Germany). The final library was prepared and sequenced by GATC Biotech AG (Konstanz, Germany) using the Illumina MiSeq® NGS system.

Bioinformatics

Demultiplexing was performed with Casava v. 1.8, and reads representing the PhiX or reads not matching indices were removed. FastQC (Andrews 2010) was used to assess the overall quality of all sample libraries, and a threshold Phred score ($Q \geq 25$) was set. The open-source software, mothur (Schloss 2009), was used to process sequences from the demultiplexed 16S rRNA gene libraries, following the online MiSeq analysis SOP (http://www.mothur.org.wiki/MiSeqs_SOP). Sequences were firstly merged using the make.contigs command. Reads containing ambiguous bases, homopolymer runs greater than eight bases, and sequences of less than 150 base pairs in length were removed from the dataset. Remaining sequences were aligned against mothur's Silva reference database, after customizing the reference alignment to concentrate on the V4 region only. Further denoising of the dataset was performed using mothur's pre clustering algorithm, allowing for up to two differences between sequences. This sorted sequences by abundance, ordering from most abundant to least and identified sequences within two nucleotides of each other. If sequences met these conditions, they were merged. Chimeric sequences were then removed from the dataset using the UCHIME algorithm in mothur as a final denoising step prior to taxonomic classification.

For taxonomic analyses, sequences were annotated using the Bayesian classifier implemented by the Ribosomal Database Project (RDP) Release 11. A minimum confidence bootstrap threshold of 80% was required for each assignment. Sample coverage, rarefaction curves, bias-corrected Chao 1 richness and Simpson's index of diversity were calculated based on assembled OTU's using mothur. Samples were rarefied to the sample with the lowest number of sequences before performing these diversity analyses, to ensure that any observed differences in diversity were not caused by uneven sampling depth.

Statistical analysis

Statistical analyses of all filtered libraries was conducted according to the mothur MiSeq protocol (Kozich et al 2013). ThetaYC (Yue and Clayton 2005) and Jaccard distance matrices were created within mothur using the dist.shared command. These matrices were calculated to examine the dissimilarity between the microbial community structure and membership of all samples respectively, and take into account the relative abundance of bacterial taxa. Microbial community structure refers to the combination of membership and the abundance of each OTU, whereas microbial community membership refers to the list of OTU's in a community and evaluates their presence/absence. PCoA was performed to visualize the resulting ThetaYC and Jaccard distances. The statistical significance of any observed distances was examined using the Analysis of Molecular Variance (AMOVA) test within the mothur MiSeq analysis protocol. Furthermore, Parsimony (Schloss and Handelsman 2006) and UniFrac (Lozupone and Knight 2005) analyses were performed in order to test whether any observed clustering between samples was statistically significant. Finally, Metastats (White et al 2009) and Indicator (McCune et al 2002) algorithms were used to determine whether any phylotypes were differentially represented between farmed and aquarium rainbow trout intestinal samples. Results were considered as statistically significant at two levels, $p<0.05$ and $p<0.01$. A one-way ANOVA was performed on the Simpson and Chao1 richness data, using Mintab 17 Statistical software (https://www.minitab.com), to test for any significant differences between the mean microbial diversity of the tested trout populations.

Establishment of predicted functional profiles

In the present study, PICRUSt (Langille at al 2013) was used to predict the functional metagenome of all samples. OTU's were firstly picked against the Greengenes v. 13_5 database and the make.biom command within mothur was used in order to produce a file compatible with the PICRUSt program. This BIOM file was uploaded to the online Galaxy terminal (http://huttenhower.sph.harvard.edu/galaxy/edu) for pre-processing before analysis

using the PICRUSt pipeline. PICRUSt was firstly used to correct OTU tables for known 16S rRNA copy numbers for each taxon and then subsequently to predict metagenomes using the precalculated KEGG (Kyoto Encyclopedia of Genes and Genomes) ortholog (KO) and Cluster of Orthologous Genes (COG) tables. Because PICRUSt relies on reference genomes that are phylogenetically similar to those represented in a community, the Nearest Sequenced Taxon Index (NSTI) values were calculated in order to quantify the availability of nearby genome representatives for each microbiome sample, and hence to determine the overall accuracy of the metagenomic predictions for all samples. The output of the PICRUSt analysis consists of a table of quantitative functional counts, i.e. KEGG pathway counts according to sample. Because some KEGG orthologs can be represented in multiple pathways, the categorize_by_function.py command within PICRUSt was used to collapse the functional predictions at the level of the individual pathways. The output files from the PICRUSt analysis were then uploaded to the Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al 2014) software package. This program permitted the further statistical interrogation of all predicted functional datasets and the production of graphical depictions of key functional pathway data.

Results

Sequencing data and microbial diversity analysis

A total of 14,088,267 reads were obtained from all sample libraries after quality filtering steps were performed. A total of 1131 OTU's were assembled from the combined libraries. After subsampling to the level of the library containing the fewest reads (DF AF1, n=142,267), rarefaction analysis revealed that all sample curves reached saturation (Figure 1). Overall, rarefaction estimates pointed to a slightly elevated level of community diversity in the fish farm samples, with the highest level of diversity noted in the aquarium tank biofilm sample. Mean Chao1 richness estimates were higher in the fish farm samples (286.15 ± 125.42) than in the aquarium samples (233.51 ± 90.44), and the estimates were even higher in the tank biofilm sample (691.89), reflecting the trend indicated in the rarefaction analysis. The mean inverse Simpson value was however greater in the aquarium fish samples (2.14 ± 0.78) versus the farm samples (1.64 ± 0.82). However, the overall microbial diversity and richness in the intestine of aquarium and farm based fish were not significantly different (Simpson: $f = 3.24$, $p = 0.088$; Chao1: $f = 1.14$, $p = 0.300$). Good's coverage estimations were on average >99% for all libraries indicating that a high level of sequence coverage was obtained. All alpha diversity statistics are detailed in Table 1.

Microbiome composition of rainbow trout distal intestine

A total of 14 separate bacterial phyla were observed across all libraries analysed. The mean distribution of OTU's at the phylum level of fish farm, aquarium and biofilm samples is depicted in Figure 2. In the fish intestinal samples from both sites, four phyla were dominant, the Tenericutes, Firmicutes, Proteobacteria and Spirochaetae. A total of 18 bacterial classes were recorded within the 14 phyla observed (Figure 3). The mean number of OTU's classified to the genus level was 143 (maximum of 274, minimum of 61) and 208 (maximum of 367, minimum of 48) in the aquarium and farmed fish intestine samples respectively.

The Tenericutes were the most dominant phylum in the fish intestinal microbiome samples, from both the aquarium and the fish farm sites. Within this phylum, the Mollicutes were the dominant class and the principal OTU classified at the genus level was *Mycoplasma*. The Mollicutes were slightly more abundant in the farmed fish samples with a mean representation of 81%, versus 68% in the intestine of the aquarium fish. The vast majority of other OTU's belonged to the classes Bacilli, Clostridia, Gammaproteobacteria and Spirochaetia. The remaining 13 classes, Alphaproteobacteria, Betaproteobacteria, Candidate Division WPS-1, Flavobacteria, Fusobacteria, Sphingobacteria, Deinococci, Negativicutes, Actinobacteria, Bacteroidia, Deltaproteobacteria, Thermodesulfobacteria and Opitutae were detected at much lower levels of sequence abundances. The next most prevalent class was the Spirochaetia, with *Brevinema* being identified as the predominant OTU identified. This class was more abundant in the aquarium fish, representing 19.7% versus 8.1% in the farmed fish samples. The phylum Firmicutes was slightly more prominent in the aquarium fish and contained OTU's that were primarily split between two bacterial classes, the Clostridia and the Bacilli. Within these classes, the principal OTU's were identified as *Lactobacillus*, *Acetanaerobacterium*, *Catellicoccus*, *Streptococcus*, *Weissella*, *Leuconostoc*, *Lactococcus*, *Enterococcus* and *Bacillus*. The phylum Proteobacteria was primarily represented by the γ subclass in both the aquarium and the farm based fish samples, with *Photobacterium*, *Pseudomonas*, *Acinetobacter*, *Maricurvus*, *Moritella* and *Pantoea* being the primary genera detected. Members of the α and β subclasses were also recorded, but were poorly represented in the fish intestinal samples.

Microbiome composition of aquarium tank biofilm and diets

In contrast to the rainbow trout intestinal samples, the tank biofilm sample was dominated by members of the Proteobacteria and Bacteroidetes, whilst the remaining OTU's were largely composed of members of the Firmicutes and Fusobacteria. The primary phylotypes within the

Proteobacteria belonged to the γ and β subclasses with the most numerous OTU's being identified as *Acidiferrobacter*, *Sedimenticola*, *Arenicella*, *Sphaerotilus*, *Polaromonas*, *Albidiferax* and *Undibacterium*. The phylum Bacteroidetes was principally represented by OTU's belonging to the class Bacteroidia with *Alkalitalea*, *Paludibacter* and *Flectobacillus* being the chief genera detected. The phylum Firmicutes was largely composed of a single OTU of the class Clostridia, identified as *Clostridium sensu stricto*. *Propionigenium* was the primary OTU assigned to the Fusobacteriaceae recorded in the tank biofilm library. The microbiome of the diet pellets was dominated by the phylum Firmicutes (mean sequence abundance 45%) and Candidate Division WPS-1 (mean sequence abundance 34%). Of the Firmicutes, the class Bacilli was well represented, with *Lactobacillus* dominating the sequence libraries in both of the diet pellet samples that were tested.

Statistical analyses

Two separate distance matrices, ThetaYC and Jaccard, were computed in order to compare the structure and membership of the intestinal microbial communities between the two rainbow trout populations sampled. PCoA of the first and second axes of the ThetaYC distances (69% of the total variation) suggested that the microbial community structure between both fish populations was similar, with both sample sets clustering close together (Figure 4a). The AMOVA analysis confirmed that any spatial separation observed in the PCoA of ThetaYC distances was not statistically different between the aquarium and farmed trout ($Fs = 1.20$, $p = 0.292$). Furthermore, Parsimony and UniFrac tests were in agreement with the AMOVA result (ParsSig = 0.085, UWSig = 0.26). The microbiome structure of the biofilm sample was however significantly different from the fish intestinal samples (UWSig = <0.001). In addition, the microbiome of the diet pellets was also found to be significantly different from the intestinal samples (WSig = <0.001, UWSig = 0.003, ParsSig = 0.025).

