Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Commensal microorganisms present at mucosal surfaces play a vital role in protecting the host organism from bacterial infection. There are multiple factors that contribute to selecting for the microbiome, key of which are host genetics. *Flavobacterium psychrophillum,* the causative agent of Bacterial Cold Water Disease in salmonids, accounts for acute losses in wild and farmed Rainbow Trout *(Oncorhynchus mykiss).* The U.S. National Center for Cool and Cold Water Aquaculture has used family-based selective breeding to generate a line of rainbow trout with enhanced resistance to *F. psychrophilum.* The goal of this study is to determine whether selective breeding impacts the gut and gill microbiome of the *F. psychrophilum*-resistant as compared to a background matched susceptible trout line. Mid-gut and gill samples were collected from juvenile fish (mean bwt 118g) and microbial diversity assessed by 16S rDNA amplicon sequencing. Results indicate that alpha diversity was significantly higher in the mid-gut of the susceptible line compared to the resistant line, while no significant differences in alpha diversity were observed in the gills. *Mycoplasma sp.* was the dominant taxon in the mid-gut of both groups, although it was present at a decreased abundance in the susceptible line. We also observed an increased abundance of taxa that could potentially be pathogenic in the susceptible line, including *Brevinema sp.* and Enterobacteriaceae members. Within the gills, both lines exhibited similar microbial profiles, with *Candidatus Branchiomonas* being the dominant taxon. Together, these results suggest that selectively bred *Flavobacterium psychrophillum*-resistant trout may

harness a more resilient gut microbiome, attributing to the disease resistant phenotype, providing a framework for future experiments.

Keywords:

Rainbow trout; Aquaculture; *Flavobacterium psychrophilum*; Bacterial Cold Water Disease; ARS-Fp-R; ARS-Fp-S; Microbiome; Disease resistance

1. Introduction

The microbiome has well established roles in pathogen exclusion and host immunity, including systemic and mucosal innate and adaptive immune responses and development of the immune system [1–3]. There is strong evidence to support a role of host genetics in the selection of the gut microbiome in humans and other mammals [4–6], although this has not been well characterized in fish. The host microbial composition is also shaped by other factors including environment, diet, and disease [7–10]. To begin to disentangle the contribution of host genetics and environmental factors shaping the fish microbiome, here we utilize a rainbow trout model in which two genetic lines of rainbow trout have been established by selective breeding that differ in susceptibility to a common environmental gram negative pathogen, *Flavobacterium psychrophilum*.

Flavobacterium psychrophilum is the causative agent of Bacterial Cold Water Disease (BCWD), which is a major concern in the United States aquaculture industry affecting a range of cold-water fish species, including the commercially relevant rainbow trout *(Oncorhynchus mykiss)*. *F. psychrophillum* is a mucosal pathogen that typically infects the skin and gills of fish [11]. Symptoms of BCWD in developed fish

69 include necrosis of the caudal region, skin lesions, eroded fin tips, and loss of
70 appetite. *F. psychrophilum* has a more pronounced effect on young fry, a condition
71 referred to as rainbow trout fry syndrome. Rainbow trout fry syndrome is
72 responsible for acute losses in trout farms worldwide, as the associated mortality
73 rate is reported to be greater than 50% [12]. BCWD is becoming an increasingly
74 difficult disease to treat, as *F. psychrophilum* strains have developed resistance to
75 several commonly used antibiotics [13–15], and there is currently no commercially
76 available licensed vaccine.
77 The National Center for Cool and Cold Water Aquaculture (NCCCWA) utilized
78 family-based selective breeding to develop two distinctive genetic lines of rainbow
79 trout that confer enhanced resistance (ARS-Fp-R), or susceptibility (ARS-Fp-S) to
80 the pathogen *F. psychrophilum* [16]. Enhanced resistance to *F. psychrophilum*-
81 induced mortality in the ARS-Fp-R line has been described, both in the laboratory
82 setting and on trout farms [17,18]. Previous studies have begun to investigate
83 possible host mechanisms that attribute to enhanced resistance. For instance, a
84 strong correlation between resistance to *F. psychrophilum* and increased spleen size
85 has been described, although this relation does not appear to translate to other
86 common fish pathogens, such as *Yersinia ruckeri* [19]. Additionally, whole-body
87 transcriptome analysis has identified numerous acute phase proteins and
88 inflammatory cytokines that are differentially expressed in each line following
89 challenge with *F. psychrophilum* [20]. Further work is needed to better characterize
90 the mechanism(s) by which enhanced resistance is achieved in the ARS-Fp-R line.

