

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

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

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9 **Running head:** Phylogeny and function of trout gut microbiome

10 **Abstract**

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

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

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

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

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

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

37 **Introduction**

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

48 Rainbow trout are reared in a number of different aquaculture settings, such as earthen ponds,
49 raceways, inshore tank systems and in freshwater/seawater cages. It has been hypothesized
50 that these different farming environments can shape the composition of the gastrointestinal
51 microbiome, with different taxa dominating according to the geographical location and
52 environmental conditions of the farm in question (Cahill 1990, Spanggaard et al 2000, Nayak
53 2010). The 'core' microbiome concept proposes that individual hosts maintained under the
54 same husbandry conditions, in the same environment and location will share similar
55 microbial taxonomic compositions (Turnbaugh and Gordon 2009, Wong et al 2013). Novel
56 nutritional strategies such as pro-, pre- and synbiotic feeds have been developed which aim to
57 modulate the gut microbiota, especially in light of the industry's commitment to reduce its
58 use of both antibiotics, and fish meal/oil sourced from wild pelagic fisheries. Therefore, it is
59 important to determine the potential existence of a core microbiome amongst rainbow trout,
60 and whether such a core is shared even amongst fish reared in different geographical

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

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

77 Furthermore, although a clearer picture has emerged of the extent of the microbial diversity
78 within the rainbow trout intestinal microbiome, thus far no reports have been documented
79 concerning the functional capability of these communities. Therefore, this study employed a
80 novel but well validated computational approach, PICRUSt (Langille et al 2013)
81 (<http://picrust.github.io/picrust>), to predict the potential functional capacity of the intestinal
82 microbiome and to complement the phylogenetic data generated. PICRUSt uses an extended
83 ancestral-state reconstruction algorithm to predict which gene families are present within 16S
84 rRNA libraries, and then combines those gene families to estimate the composite
85 metagenome. This approach has been used successfully to study the cecal microbiome of the
86 farmed broiler chicken *Gallus gallus domesticus* (Corrigan et al 2015, Pourabedin and Zhao
87 2015, Shaufi et al 2015). A detailed knowledge of the phylogenetic profile and functional
88 capacities of the intestinal microbiota is extremely important in order to aid our
89 understanding of the role of these microorganisms in fish health and digestive physiology.

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

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

97 **Materials and methods**

98 *Sample collection*

99 A total of twelve rainbow trout were collected from a freshwater fish farm based on Loch
100 Awe, Argyll, Scotland. Six fish were each randomly sampled from two separate pens,
101 identified as A and B. The water temperature at the time of sampling was 9.4°C. The fish
102 from each pen originated from different egg sources, but were raised at the same hatchery.
103 All individuals collected on the day of sampling were apparently healthy, that is, with no
104 visual signs of disease or parasites on the skin or internal organs. All fish were fed the same
105 commercial pelleted feed. The mean weight (\pm SD) of the fish from pen's A and B was $119 \pm$
106 24 g and 79 ± 10 g respectively at the time of sampling.

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

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

125 *DNA extraction*

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

143 *16S rRNA PCR and Illumina sequencing*

144 A PCR was firstly carried out using universal eubacterial primers 27F
145 AGAGTTTGATCMTGGCTAG and 1492R TACGGYTACCTTGTTACGACTT (Weisburg
146 et al 1991) that target the full length bacterial 16S rRNA gene sequence, to confirm the
147 presence of ample microbial community DNA and to rule out the presence of any potential
148 inhibitory compounds. The extraction from the sample containing buffer ASL only was
149 included in this PCR as a negative control. The PCR conditions for this confirmatory reaction
150 were as follows: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at
151 94°C for 2 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, before final
152 elongation of 72°C for 10 min. Products were then visualized on a 1.5% (w/v) agarose gel,
153 run at 100V for approximately 75 min. The presence of a single strong PCR product of
154 ~1500bp was considered to be indicative of the presence of microbial community DNA.
155 Illumina libraries were prepared following the method described by Caporaso et al (2012)
156 using the NEXTflex 16S Amplicon-Seq kit (Bioo Scientific, Austin USA). A total of 50 ng of
157 template DNA was used for each individual sample and the V4 hypervariable region of the

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

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

174 *Bioinformatics*

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

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

198 *Statistical analysis*

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

218 *Establishment of predicted functional profiles*

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

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

240 **Results**

241 *Sequencing data and microbial diversity analysis*

242 A total of 14,088,267 reads were obtained from all sample libraries after quality filtering
243 steps were performed. A total of 1131 OTU's were assembled from the combined libraries.
244 After subsampling to the level of the library containing the fewest reads (DF AF1,
245 n=142,267), rarefaction analysis revealed that all sample curves reached saturation (Figure 1).
246 Overall, rarefaction estimates pointed to a slightly elevated level of community diversity in
247 the fish farm samples, with the highest level of diversity noted in the aquarium tank biofilm
248 sample. Mean Chao1 richness estimates were higher in the fish farm samples ($286.15 \pm$
249 125.42) than in the aquarium samples (233.51 ± 90.44), and the estimates were even higher in
250 the tank biofilm sample (691.89), reflecting the trend indicated in the rarefaction analysis.
251 The mean inverse Simpson value was however greater in the aquarium fish samples ($2.14 \pm$
252 0.78) versus the farm samples (1.64 ± 0.82). However, the overall microbial diversity and
253 richness in the intestine of aquarium and farm based fish were not significantly different
254 (Simpson: $f = 3.24$, $p = 0.088$; Chao1: $f = 1.14$, $p = 0.300$). Good's coverage estimations were
255 on average >99% for all libraries indicating that a high level of sequence coverage was
256 obtained. All alpha diversity statistics are detailed in Table 1.