The Jaccard distance matrix, a further measure of dissimilarity between communities, was calculated to compare the community membership of the samples (Figure 4b). A slight separation in the clustering of both fish populations was observed in the PCoA plots created from this distance matrix. When an AMOVA was performed on this Jaccard matrix, the spatial separation was established as being statistically significant. ($Fs = 2.41$, $p = 0.001$). The Parsimony (ParsSig = <0.001) and UniFrac tests (WScore = 0.894, WSig = <0.001, UWScore = 0.981, UWSig = <0.001) confirmed this result, indicating that the microbial community membership was significantly different between the farmed and the aquarium fish. In addition, the tank biofilm sample was significantly different, in terms of community

membership, from the farmed fish samples (AMOVA Fs = 1.97, p = 0.003), but not from the aquarium fish samples (AMOVA Fs = 1.70, p = 0.096) when clustering from the PCoA was analysed.

Metastats and Indicator analyses revealed that a number of genera were discriminatory according to farming environment (Table S1). The genera *Photobacterium*, *Catellicoccus*, *Moritella*, *Ureibacillus*, *Paralactobacillus*, *Psychrilyobacter*, *Thermobacillus*, *Lactobacillus* and *Fusobacterium* were all discriminatory with the farm based fish and they were significantly more abundant in these individuals. In addition, the genera *Sphaerotilus*, *Maricurvus* and *Weissella* were differentially represented in the aquarium fish (Figure S1a, b).

Predicted functional metagenomes of the rainbow trout intestinal microbiome

PICRUSt was used to predict the functional potential of the intestinal microbiome of rainbow trout. Mean NSTI values were 0.114 ± 0.157 and 0.064 ± 0.116 for the aquarium and farm samples respectively indicating that all samples were tractable for PICRUSt analysis (Langille et al 2013). KEGG orthologs were classified to level 3. The majority of the predicted functional pathways were found to belong to four main categories. These were as follows: 1) metabolism 2) environmental information processing 3) genetic information processing and 4) cellular processes (Figure 5). No significant differences were noted in predicted functional potential between both populations of fish sampled (Figure S2). Within the metabolism pathways, increases in genes associated with carbohydrate, protein and amino acid metabolism were noted, and to a lesser extent pathways associated with energy, vitamin and lipid metabolism. The environmental information processing category was dominated by genes associated with membrane transport and signal transduction. Genes associated with transporters, ABC transporters, the bacterial secretion system, the phosphotransferase system and the two component system were identified. Genetic information processing pathways contained genes involved in protein folding and export, transcription, translation, and DNA replication and repair.

Discussion

The geographical location of fish farms has been posited to have an impact upon the composition of the intestinal microbiome of the cultured individuals, due to the influence of the native microbial ecology of each site (Ringø et al 1995, Holben et al 2002, Lozupone and Knight 2007, Sullam et al 2012, Giatsis et al 2015). To date, most studies have focused on

355 establishing the diversity and stability of salmonid gut microbiomes from single aquaculture
356 facilities (Wong et al 2013, Zarkasi et al 2014). Furthermore, there is a paucity of information
357 relating to the functional potential of these bacteria and how they might influence the overall
358 health of the fish. The study reported here is, to the author's knowledge, the first to employ
359 high throughput sequencing methods to characterize the phylogeny and functionality of the
360 intestinal microbiome of rainbow trout at two different rearing locations. The results of this
361 research have revealed that the overall structure of the microbiome between the farm and
362 aquarium raised fish analysed in this study was very similar, however the community
363 membership was significantly different between the two populations. The data generated
364 using PICRUSt revealed that the predicted functional potential of these communities was
365 similar between both groups, and suggests that these communities might play an active role
366 in the metabolism of dietary ingredients.

367 Phylum level assignment of OTU's indicated a dominance of Tenericutes among all of the
368 fish sampled from both locations. The genus *Mycoplasma* was especially prevalent in all of
369 the rainbow trout intestinal libraries analysed in this study. The phylum Tenericutes was also
370 present in the aquarium biofilm and diet samples tested, but at very low levels of detection
371 when compared with the fish intestinal samples, suggesting that members of this phylum
372 might be specifically adapted to the gastrointestinal environment of farmed rainbow trout.
373 However, further samples of the tank/cage biofilm and diet pellets would need to be collected
374 and analysed from both environments in future studies in order to confirm this hypothesis.
375 *Mycoplasma* were first reported to be a major component of the intestinal microbiome of wild
376 Atlantic salmon (Holben et al 2002) and then the Californian mudsucker (Bano et al 2007)
377 and have since been observed in the GI tract other fish and shellfish species (Moran et al
378 2005, Kim et al 2007, King et al 2012). An increasing number of studies are currently
379 revealing its dominance within the intestine of farmed salmonids (Abid et al 2013, Green et al
380 2013, Zarkasi et al 2014, Llewellyn et al 2015, Lowrey et al 2015, Ozório et al 2015) and yet
381 its function within the GI tract of these fish remains poorly understood. The prevalence of
382 this phylotype in both the aquarium and farm-based fish may suggest that the geographical
383 location of the rearing environment does not impact upon its presence, and that rainbow trout
384 could be a specific host for this microbe. The Mycoplasmataceae are fastidious organisms,
385 and are difficult to grow on conventional microbiological isolation media. This might explain
386 why their abundance in the rainbow trout intestine is now being reported more frequently, as
387 studies that employ high throughput sequencing methods are published.

There was no significant difference in mean microbial diversity between the farm and the aquarium samples. A higher diversity in the farm samples was initially expected, given that the aquarium based fish were maintained in a single aquaculture facility, in flow-through tanks without water recirculation. Furthermore, the aquarium reared trout were obtained from a single supplier and from the same egg source. These combined factors would likely have limited the environmental variation and may have increased the probability of a similar microbiome structure and membership. In contrast, the farm samples were obtained from cages situated in a Scottish loch, and hence these fish were more likely to have been exposed to a greater diversity of microorganisms. However, the mean microbial diversity, whilst slightly higher in the farm samples, was remarkably similar between both populations in spite of the different environmental conditions of each site. This suggests that other factors aside from the geographical location of the culture system may be more influential drivers of microbial diversity in the rainbow trout intestine.

Some studies of the intestinal microbiota of rainbow trout have hypothesized that the composition could mirror that of the surrounding aquatic environment (Trust and Sparrow 1974, Yoshimizu and Kimura 1976, Sugita et al 1982, Ringø and Strom 1994, Nayak 2010, Semova et al 2012, Xing et al 2013, Sullam et al 2015). However, the microbiome structures of the aquarium tank biofilm and the diet samples were significantly different from the intestinal libraries in the present study. These data suggest that the intestinal microbiome may be specialized, and may not simply be a reflection of the microbial flora of the surrounding environment. Future studies should include analyses of the microbiome of the farm and aquarium water in order to further explore this theory. The PCoA revealed a homogeneity between the structure of the intestinal microbiome in the farm and aquarium based fish. However, the community membership was significantly different between the groups. This suggests that the 'core' microbial phyla and classes are somewhat stable in the rainbow trout intestine, regardless of geographical location, but that other assemblages of more sporadic OTU's can vary accordingly. These results reflect those reported in similar studies. Roeselers et al (2011) revealed that individual zebrafish (*Danio rerio*), sampled from wild and domesticated populations, shared a stable core gut microbiome independent of their origin. Another recent study on the wild Atlantic salmon intestinal microbiome found that community composition was not significantly impacted by geography and that individual fish, at different life stages, possessed remarkably similar intestinal microbiome structures which were distinct from those found in the environment (Llewellyn et al 2015).

Furthermore, Bakke et al (2015) reported that cod (*Gadhus morhua*) larvae shared a gut microbiome structure significantly different to that of their rearing water and diet. Taken together, these findings are suggestive of specialized and potentially co-evolved associations between fish species and their intestinal microbiota.

The presence of a number of OTU's that were discriminatory according to geographical location most likely explains the spatial separation observed in the community membership plots. The genus *Lactobacillus* was significantly more abundant in the farm based fish. Its elevated levels suggest that this organism may have been enriched by the diet, possibly as it is known that the relative abundance of this bacterium is affected by diet type (Desai et al 2012, Wong et al 2013, Ingerslev et al 2014), and both populations of fish sampled in this study were fed different diets. Lactobacilli are commonly observed inhabitants of the teleost fish gut, but usually represent a minor proportion of the overall microbial community (Desai et al 2012, Merrifield et al 2014). Other organisms such as *Moritella*, *Photobacterium* and *Psychrilyobacter* were also found to be discriminatory according to location, and were significantly more abundant in the farm raised fish. The exact reason for this is unclear, but some species of *Moritella* and *Photobacterium* are fish pathogens known to cause conditions such as winter ulcer disease and pasteurellosis respectively (Fouz et al 1992, Gauthier et al 1995, Lunder et al 1995, Pedersen at al 1997, Benediktsdottir et al 1998, Bruno et al 1998, Lovoll et al 2009) in farmed salmonids, and all three of these genera are primarily associated with cold water temperatures. It should be noted that at the time of sampling, the fish farm was experiencing the lowest average water temperature recorded for that calendar month in over a decade. This could perhaps explain the enrichment of these psychrophilic bacterial taxa within the intestine of these particular fish.

The principal functional pathways expressed in both populations of fish were primarily associated with metabolism, transport and cellular processes. Membrane transport pathways, such as ABC transporters, utilize the energy of ATP binding and hydrolysis to transport substrates across cellular membranes (Rees et al 2009). They are essential to cell viability and growth and therefore vital for bacterial survival in the intestinal ecosystem. Genes affiliated with the phosphotransferase system (PTS) were found to be abundant in the intestinal microbiomes of both farmed and aquarium reared trout, and this system is used by bacteria for sugar uptake where the source of energy is from phosphoenolpyruvate (PEP), a key intermediate in glycolysis (Meadow et al 1985, Erni 2012). The PTS is a multicomponent network that always involves enzymes of the both the plasma membrane and the cytoplasm,

454 and is involved in transporting many different sugars into bacterial cells, including glucose,
455 mannose, fructose and cellobiose. Two component system pathways, that are commonly
456 found in all prokaryotes, were also enhanced, and modulate gene expression based on
457 environmental stimuli such as temperature, pH and nutrient availability (Mitrophanov and
458 Groisman 2008). The enhancement of these gene pathways suggests that the intestinal
459 microbiome could play an active role in sensing and utilizing sugars as resources for energy
460 production and for the biosynthesis of cellular components.