91 In this paper, we begin to investigate the hypothesis that the higher innate disease
92 resistance of the ARS-Fp-R line is due to differences in the microbial composition at
93 host mucosal surfaces. Using 16S rDNA amplicon sequencing, we determine and
94 compare the mid-gut and gill microbiomes of rainbow trout lines selectively bred
95 for *F. psychrophilum* resistance and susceptibility.
96

97 **2. Materials and Methods**

98 **2.1. Animals and sampling**

99 The Institutional Animal Care and Use Committee (Leetown, WV) reviewed and
100 approved all animal husbandry practices and disease challenge protocols per
101 standards set forth in the USDA, ARS Policies and Procedures 130.4.v.3 titled
102 'Institutional Animal Care and Use 84 Committee'. Fish used in these experiments
103 were from the 2017 Year Class and maintained as specific pathogen free as
104 determined by biannual testing as previously described [17]. A total of 33 single
105 sire-dam matings contributed to the pool of ARS-Fp-R line fish and 31 matings
106 contributed to the pool of ARS-Fp-S line. The disease resistant phenotype of each
107 genetic line was evaluated at two time-points, 75 and 276 days post-hatch. Fish
108 were challenged with *F. psychrophilum* strain CSF259-93 and survival recorded over
109 21 days as previously described [19]. Mean fish body weight at the first evaluation
110 was 1.9 g and a total of 120 ARS-Fp-R and 119 ARS-Fp-S fish (n=3 tanks per line)
111 were challenged by intraperitoneal injection with a dose of 1.4E+07 CFU g^{-1} in a
112 total volume of 50 µL using a 26g needle fitted onto an Eppendorf repeating pipette.
113 Mean body weight at the second evaluation was 194 g and a total of 70 ARS-Fp-R

114 and 70 ARS-Fp-S fish (n=2 tanks per line) were challenged by intramuscular
115 injection with a dose of 3.5E+06 CFU g^{-1} body weight in a total volume of 50 µL using
116 a 26g needle fitted onto an Eppendorf repeating pipette. The fish utilized in the
117 second experiment were part of a larger study evaluating experimental vaccination
118 and these fish had been sham vaccinated with PBS 35 days prior to challenge. In
119 both challenges, *F. psychrophilum* was isolated from mortalities and confirmed by
120 PCR genotyping.
121 At the time of microbiome sampling, fish were reared under two different tank
122 conditions as described in Supplemental data file 1. Ten fish from each tank system
123 per genetic line were euthanized using 200 mg L^{-1} MS222 for 5 minutes. Each fish
124 was photographed, weighted, gill tissue sampled, intestinal mid-gut sampled, and
125 spleen weighed within 30 minutes of euthanasia. Sample were placed in SLB [21]
126 on ice and then moved to storage at -80˚ C. Instruments were cleaned between each
127 fish and gloves changed between tank groups. Three control tubes containing SLB
128 alone were included as negative controls.

129 **2.2. DNA Extraction, 16S rDNA PCR Amplification, and Sequencing**

130 Whole genomic DNA was extracted from skin and gill samples by first lysing tissue
131 samples using sterile 3 mm tungsten beads (Qiagen) and Qiagen TissueLyser II.
132 Next, using the cetyltrimethylammonium bromide method as previously described
133 [21], DNA was isolated and suspended in 50 µL RNase and DNase free molecular
134 biology grade water. DNA concentration and purity was then assessed using a
135 Nanodrop ND 1000 (Thermo Scientific).
136 Bacterial DNA was then replicated by PCR using Illumina adapter fused primers