257 *Microbiome composition of rainbow trout distal intestine*

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

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

286 *Microbiome composition of aquarium tank biofilm and diets*

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

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

301 *Statistical analyses*

302 Two separate distance matrices, ThetaYC and Jaccard, were computed in order to compare
303 the structure and membership of the intestinal microbial communities between the two
304 rainbow trout populations sampled. PCoA of the first and second axes of the ThetaYC
305 distances (69% of the total variation) suggested that the microbial community structure
306 between both fish populations was similar, with both sample sets clustering close together
307 (Figure 4a). The AMOVA analysis confirmed that any spatial separation observed in the
308 PCoA of ThetaYC distances was not statistically different between the aquarium and farmed
309 trout ($F_s = 1.20$, $p = 0.292$). Furthermore, Parsimony and UniFrac tests were in agreement
310 with the AMOVA result (ParsSig = 0.085, UWSig = 0.26). The microbiome structure of the
311 biofilm sample was however significantly different from the fish intestinal samples (UWSig
312 = <0.001). In addition, the microbiome of the diet pellets was also found to be significantly
313 different from the intestinal samples (WSig = <0.001 , UWSig = 0.003, ParsSig = 0.025).

314 The Jaccard distance matrix, a further measure of dissimilarity between communities, was
315 calculated to compare the community membership of the samples (Figure 4b). A slight
316 separation in the clustering of both fish populations was observed in the PCoA plots created
317 from this distance matrix. When an AMOVA was performed on this Jaccard matrix, the
318 spatial separation was established as being statistically significant. ($F_s = 2.41$, $p = 0.001$). The
319 Parsimony (ParsSig = <0.001) and UniFrac tests (WScore = 0.894, WSig = <0.001 , UWScore
320 = 0.981, UWSig = <0.001) confirmed this result, indicating that the microbial community
321 membership was significantly different between the farmed and the aquarium fish. In
322 addition, the tank biofilm sample was significantly different, in terms of community

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

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

333 *Predicted functional metagenomes of the rainbow trout intestinal microbiome*

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

350 **Discussion**

351 The geographical location of fish farms has been posited to have an impact upon the
352 composition of the intestinal microbiome of the cultured individuals, due to the influence of
353 the native microbial ecology of each site (Ringø et al 1995, Holben et al 2002, Lozupone and
354 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.

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

401 Some studies of the intestinal microbiota of rainbow trout have hypothesized that the
402 composition could mirror that of the surrounding aquatic environment (Trust and Sparrow
403 1974, Yoshimizu and Kimura 1976, Sugita et al 1982, Ringø and Strom 1994, Nayak 2010,
404 Semova et al 2012, Xing et al 2013, Sullam et al 2015). However, the microbiome structures
405 of the aquarium tank biofilm and the diet samples were significantly different from the
406 intestinal libraries in the present study. These data suggest that the intestinal microbiome may
407 be specialized, and may not simply be a reflection of the microbial flora of the surrounding
408 environment. Future studies should include analyses of the microbiome of the farm and
409 aquarium water in order to further explore this theory. The PCoA revealed a homogeneity
410 between the structure of the intestinal microbiome in the farm and aquarium based fish.
411 However, the community membership was significantly different between the groups. This
412 suggests that the 'core' microbial phyla and classes are somewhat stable in the rainbow trout
413 intestine, regardless of geographical location, but that other assemblages of more sporadic
414 OTU's can vary accordingly. These results reflect those reported in similar studies. Roeselers
415 et al (2011) revealed that individual zebrafish (*Danio rerio*), sampled from wild and
416 domesticated populations, shared a stable core gut microbiome independent of their origin.
417 Another recent study on the wild Atlantic salmon intestinal microbiome found that
418 community composition was not significantly impacted by geography and that individual
419 fish, at different life stages, possessed remarkably similar intestinal microbiome structures
420 which were distinct from those found in the environment (Llewellyn et al 2015).

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

425 The presence of a number of OTU's that were discriminatory according to geographical
426 location most likely explains the spatial separation observed in the community membership
427 plots. The genus *Lactobacillus* was significantly more abundant in the farm based fish. Its
428 elevated levels suggest that this organism may have been enriched by the diet, possibly as it
429 is known that the relative abundance of this bacterium is affected by diet type (Desai et al
430 2012, Wong et al 2013, Ingerslev et al 2014), and both populations of fish sampled in this
431 study were fed different diets. Lactobacilli are commonly observed inhabitants of the teleost
432 fish gut, but usually represent a minor proportion of the overall microbial community (Desai
433 et al 2012, Merrifield et al 2014). Other organisms such as *Moritella*, *Photobacterium* and
434 *Psychrilyobacter* were also found to be discriminatory according to location, and were
435 significantly more abundant in the farm raised fish. The exact reason for this is unclear, but
436 some species of *Moritella* and *Photobacterium* are fish pathogens known to cause conditions
437 such as winter ulcer disease and pasteurellosis respectively (Fouz et al 1992, Gauthier et al
438 1995, Lunder et al 1995, Pedersen et al 1997, Benediksdottir et al 1998, Bruno et al 1998,
439 Lovoll et al 2009) in farmed salmonids, and all three of these genera are primarily associated
440 with cold water temperatures. It should be noted that at the time of sampling, the fish farm
441 was experiencing the lowest average water temperature recorded for that calendar month in
442 over a decade. This could perhaps explain the enrichment of these psychrophilic bacterial
443 taxa within the intestine of these particular fish.