461 It is well documented that rainbow trout exhibit poor utilization of dietary carbohydrates
462 (Lovell 1989, Guillaume and Choubert 1999, Geurden et al 2014), but the precise reasons for
463 this remain unclear. The involvement of gene pathways dictating carbohydrate metabolism
464 suggests that members of the microbiome may actively carry out fermentative processes
465 within the intestine. Members of the phylum Firmicutes and Spirochaetes are known to play
466 important roles in the fermentation of dietary carbohydrates, transporting non-digestible
467 sugars across their cellular membranes (Corrigan et al 2015). For most microbial
468 fermentations, glucose dissimilation occurs through the glycolytic pathway. The most
469 commonly produced molecule from this process is pyruvate. Therefore, the elevation of the
470 glycolysis/gluconeogenesis and pyruvate metabolism pathways represents a further indication
471 of the fermentative potential of the intestinal microbiome of trout, and may be correlated with
472 the presence of Firmicutes as one of the core microbial phyla observed in the rainbow trout
473 intestine. The fermentation of dietary carbohydrate by members of the intestinal microbiota
474 results in the formation of SCFA such as acetate, propionate and butyrate, which can be
475 utilized in energy metabolism and which have also been shown to promote the health of
476 intestinal enterocytes (Hamer et al 2008, Louis and Flint 2009). Moreover, high
477 concentrations of SCFA have previously been recorded in a variety of fish species, including
478 rainbow trout (Smith et al 1996, Clements et al 2014). The ability of the rainbow trout
479 intestinal microbiome to utilize dietary carbohydrate as an energy yielding substrate is thus
480 an interesting avenue for future research, and may improve our understanding of
481 carbohydrate digestibility in fish.

482 The elevation of gene pathways responsible for amino acid fermentation and peptidase
483 production could be linked to the high protein nature of rainbow trout aquafeeds. Rainbow
484 trout require high levels of dietary protein, i.e. more than 35% of diet dry matter (National
485 Research Council 2011). This is most likely linked to persistent amino acid catabolism for
486 their use as an energy source (Kaushik and Seiliez 2010, Geurden et al 2014). Dietary

proteins that escape digestion by key endogenous digestive enzymes such as chymotrypsin and trypsin are made available to bacteria for fermentation. These enzymes originate in the pancreas and are not produced by the intestine itself (Guillaume and Choubert 1999). Therefore, the fermentative activity of the microbiome may be particularly important in the distal intestinal region, where such enzymes are likely to be less influential. The Clostridia were abundant in all fish, and are recognized as being proteolytic bacteria that can ferment amino acids (Neis et al 2015). The amino sugar metabolic pathway, expressed by the intestinal microbiome of the fish in this study, is specifically responsible for breaking down protein into its constituent di- and tri-peptides and amino acids (Miska et al 2014, Shaufi et al 2015). These can then be utilized in energy metabolism, used to form the structural components of intestinal epithelial cells or exported to the liver for further processing. There is evidence that symbiotic intestinal microbes of other animals manufacture peptidases and amino acids that are then provided to the host (Douglas 2013, Neis et al 2015). Moreover, Clements et al (2014) recently speculated on the involvement of the intestinal microbiota of fish in protein metabolism, and Kuz'mina et al (2015) demonstrated that the intestinal microflora of crucian carp contributed ~45% of total peptidase production in this species. Additionally, Zarkasi et al (2016) reported a progressive enrichment of proteolytic bacteria in the distal intestine of cage farmed Atlantic salmon, concurrent with increasing levels of dietary protein inclusion. The metagenomic data indicate that similar microbially mediated mechanisms of protein breakdown may occur in the rainbow trout intestinal tract, which could supplement the action of endogenous digestive enzymes. Protein fermentation pathways, similar to those for carbohydrate fermentation, can also result in the production of SCFA, especially branched chain fatty acids (BCFA), which can then be metabolized by the host (Jha and Berrocoso 2016).

In summary, the results show that the core microbiome structure between the two populations of rainbow trout remained similar, regardless of the differences in their rearing environment. Five bacterial phyla, the Tenericutes, Firmicutes, Spirochaetes, Proteobacteria and Bacteroidetes were dominant in all of the fish intestine samples. The Tenericutes, and in particular, the genus *Mycoplasma* was the most dominant genus in all read libraries. The pattern of dominance of this microbe, in conjunction with its streamlined genome, is suggestive of an obligate symbiotic relationship with the rainbow trout intestine. No significant differences were observed in microbial community diversity or structure between both groups, indicating that the overall composition of the rainbow trout intestinal

microbiome may be conserved irrespective of the location of the farming system. Significant differences in community membership were however observed, which suggests that more sporadic taxa unique to each environment may successfully inhabit the intestinal tract of the trout. The functional data obtained in this study demonstrate that the rainbow trout intestinal microbiome possesses the capability to influence protein and carbohydrate metabolism, and may therefore complement the action of endogenous digestive enzymes. Future studies should focus on the profiling of metabolites from pathways identified by functional metagenomics, in order to further evaluate the overall contribution of these microbes to the digestive and energetic processes of farmed fish. Such additional research will enhance our ability to exploit the functional potential of the intestinal microbiome, and could aid in the development of novel nutritional strategies that improve the gut health of rainbow trout.

Acknowledgements

The authors would like to thank Mr. Niall Auchinachie of the University of Stirling Institute of Aquaculture for his technical assistance during the aquarium phase of this research. Furthermore, we would like to thank Mr. John Anderson, farm manager at Braevallich fish farm, for kindly providing farm access for the collection of samples.

Conflicts of interest

The authors declare no conflicts of interest.

References

Abid, A., Davies, S.J., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J. and Merrifield, D.L. (2013) Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish and Shellfish Immunology* **35**, 1948-1956

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Bakke, I., Coward, E., Andersen, T. and Vadstein, O. (2015). Selection in the host structures the microbiota associated with developing cod larvae (*Gadhus morhua*). *Environmental Microbiology* **17**, 3914-3924

548 Bano, N., Derae Smith, A., Bennett, W., Vasquez, L. and Hollibaugh, J.T. (2007) Dominance
549 of *Mycoplasma* in the guts of the long-jawed mudsucker, *Gillichthys mirabilis*, from five
550 California salt marshes. *Environmental Microbiology* **9**, 2636-2641

551 Benediktsdottir, E., Helgason, S. and Sigurjonsdottir, H. (1998) *Vibrio* spp. isolated from
552 salmonids with shallow skin lesions and reared at low temperature. *Journal of Fish Diseases*
553 **21**, 19-28

554 Bruno, D.W., Griffiths, J., Petrie, J. and Hastings, T.S. (1998) *Vibrio viscosus* in farmed
555 Atlantic salmon *Salmo salar* in Scotland: field and experimental observations. *Diseases of*
556 *Aquatic Organisms* **34**, 161-166

557 Cahill, M.M. (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41

558 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens,
559 S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G. and Knight, R.
560 (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and
561 MiSeq platforms. *The ISME Journal* **6**, 1621-1624

562 Carda-Dieguez, M., Mira, A. and Fouz, B. (2014) Pyrosequencing survey of intestinal
563 microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets. *FEMS*
564 *Microbiology Ecology* **87**, 451-459

565 Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal
566 microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898

567 Corrigan, A., De Leeuw, M., Penaud-Frezet, S., Dimova, D. and Murphy, R.A. (2015)
568 Phylogenetic and functional alterations in bacterial community compositions in broiler ceca
569 as a result of mannan oligosaccharide supplementation. *Applied and Environmental*
570 *Microbiology* **81**, 3460-3470

571 Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and
572 Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout
573 (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

574 Douglas, A.E. (2013) Microbial brokers of insect-plant interactions revisited. *Journal of*
575 *Chemical Ecology* **39**, 952-961

576 Erni, B. (2012) The bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS):
577 an interface between energy and signal transduction. *Journal of the Iranian Chemical Society*
578 **10**, 593-630

579 Fouz, B., Larsen, J.L., Nielsen, B., Barja, J.L. and Toranzo, A.E. (1992) Characterization of
580 *Vibrio damsela* strains isolated from turbot *Scopthalmus maximus* in Spain. *Diseases of*
581 *Aquatic Organisms* **12**, 155-156

582 Gauthier, G., Lafay, B., Ruimy, R., Breittmayer, V., Nicholas, J.L., Gauthier, M. and
583 Christen, R. (1995) Small sub-unit rRNA sequences and whole DNA relatedness concur for
584 the reassignment of *Pasteurella piscicida* (Snieszko et al.) Janssen and Surgalla to the genus
585 *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. *International*
586 *Journal of Systematic Bacteriology* **45**, 139-144

587 Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D.,
588 Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S. and Panserat, S. (2014) High or low
589 dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and
590 intestinal microbiota in juvenile rainbow trout. *The Journal of Experimental Biology* **217**,
591 3396-3406

592 Ghanbari, M., Kneifel, W. and Domig, K. (2015) A new view of the fish gut microbiome:
593 Advances from next-generation sequencing. *Aquaculture* **448**, 464-475

594 Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J. and Verdegem, M.
595 (2015) The impact of rearing environment on the development of gut microbiota in tilapia
596 larvae. *Scientific Reports* **5**: 18206, **doi**: 10.1038/srep18206

597 Green, T.J., Smullen, R. and Barnes, A.C. (2013) Dietary soybean protein concentrate-
598 induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with
599 alterations in gut microbiota. *Veterinary Microbiology* **166**, 286-292

600 Guillaume, J. and Choubert, G. (1999) Digestive physiology and nutrient digestibility in
601 fishes. In: *Nutrition and Feeding of Fish and Crustaceans*. (translated by Watson, J.)
602 (Guillaume, G., Kaushik, S., Bergot, P. and Metailler, R. Eds.) pp. 27-58, Springer-Praxis
603 Publishing, Chichester, UK

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J. and Brummer, R.J. (2008) Review article: the role of butyrate on colonic function. *Alimentary Pharmacology and Therapeutics* **27**, 104-119