444 The principal functional pathways expressed in both populations of fish were primarily
445 associated with metabolism, transport and cellular processes. Membrane transport pathways,
446 such as ABC transporters, utilize the energy of ATP binding and hydrolysis to transport
447 substrates across cellular membranes (Rees et al 2009). They are essential to cell viability and
448 growth and therefore vital for bacterial survival in the intestinal ecosystem. Genes affiliated
449 with the phosphotransferase system (PTS) were found to be abundant in the intestinal
450 microbiomes of both farmed and aquarium reared trout, and this system is used by bacteria
451 for sugar uptake where the source of energy is from phosphoenolpyruvate (PEP), a key
452 intermediate in glycolysis (Meadow et al 1985, Erni 2012). The PTS is a multicomponent
453 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

487 proteins that escape digestion by key endogenous digestive enzymes such as chymotrypsin
488 and trypsin are made available to bacteria for fermentation. These enzymes originate in the
489 pancreas and are not produced by the intestine itself (Guillaume and Choubert 1999).
490 Therefore, the fermentative activity of the microbiome may be particularly important in the
491 distal intestinal region, where such enzymes are likely to be less influential. The Clostridia
492 were abundant in all fish, and are recognized as being proteolytic bacteria that can ferment
493 amino acids (Neis et al 2015). The amino sugar metabolic pathway, expressed by the
494 intestinal microbiome of the fish in this study, is specifically responsible for breaking down
495 protein into its constituent di- and tri-peptides and amino acids (Miska et al 2014, Shaufi et al
496 2015). These can then be utilized in energy metabolism, used to form the structural
497 components of intestinal epithelial cells or exported to the liver for further processing. There
498 is evidence that symbiotic intestinal microbes of other animals manufacture peptidases and
499 amino acids that are then provided to the host (Douglas 2013, Neis et al 2015). Moreover,
500 Clements et al (2014) recently speculated on the involvement of the intestinal microbiota of
501 fish in protein metabolism, and Kuz'mina et al (2015) demonstrated that the intestinal
502 microflora of crucian carp contributed ~45% of total peptidase production in this species.
503 Additionally, Zarkasi et al (2016) reported a progressive enrichment of proteolytic bacteria in
504 the distal intestine of cage farmed Atlantic salmon, concurrent with increasing levels of
505 dietary protein inclusion. The metagenomic data indicate that similar microbially mediated
506 mechanisms of protein breakdown may occur in the rainbow trout intestinal tract, which
507 could supplement the action of endogenous digestive enzymes. Protein fermentation
508 pathways, similar to those for carbohydrate fermentation, can also result in the production of
509 SCFA, especially branched chain fatty acids (BCFA), which can then be metabolized by the
510 host (Jha and Berrocoso 2016).

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

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

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536 **Conflicts of interest**

537 The authors declare no conflicts of interest.

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766 **Supporting data legends**

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

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

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

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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.

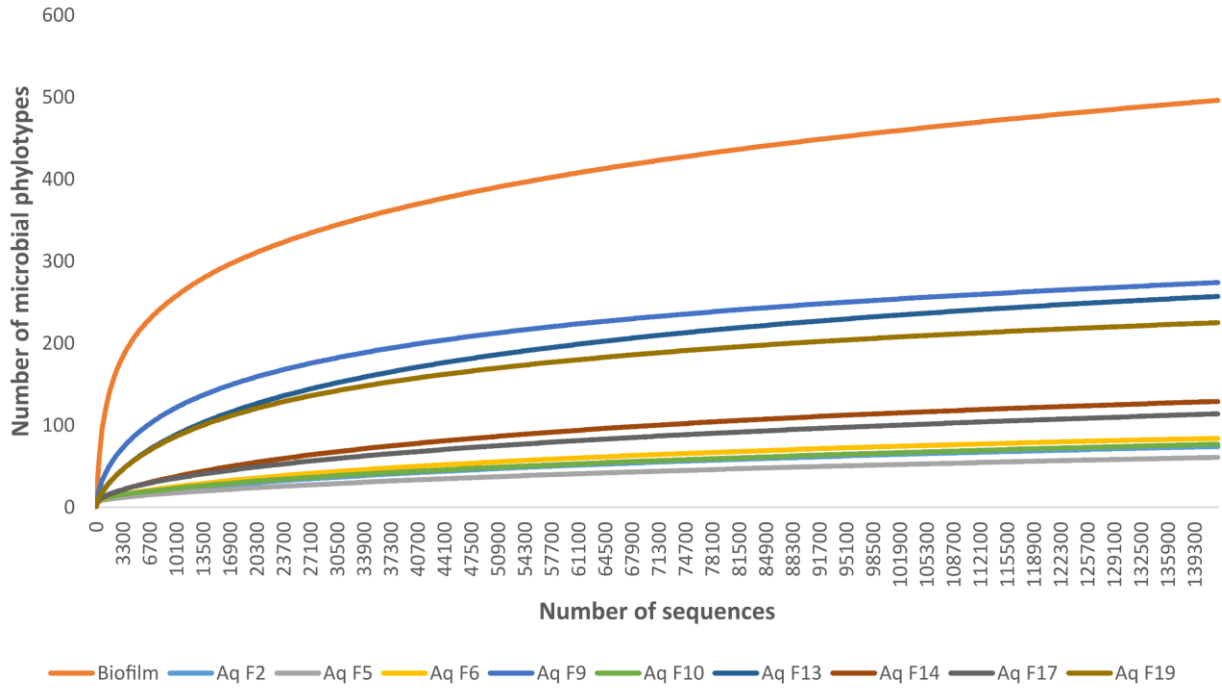
Figure 3: Relative % sequence abundance of aquarium tank biofilm, diet and intestinal microbial classes observed in individual fish sampled from a) aquarium (n=9) and b) farm (n = 12).

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.

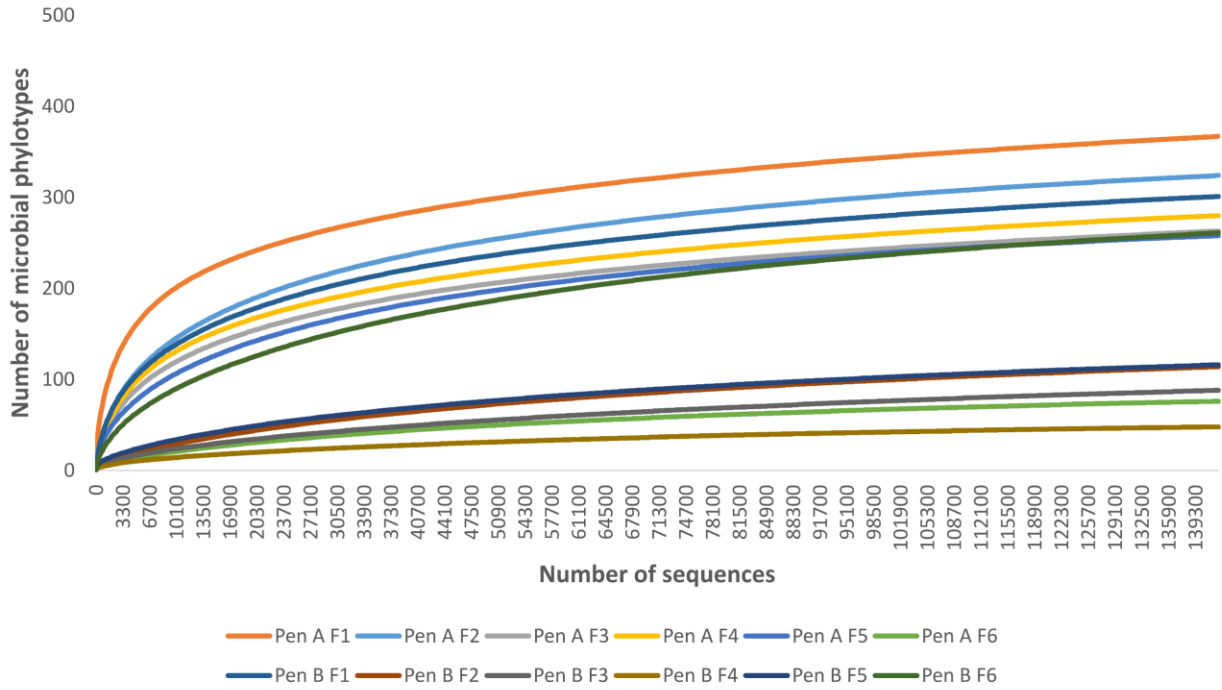
Figure 5: Predicted functional metagenomic pathways of rainbow trout intestinal microbiome, as identified by PICRUSt and STAMP analyses.

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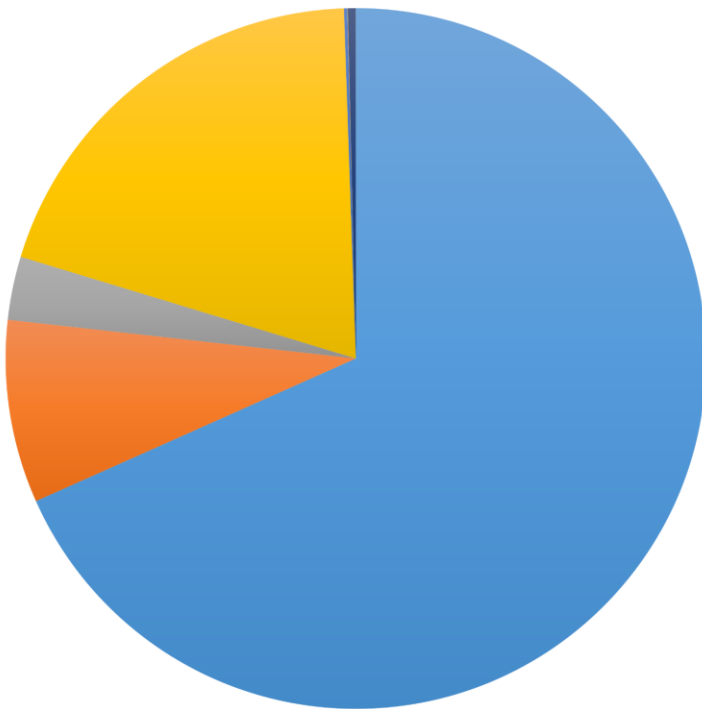
a)



b)

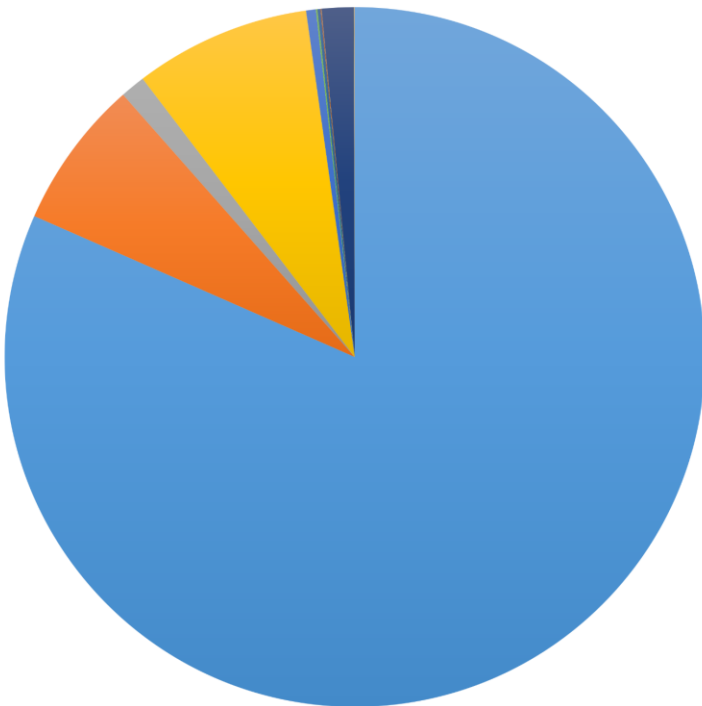


a)