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H., (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Ingerslev, H., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **424**, 24-34

Jha, R. and Berrocoso, J.F.D. (2016) Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Animal Feed Science and Technology* **212**, 18-26

Kaushik, S.J. and Seiliez, I. (2010) Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research* **41**, 322-332

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

King, G.M., Judd, C., Kuske, C.R. and Smith, C. (2012) Analysis of stomach and gut microbiomes of the eastern oyster (*Crossotrea virginica*) from coastal Louisiana, USA. *PLOS ONE* **7,** e51475

Kormas, K.A., Meziti, A., Mente, E. and Frentzos, A. (2014) Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open* **3**, 718-728

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. (2013) Development of a dual indexing sequencing strategy and curation pipeline for analyzing amplicon sequencing data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79**, 5112-5120

Kuz'mina, V.V., Skvortsova, E.G., Shalygin, M.V. and Kovalenko, K.E. (2015) Role of peptidases of the intestinal microflora and prey in temperature adaptations of the digestive

633 system in planktivorous and benthivorous fish. *Fish Physiology and Biochemistry* **41**, 1359-
634 1368

635 Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A.,
636 Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G. and
637 Huttenhower, C. (2013) Predictive functional profiling of microbial communities using 16S
638 rRNA marker gene sequences. *Nature Biotechnology* **31**, 814-821

639 Letunic, I., Yamada, T., Kanehisa, M. and Bork, P. (2008) iPath: interactive exploration of
640 biochemical pathways and networks. *Trends in Biochemical Sciences* **33**, 101-103

641 Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N. (2014) Teleost microbiomes:
642 the state of the art in their characterization, manipulation and importance in aquaculture and
643 fisheries. *Frontiers in Microbiology* **5**: 207, **doi**: 10.3389/fmicb.2014.00207

644 Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R.,
645 Creer, S. and Derome, N. (2015) The biogeography of the Atlantic salmon (*Salmo salar*) gut
646 microbiome. *The ISME Journal* **10**, 1280-1284

647 Louis, P. and Flint, H.J. (2009) Diversity, metabolism and microbial ecology of butyrate
648 producing bacteria from the human large intestine. *FEMS Microbiology Letters* **294**, 1-8

649 Lovell, T. (1989) Feed formulation and processing. In: *Nutrition and Feeding of Fish*.
650 pp.175-192, Springer, US

651 Lovoll, M., Wiik-Nielsen, C.R., Tunsjo, H.S., Colquhoun, D., Lunder, T., Sorum, H. and
652 Grove S. (2009) Atlantic salmon bath challenged with *Moritella viscosa* – pathogen invasion
653 and host response. *Fish and Shellfish Immunology* **26**, 877-884

654 Lowrey, L., Woodhams, D.C., Tacchi, L. and Salinas, I. (2015) Topographical mapping of
655 the rainbow trout microbiome reveals a diverse bacterial community in the skin with
656 antifungal properties. *Applied and Environmental Microbiology* **81**, 6915-6925

657 Lozupone, C.A. and Knight, R. (2005) UniFrac: A new phylogenetic method for comparing
658 microbial communities. *Applied and Environmental Microbiology* **71**, 8228-8235

659 Lozupone, C.A. and Knight, R. (2007) Global patterns in bacterial diversity. *Proceedings of*
660 *the National Academy of Sciences* **104**, 11436-11440

Lunder, T., Evensen, O., Holstad, G. and Halstein, T. (1995) 'Winter ulcer' in the Atlantic salmon *Salmo salar*. Pathological and bacteriological investigations and transmission experiments. *Diseases of Aquatic Organisms* **23**, 39-49

McCune, B., Grace, J.B. and Urban, D.L. (2002) Analysis of ecological communities. MjM Software, Gleneden Beach, Oregon USA

Meadow, N.D., Kukuruzinska, M.A. and Roseman, S. (1985) The bacterial phosphoenolpyruvate: sugar phosphotransferase system. In: *The Enzymes of Biological Membranes* (2nd edn.) (Martonosi, A. Ed.) pp. 523-559, Springer, US

Merrifield, D.L., Balcazar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.Z., Hoseinifar, S.H. and Ringø, E. (2014) Indigenous lactic acid bacteria in fish and crustaceans. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (Merrifield, D. and Ringø, E., Eds.) pp. 128-168, WILEY Blackwell, Oxford, UK

Miska, K.B., Fetterer, R.H. and Wong, E.A. (2014) The expression of amino acid transporters, aminopeptidase N, and the di- and tri-peptide transporter PepT1 in the embryo of the domesticated chicken (*Gallus gallus*) shows developmental regulation. *Poultry Science* **93**, 2262-2270

Mitrophanov, A.Y. and Groisman, E. (2008) Signal integration in bacterial two-component regulatory systems. *Genes and Development* **22**, 2601-2611

Miyake, S., Ngugi, D.K. and Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology* **24**, 656-672

Moran, D., Turner, S.J. and Clements, K.D. (2005) Ontogenetic development of the gastrointestinal microbiota in the marine herbivorous fish *Kyphosus sydneyanus*. *Microbial Ecology* **49**, 590-597

National Research Council (2011) *Nutrient Requirements of Fish and Shrimp*. National Academy Press, Washington D.C.

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41,** 1553-1573

Neis, E.P.J.G., Dejong, C.H.C. and Rensen, S.S. (2015) The role of microbial amino acid metabolism in host metabolism. *Nutrients* **7**, 2930-2946

690 Ozório, R.O.A., Kopecka-Pilarczyk, J., Peixoto, M.J., Lochmann, R., Santos, R.J., Santos, G.,
691 Weber, B., Calheiros, J., Ferrez-Arruda, L., Vaz-Pires, P. and Goncalves, J.F.M. (2015)
692 Dietary probiotic supplementation in juvenile rainbow trout (*Oncorhynchus mykiss*) reared
693 under cage culture production: effects on growth, fish welfare, flesh quality and intestinal
694 microbiota. *Aquaculture Research* **doi:** 10.1111/are.12724

695 Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. (2014) STAMP: Statistical
696 analysis of taxonomic and functional profiles. *Bioinformatics* **30**, 3123-3124

697 Pedersen, K., Dalsgaard, I. and Larsen, J.L. (1997) *Vibrio damsela* associated with diseased
698 fish in Denmark. *Applied and Environmental Microbiology* **63**, 711-715

699 Pourabedin, M. and Zhao, X. (2015) Prebiotics and gut microbiota in chickens. *FEMS*
700 *Microbiology Letters* **362**, **doi**:10.1093/femsle/fnv122

701 Rees, D.C., Johnson, E. and Lewinson, O. (2009) ABC transporters: the power to change.
702 *Nature Reviews Molecular Cell Biology* **10**, 218-227

703 Ringø, E. and Strom, E. (1994) Microflora of Arctic charr, *Salvelinus alpinus* (L):
704 gastrointestinal microflora of free living fish and effect of diet and salinity on intestinal
705 microflora. *Aquaculture and Fish Management* **25**, 623-629

706 Ringø, E., Strom, E. and Tabachek, J.A. (1995) Intestinal microflora of salmonids: a review.
707 *Aquaculture Research* **26**, 773-789

708 Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K.
709 and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*
710 **5**, 1595-1608

711 Schloss, P.D. (2009) Introducing mothur: Open-source, platform-independent, community-
712 supported software for describing and comparing microbial communities. *Applied and*
713 *Environmental Microbiology* **75**, 7537-7541

714 Schloss, P.D. and Handelsman, J. (2006) Introducing TreeClimber, a test to compare
715 microbial community structures. *Applied and Environmental Microbiology* **72**, 2379-2384

716 Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A. and Rawls,
717 J.F. (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the
718 zebrafish. *Cell Host and Microbe* **12**, 277-288

719 Shaufi, M.A.M, Sieo, C.C., Chong, C.W., Gan, H.M. and Ho, Y.W. (2015) Deciphering
720 chicken gut microbial dynamics based on high throughput 16S r RNA metagenomics
721 analyses. *Gut Pathogens* **7**:4, **doi:** 10.1186/s13099-015-0051-7

722 Smith, T.B., Wahl, D.H. and Mackie, R.I. (1996) Volatile fatty acids and anaerobic
723 fermentation in temperate piscivorous and omnivorous freshwater fish. *Journal of Fish*
724 *Biology* **48**, 829-841

725 Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The
726 microflora of rainbow trout intestine: a comparison of traditional and molecular
727 identification. *Aquaculture* **182**, 1-15

728 Sugita, H., Isida, Y., Deguchi, Y. And Kadota, H. (1982) Bacterial flora in the gastrointestine
729 of *Tilapia nilotica* adapted in sea water. *Bulletin of the Japanese Society of Scientific*
730 *Fisheries* **49**, 987-991

731 Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R.,
732 Kilham, S.S. and Russell, J.A. (2012) Environmental and ecological factors that shape the gut
733 bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378

734 Sullam, K.E., Rubin, B.E., Dalton, C.M., Kilham, S.S., Flecker, A.S., and Russell, J.A.
735 (2015) Divergence across diet, time and populations rules out parallel evolution in the gut
736 microbiomes of Trinadadian guppies. *The ISME Journal* **9,** 1508-1522

737 Trust, T.J. and Sparrow, R.A.H. (1974) The bacterial flora in the alimentary tract of
738 freshwater salmonid fishes. *Canadian Journal of Microbiology* **20**, 1219-1228

739 Turnbaugh, P.J. and Gordon, J.I. (2009). The core gut microbiome, energy balance and
740 obesity. *The Journal of Physiology* **587**, 4153-4158.

741 Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA
742 amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703

743 White, J.R., Nagarajan, N. and Pop, M. (2009) Statistical methods for detecting differentially
744 abundant features in clinical metagenomics samples. *PLOS Computational Biology* **5**,
745 e1000352

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y. and Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scopthalmus maximus*) *FEMS Microbiology Ecology* **86**, 432-443

Yamada, T., Letunic, I., Okuda, S., Kanehisa, M. and Bork, P. (2011) iPath 2.0: interactive pathway explorer. *Nucleic Acids Research* **39,** 412-415.