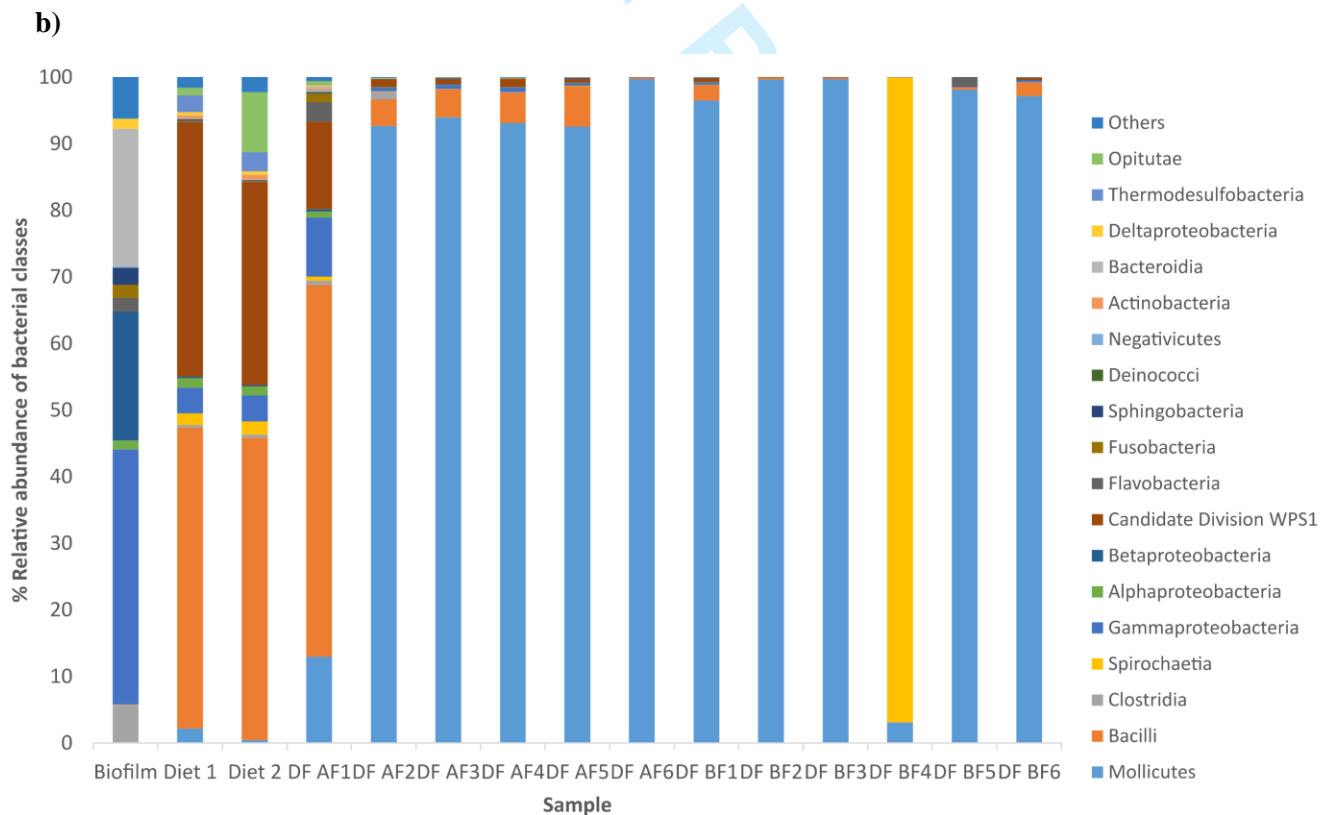
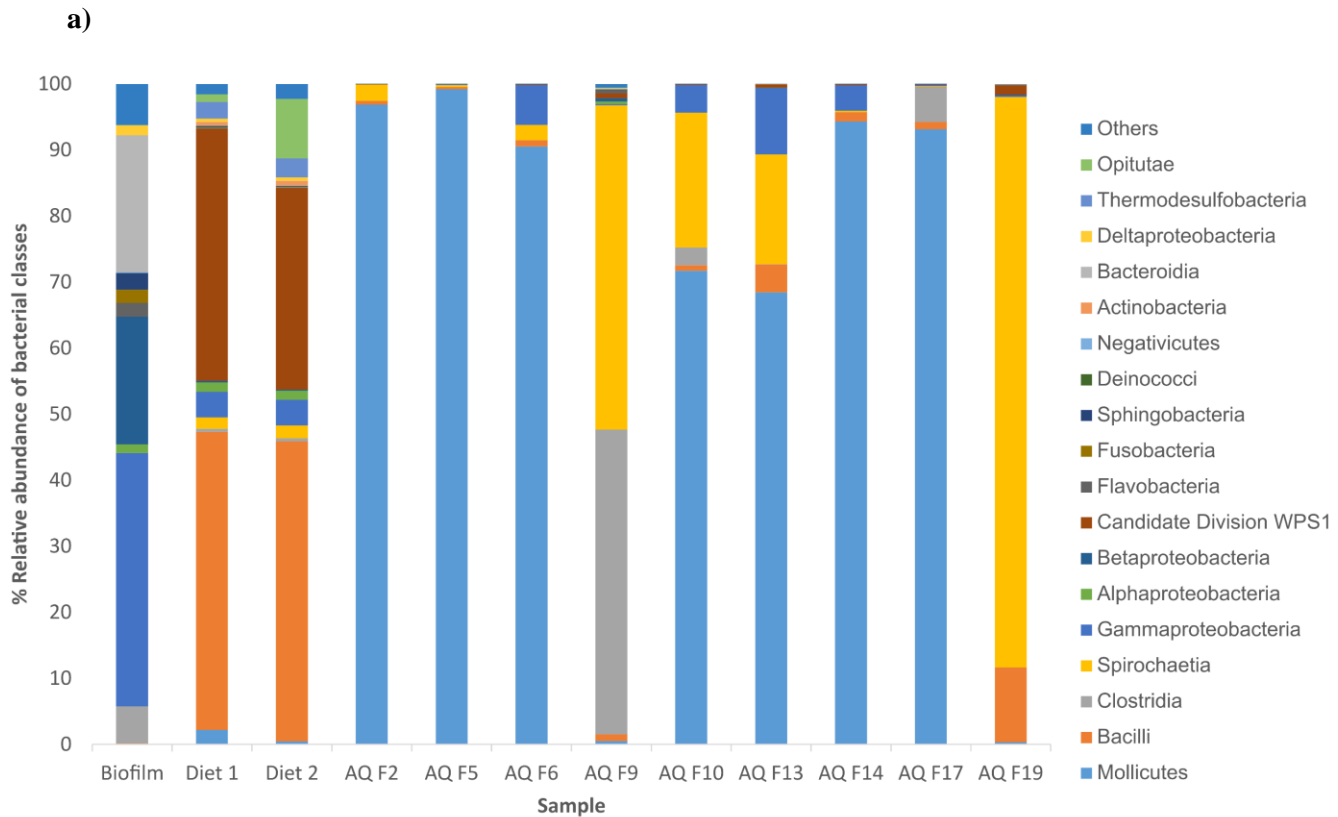


- Tenericutes (68.32%)
- Firmicutes (8.43%)
- Proteobacteria (2.92%)
- Spirochaetes (19.77%)
- Bacteroidetes (0.14%)
- Verrucomicrobia (0.01%)
- Fusobacteria (0.001%)
- Actinobacteria (0.01%)
- Deinococcus-Thermus (0.002%)
- Planctomycetes (0.002%)
- Candidate Division WPS-1 (0.35%)
- Candidate Division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

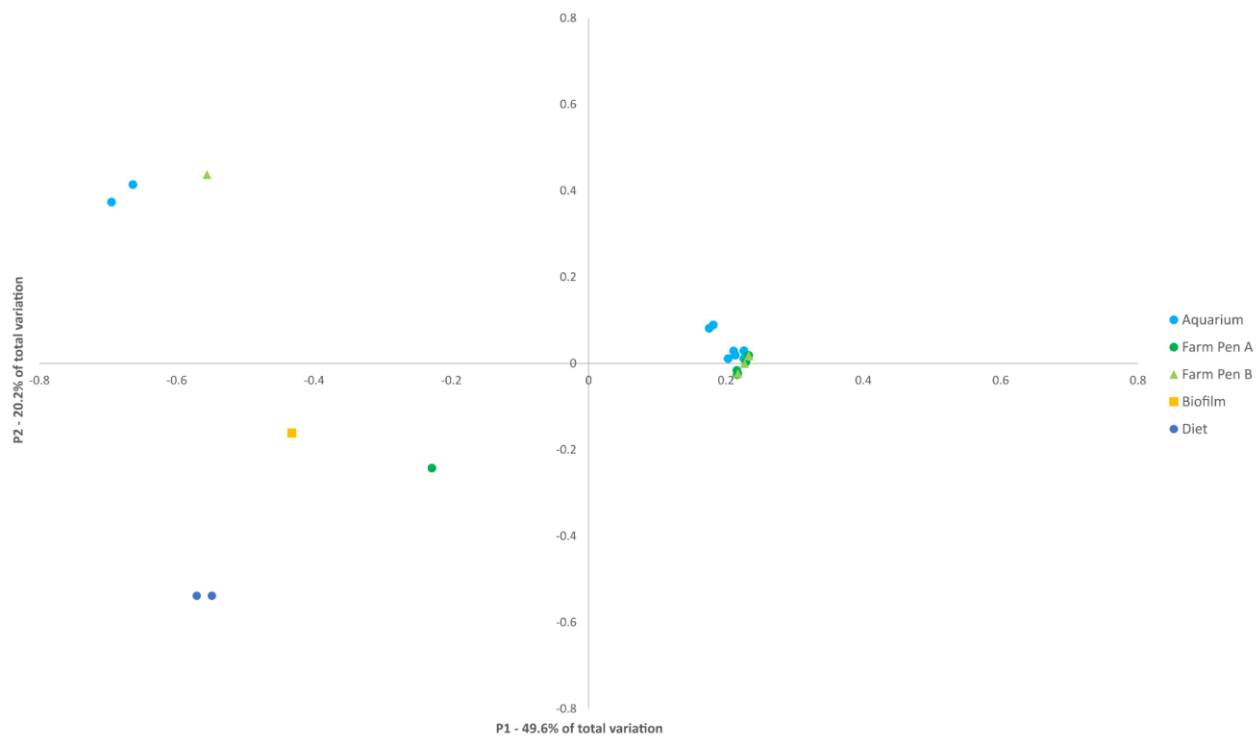
b)



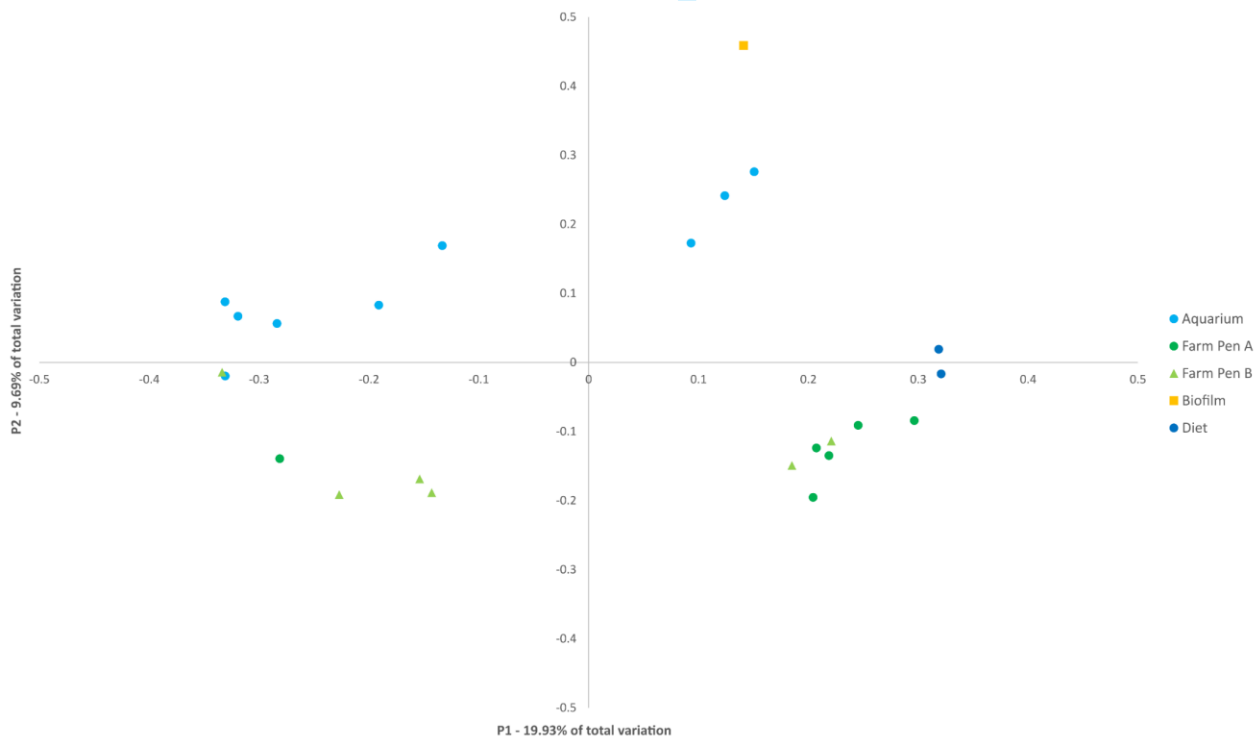
- Tenericutes (81.59%)
- Firmicutes (6.89%)
- Proteobacteria (1.15%)
- Spirochaetes (8.13%)
- Bacteroidetes (0.45%)
- Verrucomicrobia (0.06%)
- Fusobacteria (0.13%)
- Actinobacteria (0.04%)
- Deinococcus-Thermus (0.003%)
- Planctomycetes (0.01%)
- Candidate Division WPS-1 (1.46%)
- Candidate Division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