Yoshimizu, M. and Kimura, T. (1976) Studies of the intestinal microflora of salmonids. *Fish Pathology* **10**, 243-259

Yue, J.C. and Clayton, M.K. (2005) A similarity measure based on species proportions. *Communications in Statistics Theory and Methods* **34**, 2123-2131

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

Zarkasi, K., Taylor, R.S., Abell, G.C.J, Tamplin, M.L., Glencross, B.D. and Bowman, J.P. (2016) Atlantic Salmon (*Salmo salar* L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet. *Microbial Ecology* **71,** 589-603

Supporting data legends

Table S1. **Phylotypes identified as discriminatory according to rearing environment by both Metastats and Indicator analyses. Statistical significance was accepted on two levels (p<0.05, p<0.01**)

Figure S1. Bacterial taxa identified by Metastats and Indicator analysis as discriminatory between aquarium and farm based rainbow trout intestinal samples. The data are plotted as mean relative percentage sequence abundance ± SEM *P<0.05 **P<0.01. Data are split into **a** and **b** to improve interpretation.

Figure S2. Principal coordinate analysis (PCoA) of predicted functional metagenomes between intestinal microbiomes of aquarium and farm-based rainbow trout. Each dot represents an individual sample.

779

780

Figure Legends

Figure 1: Rarefaction analysis of a) aquarium and b) farm based rainbow trout intestinal microbiome samples. Samples were rarefied according to the library with the lowest number of reads (n = 142267, Sample DF AF1).

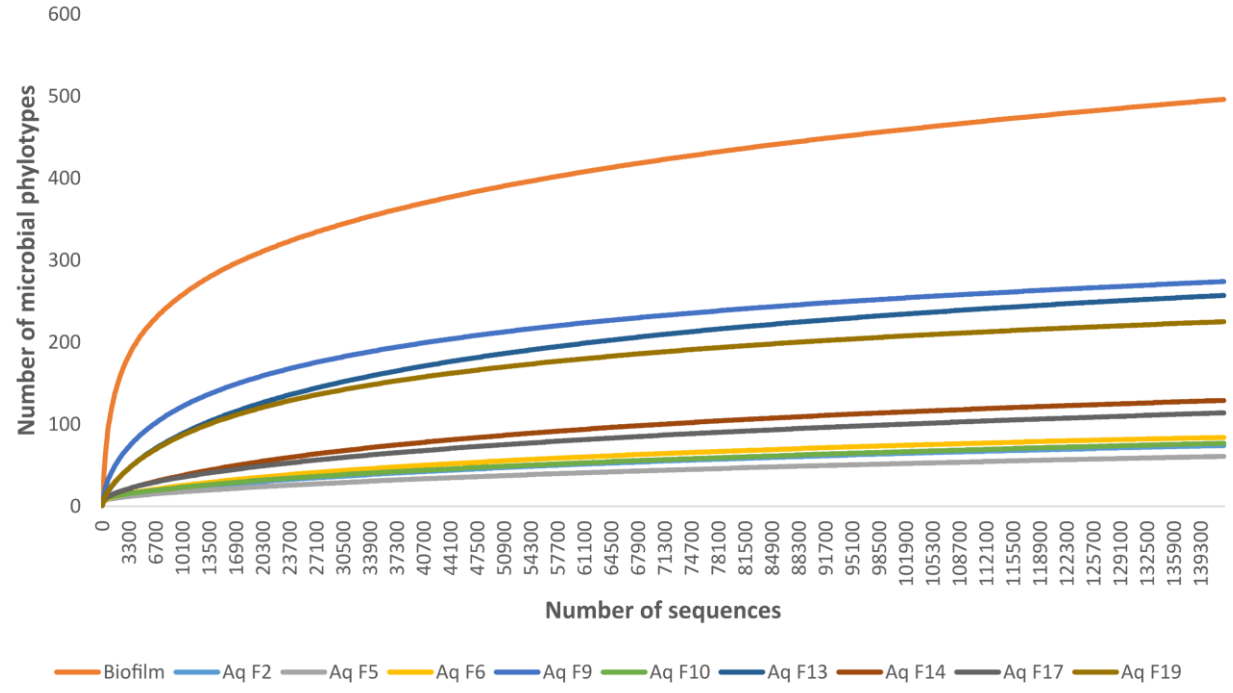
Figure 2: Mean relative % sequence abundance of microbial phyla recorded in the distal intestine of a) aquarium and b) farm-based fish.

Figure 3: Relative % sequence abundance of aquarium tank biofilm, diet and intestinal microbial classes observed in individual fish sampled from a) aquarium (n=9) and b) farm (n = 12).

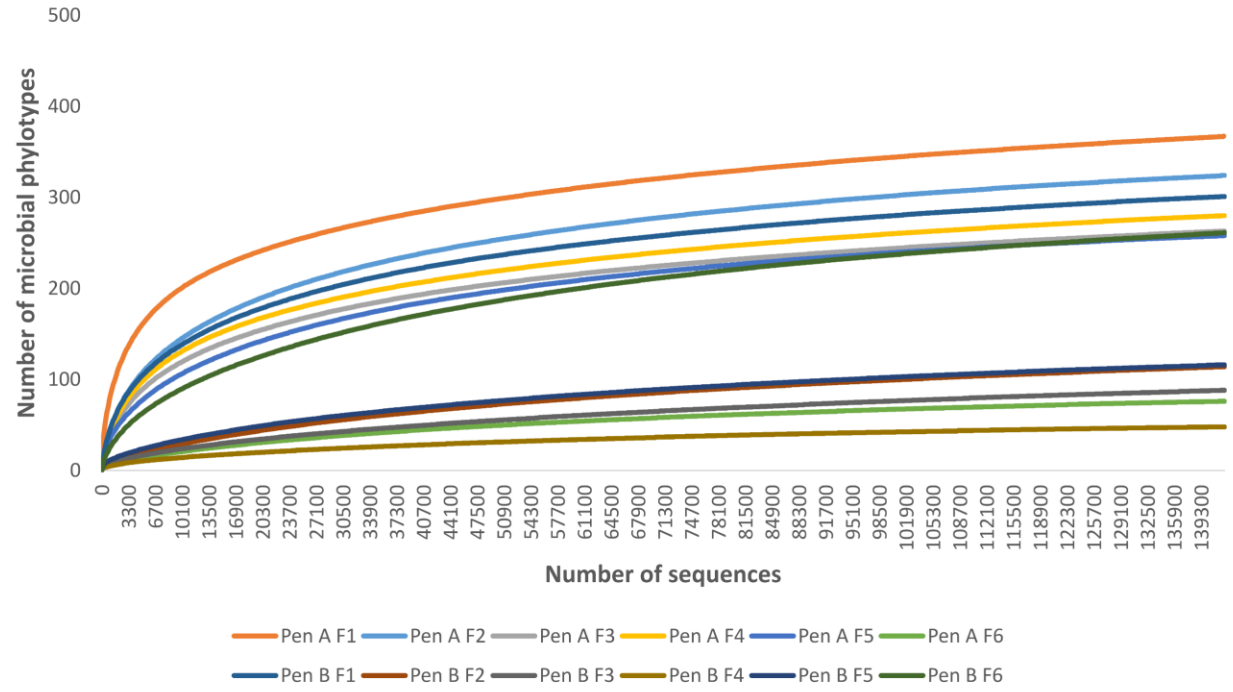
Figure 4: Principal coordinate analysis (PCoA) depicting differences in microbial community structure and membership between aquarium fish, farm-based fish, tank biofilm and diet pellet samples based on a) ThetaYC and b) Jaccard distances respectively. Each dot represents an individual sample.

Figure 5: Predicted functional metagenomic pathways of rainbow trout intestinal microbiome, as identified by PICRUSt and STAMP analyses.

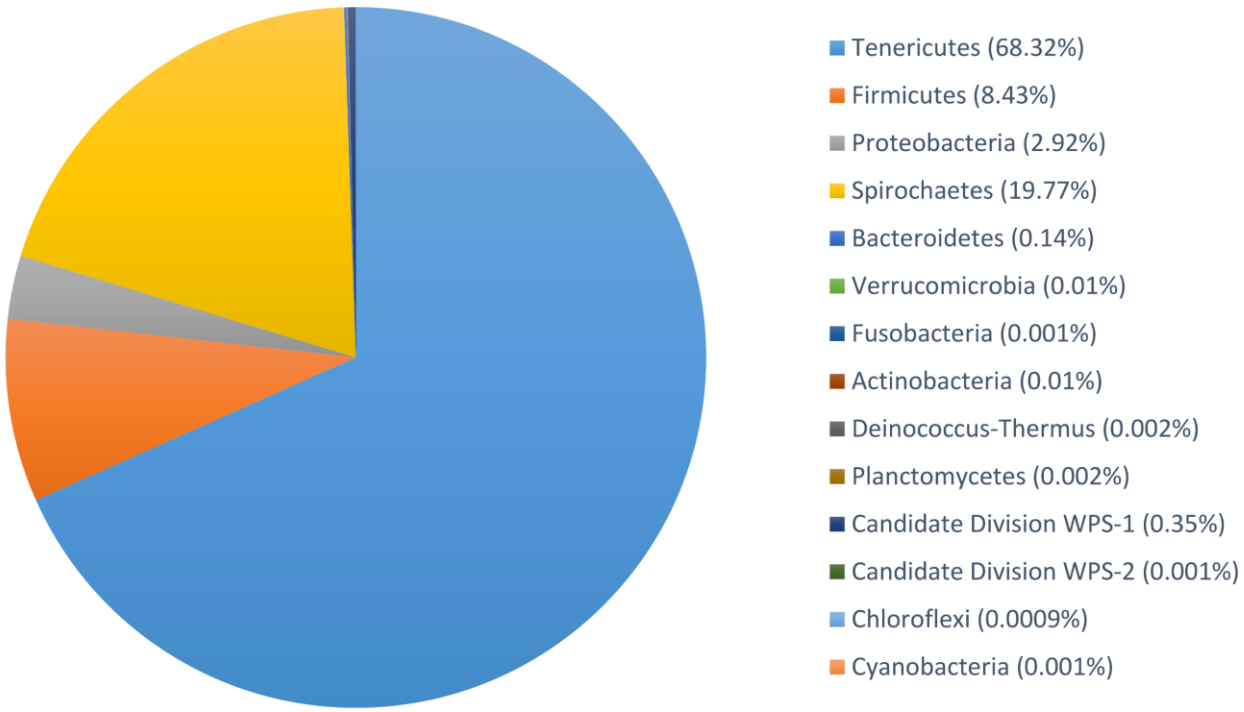
a)