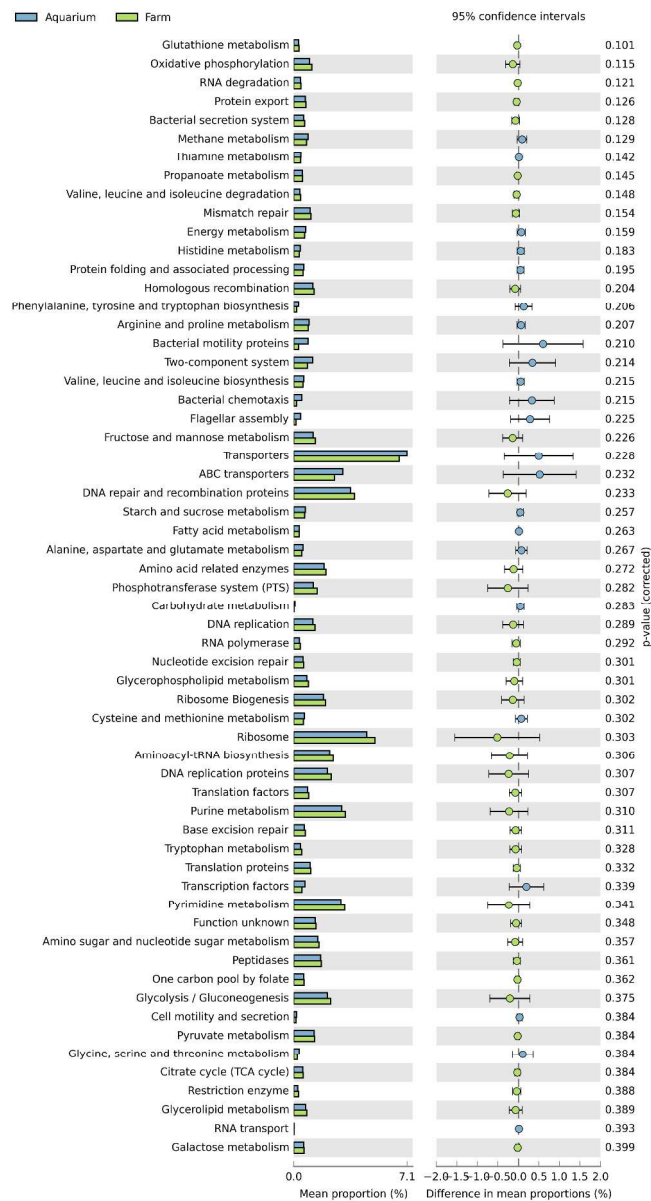


a)



b)





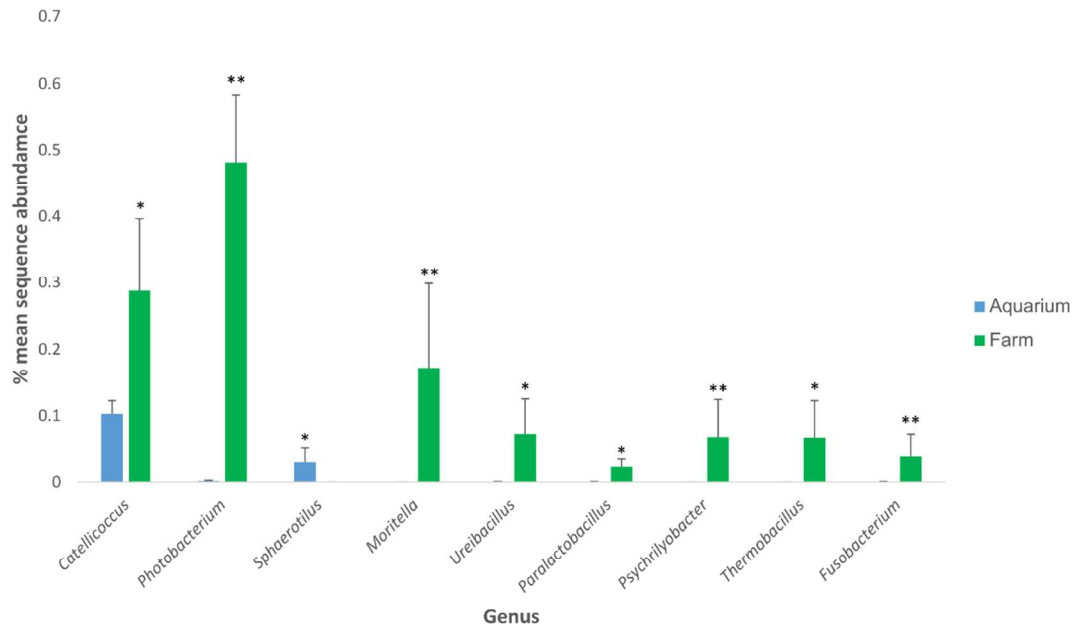
Predicted functional metagenomic pathways of rainbow trout intestinal microbiome, as identified by PICRUST and STAMP analyses.

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 ($p < 0.05$, $p < 0.01$)

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

a)



b)

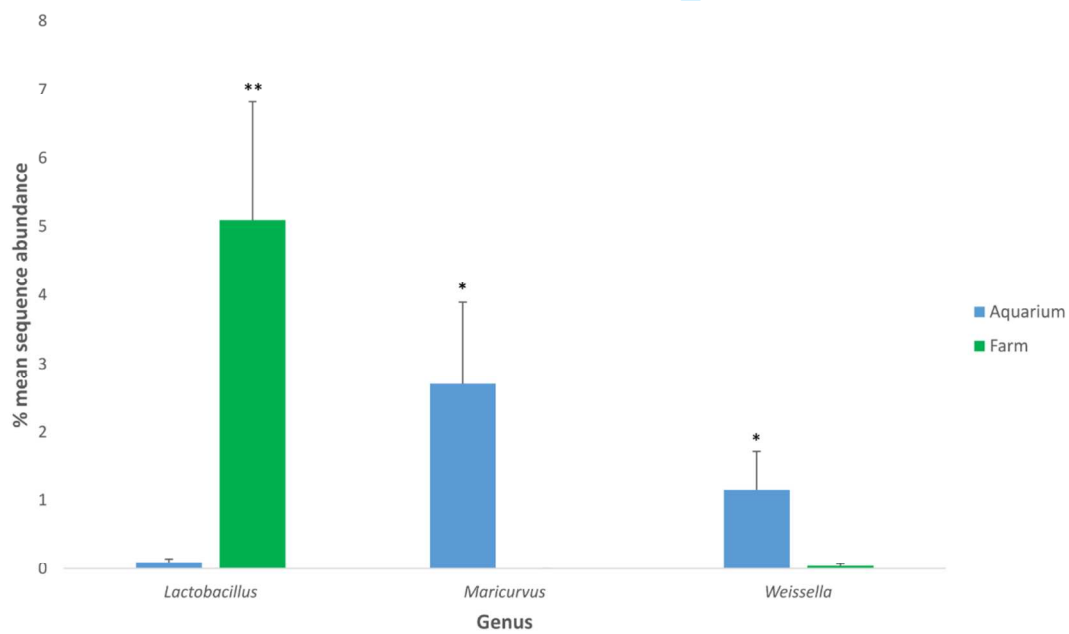


Figure S1. Bacterial taxa identified by Metastats and Indicator analysis as discriminatory between aquarium and farm based rainbow trout intestinal samples. The data are plotted as mean relative percentage sequence abundance \pm SEM * $P < 0.05$ ** $P < 0.01$. Data are split into **a** and **b** to improve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

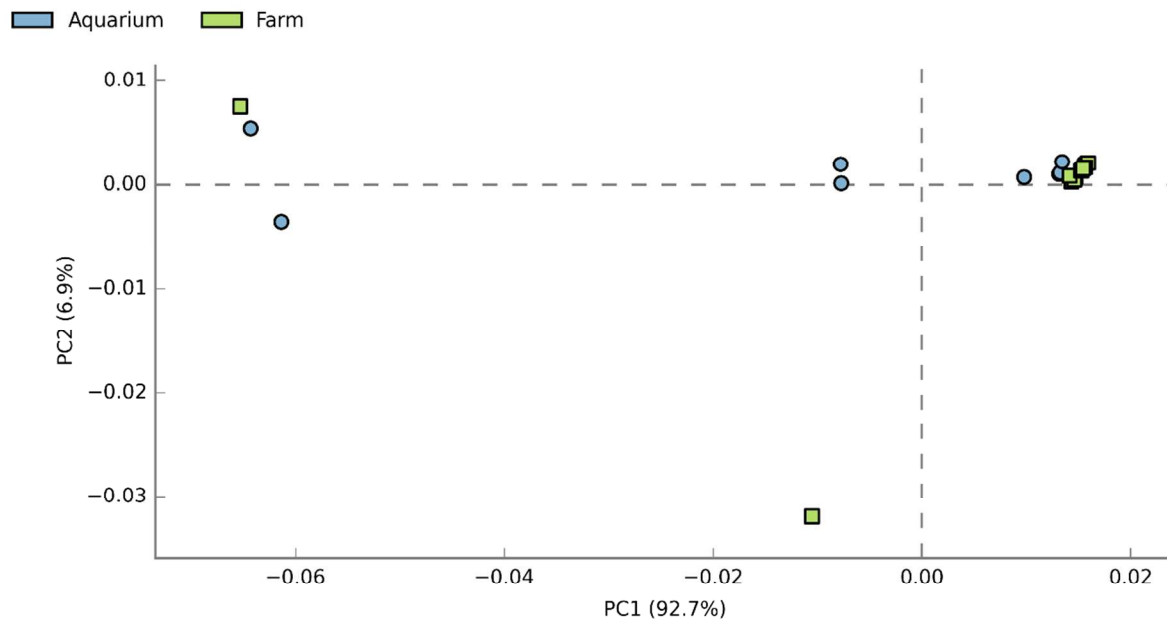


Figure S2. Principal coordinate analysis (PCoA) of predicted functional metagenomes between intestinal microbiomes of aquarium and farm-based rainbow trout. Each dot represents an individual sample.