Number of microbial phylotypes
600
500
400
300
200
100
0
0
3300
6700
10100
13500
16900
20300
23700
27100
30500
33900
37300
40700
44100
47500
50900
54300
57700
61100
64500
67900
71300
74700
78100
81500
84900
88300
91700
95100
98500
101900
105300
108700
112100
115500
118900
122300
125700
129100
132500
135900
139300
Number of sequences
Biofilm
Aq F2
Aq F5
Aq F6
Aq F9
Aq F10
Aq F13
Aq F14
Aq F17
Aq F19

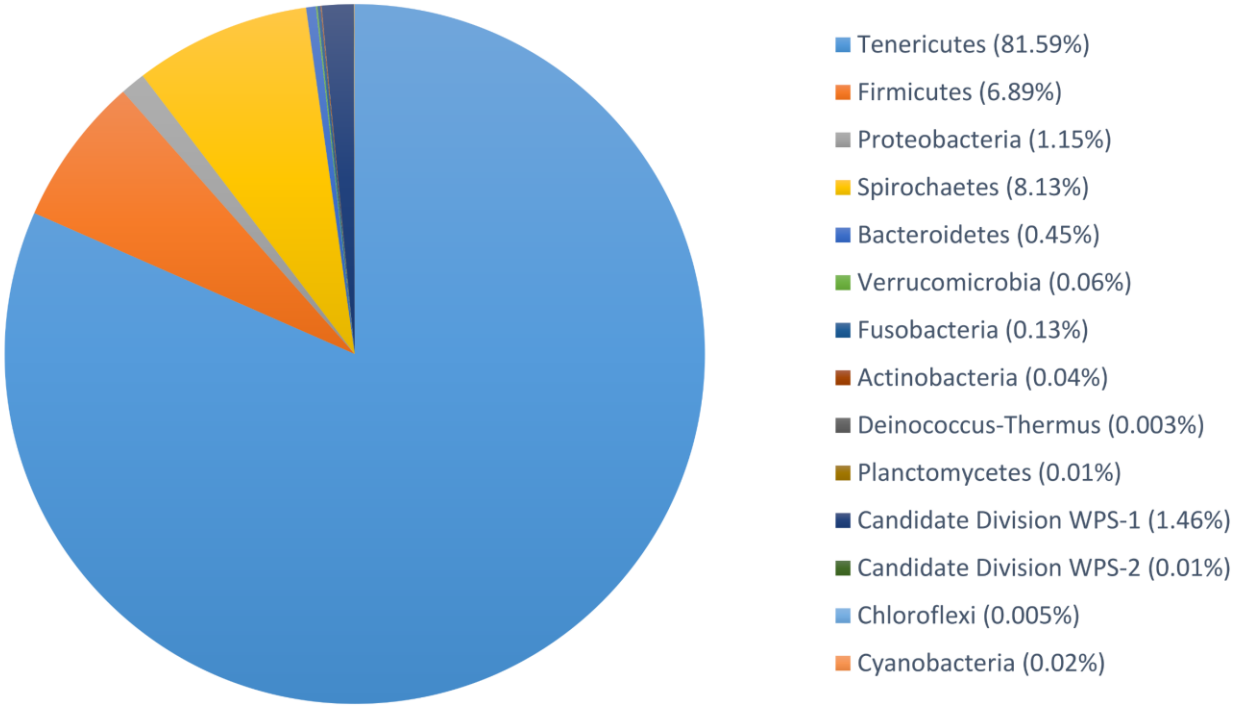
b)


Number of microbial phylotypes
500
400
300
200
100
0
0
3300
6700
10100
13500
16900
20300
23700
27100
30500
33900
37300
40700
44100
47500
50900
54300
57700
61100
64500
67900
71300
74700
78100
81500
84900
88300
91700
95100
98500
101900
105300
108700
112100
115500
118900
122300
125700
129100
132500
135900
139300
Number of sequences
Pen A F1
Pen A F2
Pen A F3
Pen A F4
Pen A F5
Pen A F6
Pen B F1
Pen B F2
Pen B F3
Pen B F4
Pen B F5
Pen B F6

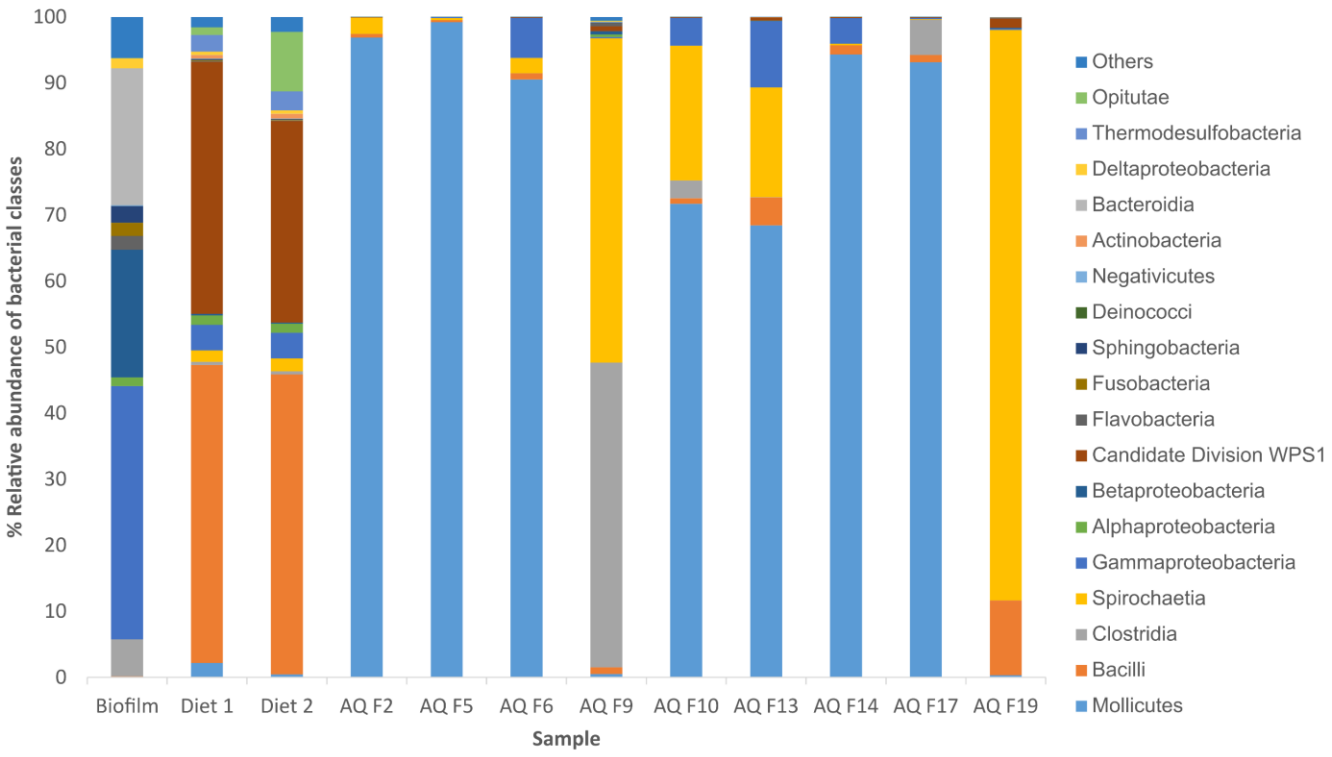
a)


Tenericutes (68.32%)
Firmicutes (8.43%)
Proteobacteria (2.92%)
Spirochaetes (19.77%)
Bacteroidetes (0.14%)
Verrucomicrobia (0.01%)
Fusobacteria (0.001%)
Actinobacteria (0.01%)
Deinococcus-Thermus (0.002%)
Planctomycetes (0.002%)
Candidate Division WPS-1 (0.35%)
Candidate Division WPS-2 (0.001%)
Chloroflexi (0.0009%)
Cyanobacteria (0.001%)

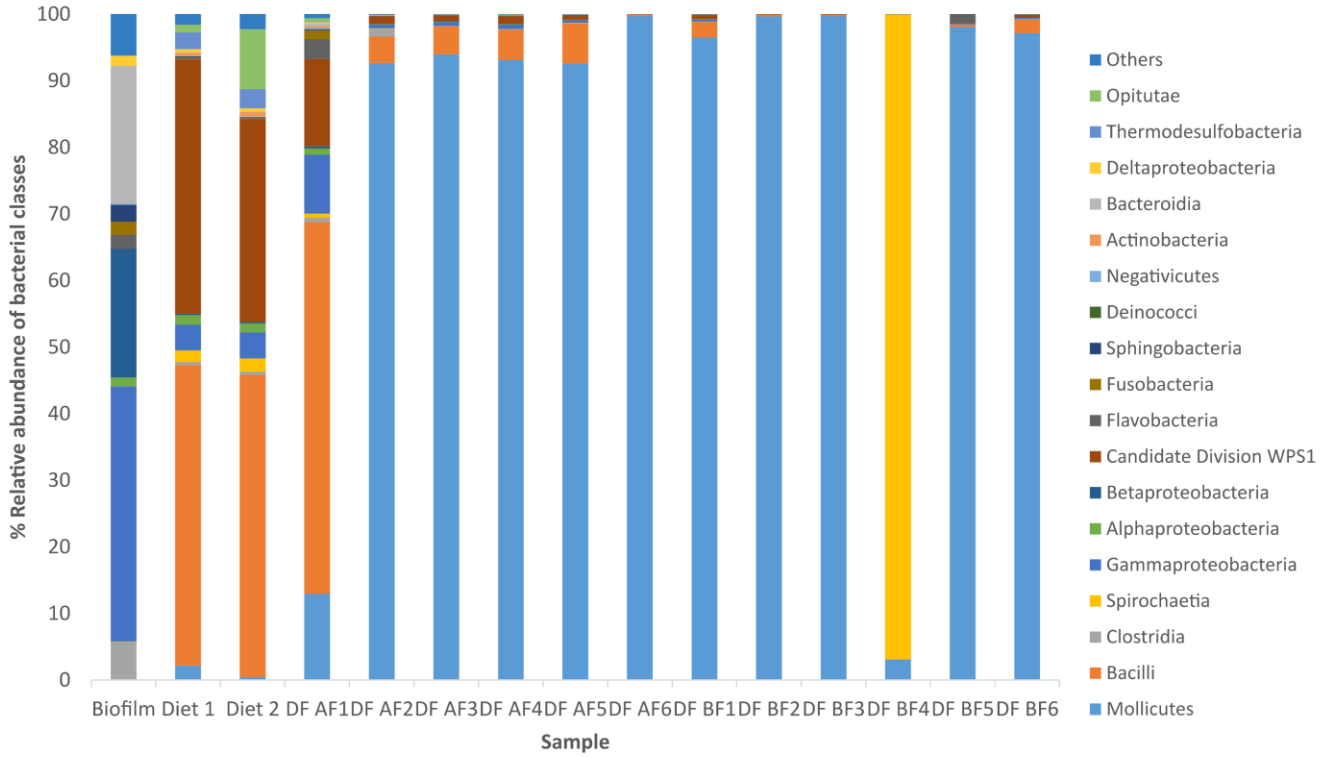
b)


Tenericutes (81.59%)
Firmicutes (6.89%)
Proteobacteria (1.15%)
Spirochaetes (8.13%)
Bacteroidetes (0.45%)
Verrucomicrobia (0.06%)
Fusobacteria (0.13%)
Actinobacteria (0.04%)
Deinococcus-Thermus (0.003%)
Planctomycetes (0.01%)
Candidate Division WPS-1 (1.46%)
Candidate Division WPS-2 (0.01%)
Chloroflexi (0.005%)
Cyanobacteria (0.02%)

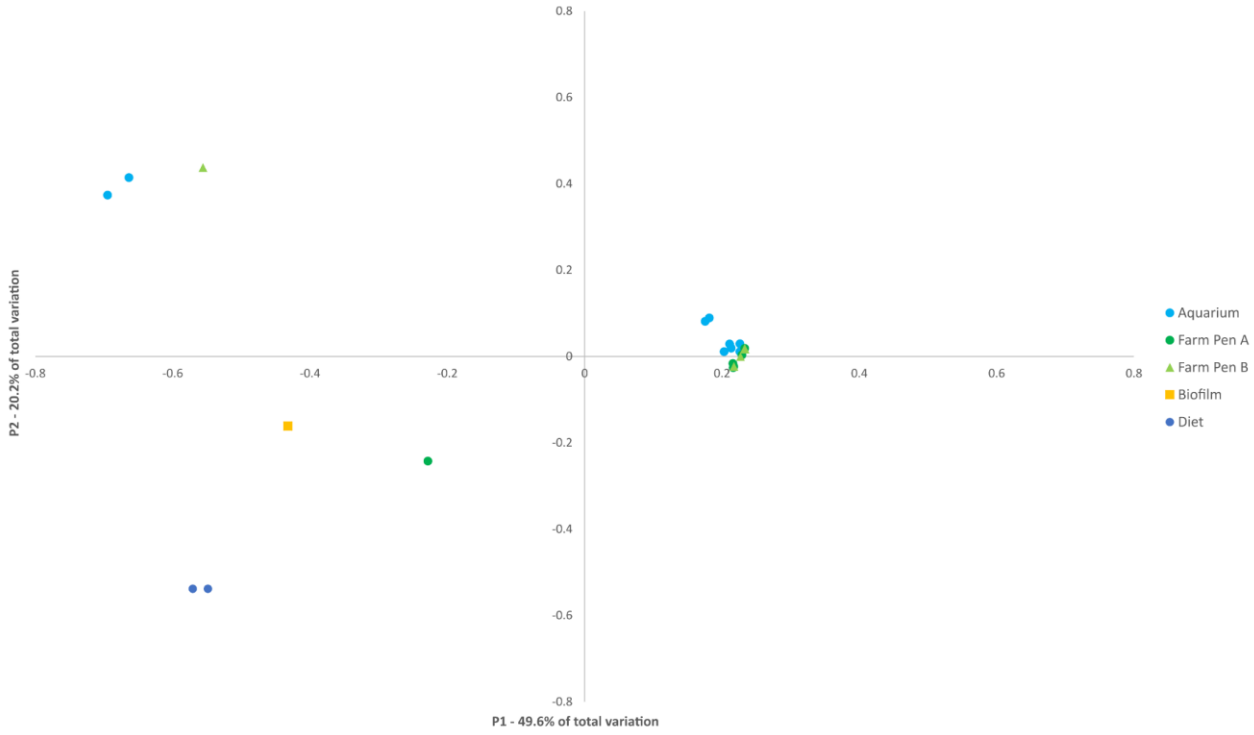
a)


% Relative abundance of bacterial classes
100
90
80
70
60
50
40
30
20
10
0
Biofilm
Diet 1
Diet 2
AQ F2
AQ F5
AQ F6
AQ F9
AQ F10
AQ F13
AQ F14
AQ F17
AQ F19
Sample
Others
Opitutae
Thermodesulfobacteria
Deltaproteobacteria
Bacteroidia
Actinobacteria
Negativicutes
Deinococci
Sphingobacteria
Fusobacteria
Flavobacteria
Candidate Division WPS1
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Spirochaetia
Clostridia
Bacilli
Mollicutes

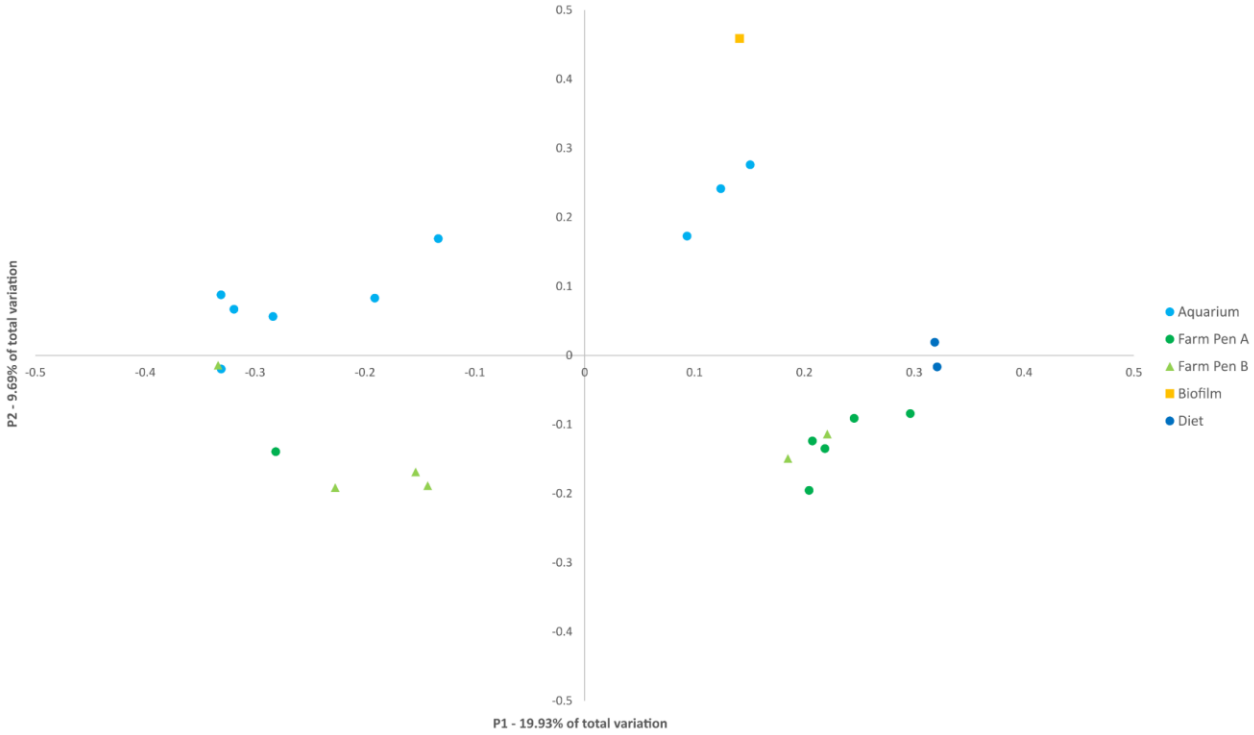
b)

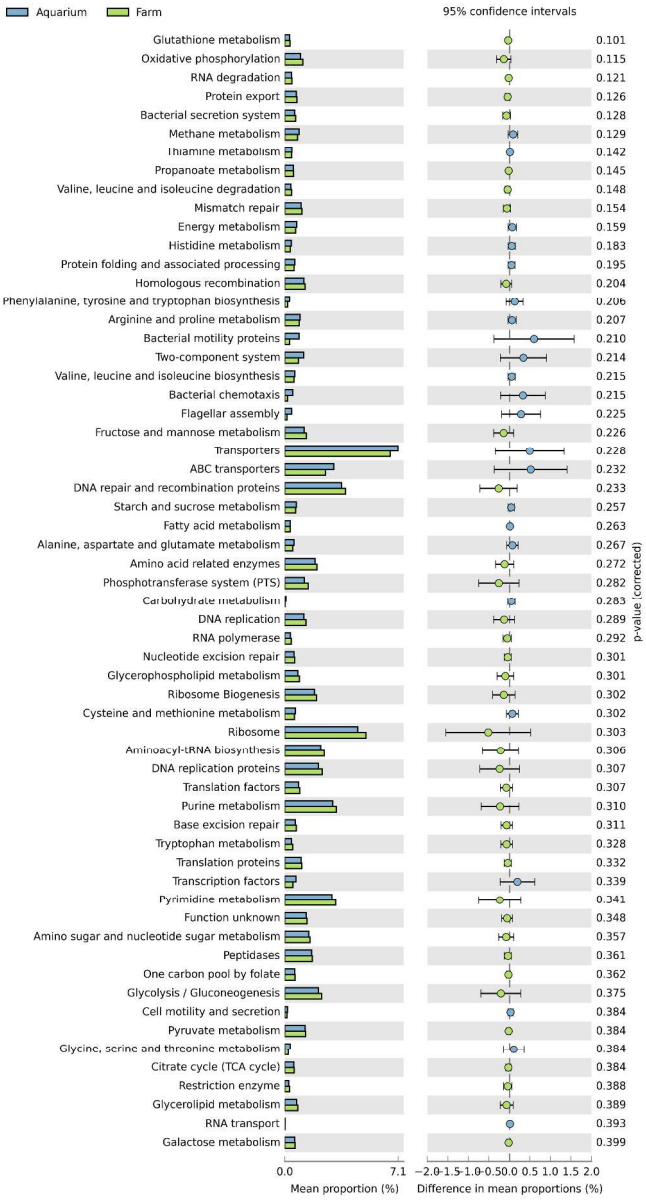

% Relative abundance of bacterial classes
100
90
80
70
60
50
40
30
20
10
0
Biofilm
Diet 1
Diet 2
DF AF1
DF AF2
DF AF3
DF AF4
DF AF5
DF AF6
DF BF1
DF BF2
DF BF3
DF BF4
DF BF5
DF BF6
Sample
Others
Opitutae
Thermodesulfobacteria
Deltaproteobacteria
Bacteroidia
Actinobacteria
Negativicutes
Deinococci
Sphingobacteria
Fusobacteria
Flavobacteria
Candidate Division WPS1
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Spirochaetia
Clostridia
Bacilli
Mollicutes

a)


0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
P2 - 20.2% of total variation
P1 - 49.6% of total variation
Aquarium
Farm Pen A
Farm Pen B
Biofilm
Diet

b)


0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
P2 - 9.69% of total variation
P1 - 19.93% of total variation
Aquarium
Farm Pen A
Farm Pen B
Biofilm
Diet



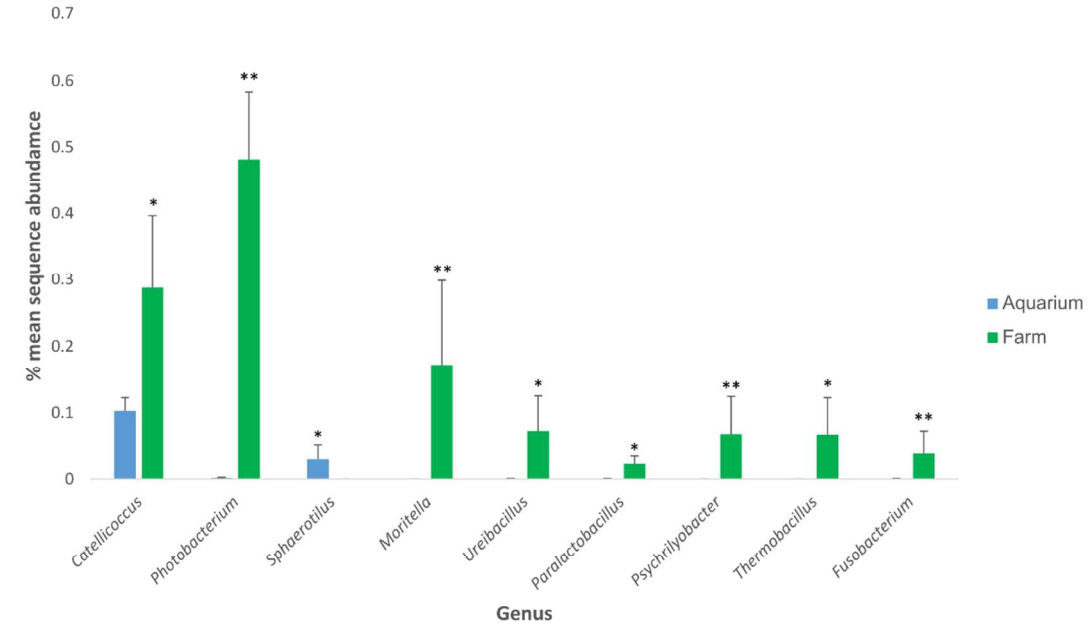
Predicted functional metagenomic pathways of rainbow trout intestinal microbiome, as identified by PICRUSt and STAMP analyses.

321x580mm (300 x 300 DPI)

Table S1. Phylotypes identified as discriminatory according to rearing environment by both Metastats and Indicator analyses. Statistical significance was accepted on two levels ($p<0.05$, $p<0.01$)

	p value		
Phylotype	**Metastats**	**Indicator**	**Discriminator**
Photobacterium	0.0009	0.0009	Farm
Catellicoccus	0.001	0.034	Farm
Moritella	0.0009	0.0009	Farm
Ureibacillus	0.001	0.014	Farm
Paralactobacillus	0.0009	0.007	Farm
Psychrilyobacter	0.0009	0.002	Farm
Thermobacillus	0.0009	0.044	Farm
Lactobacillus	0.0009	0.006	Farm
Fusobacterium	0.0009	0.0009	Farm
Maricurvus	0.001	0.049	Aquarium
Weissella	0.0009	0.003	Aquarium
Sphaerotilus	0.001	0.031	Aquarium

a)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% mean sequence abundamce
Catellicoccus
Photobacterium
Sphaerotilus
Moritella
Ureibacillus
Paralactobacillus
Psychrilyobacter
Thermobacillus
Fusobacterium
Genus
Aquarium
Farm

b)

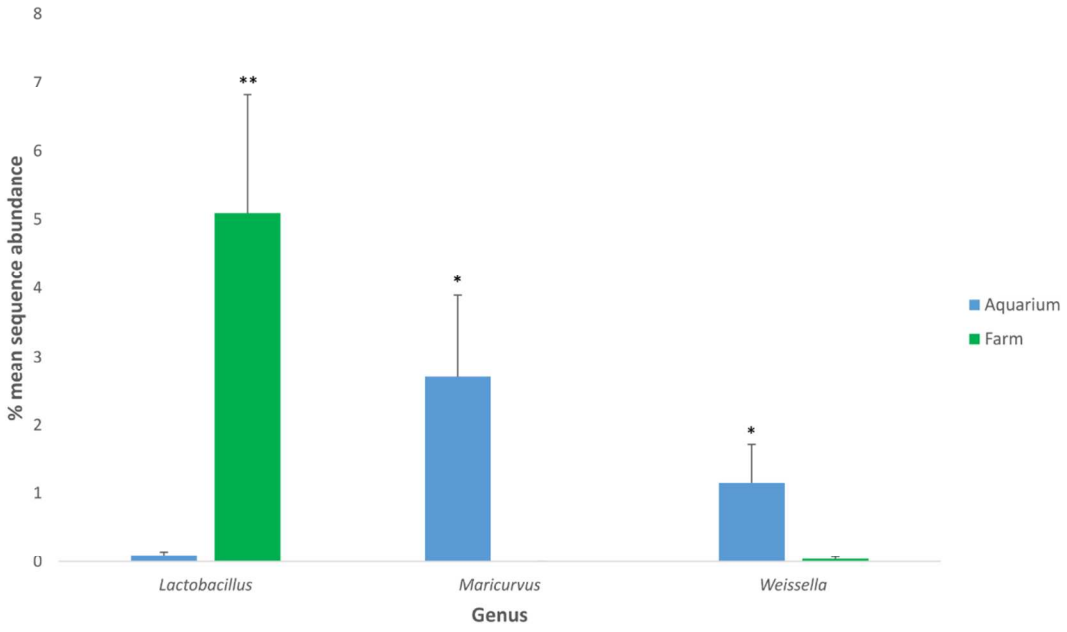

8
7
6
5
4
3
2
1
0
% mean sequence abundance
Lactobacillus
Maricurvus
Weissella
Genus
Aquarium
Farm

Figure S1. Bacterial taxa identified by Metastats and Indicator analysis as discriminatory between aquarium and farm based rainbow trout intestinal samples. The data are plotted as mean relative percentage sequence abundance ± SEM *P<0.05 **P<0.01. Data are split into **a** and **b** to improve interpretation.

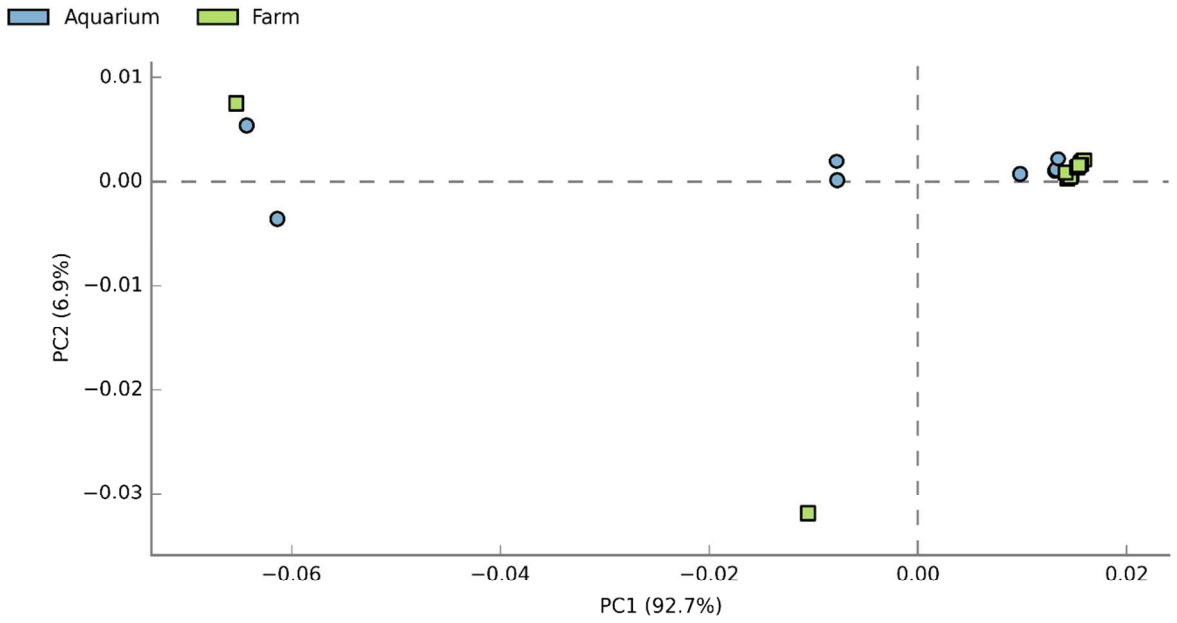


Figure S2. Principal coordinate analysis (PCoA) of predicted functional metagenomes between intestinal microbiomes of aquarium and farm-based rainbow trout. Each dot represents an individual sample.