137 targeting the V1-V3 region of the prokaryotic 16S rDNA gene. The primer sequences
138 were as follows: 28F 5'-GAGTTTGATCNTGGCTCAG-3' and 519R
139 5'GTNTTACNGCGGCKGCTG-3' (where N = any nucleotide, and K = T or G). DNA
140 samples were diluted 1:10 or 1:100, and Quantabio 5PRIME HotMasterMix was
141 used. 16S amplicons were generated using the following conditions: 94° C for 90s;
142 33 cycles of 94° C for 30s, 52° C for 30s, 72° C for 90s; and a final extension of 72° C
143 for 7 min A positive control of a verified 16S V1-V3 amplicon, and a negative control
144 of molecular biology grade water was included in every PCR reaction. In addition,
145 we included a mock community positive control with each sequencing run, which
146 consisted of equal DNA amounts of 7 different bacterial isolates previously cloned
147 and a negative control that consisted of SLB handled in the same way as the rest of
148 the tubes during sampling to which no tissue was added. Amplicons were purified
149 using the Axygen AxyPrep Mag PCR Clean-up Kit (Thermo Scientific), and eluted into
150 30 µL molecular biology grade water. Unique oligonucleotide barcodes were ligated
151 to the 5' and 3' ends of each sample, as well as the Nextera adaptor sequences, using
152 the Nextera XT Index Kit v2 set A (Illumina). DNA concentrations were quantified
153 using a Qubit, and normalized to a concentration of 200 ng/uL for DNA library
154 pooling. Pooled samples were cleaned once more using the Axygen PCR clean-up kit
155 before being sent off for sequencing. Sequencing was performed on the Illumina
156 Miseq platform using the MiSeq Reagent Kit v3 (600 cycle) at the Clinical and
157 Translational Sciences Center at the University of New Mexico Health Sciences
158 Center.

159 **2.3. Data Analysis and statistics**

160 Differences in survival between genetic lines were determined using the product
161 limit method of Kaplan and Meier and calculations were performed using GraphPad
162 v4.0 software. Log-rank (Mantel-Cox) test was used to compare survival curves.
163 Initial sequence data was analyzed using the latest version of Quantitative Insights
164 Into Microbial Ecology 2 (Qiime2 v2018.6) [22]. Demultiplexed sequence reads
165 were quality filtered using DADA2 [23]. Samples were then rarefied to a sampling
166 depth of 12,603 sequences per sample for mid-gut reads, and 2020 sequences per
167 sample for gill reads. Amplicon sequence variants (ASVs) were picked by aligning to
168 the latest Silva 16S database (version 132). Core diversity metrics were analyzed,
169 including number of ASVs and Shannon's diversity index for alpha diversity, and
170 PERMANOVA for beta diversity. Nonmetric multidimensional scaling and
171 generation of heat maps were performed in RStudio [24] using the phyloseq
172 package [25]. Random forest modeling was performed in Qiime2. Differential
173 abundance testing was performed in Qiiime2 using ANCOM [26], as well as in
174 RStudio using the DESeq2 package [27]. For all statistical analyses, fish were split
175 into groups based on 'Treatment' (ARS-Fp-R, ARS-Fp-S) or 'Tank Treatment'.
176

177 **3. Results**

178 **3.1. Phenotype Confirmation of Disease Resistance/Susceptibility**

179 The relative phenotype of the two genetic lines was evaluated at time-points either
180 before or after microbiome sampling. At both time points, the survival of the ARS-
181 Fp-R genetic line was significantly higher ($P<0.001$) than the ARS-Fp-S line and
182 consistent with estimated mid-point breeding values. In the first evaluation, a total

183 of 3/120 (3%) ARS-Fp-R line fish died compared to 82/119 (69%) ARS-Fp-S line
184 fish. In the second evaluation, 2/70 (3%) ARS-Fp-R fish died, while 58/70 (83%)
185 ARS-Fp-S line fish died within the 21-day challenge period.

186 **3.2. High throughput sequencing analysis**

187 A total number of 3,598,038 raw reads were obtained from all mid-gut samples.
188 After merging paired ends, quality filtering, and removal of chimeric reads with
189 DADA2, as well as filtering out non-specific trout genomic reads, a total of 1,413,104
190 reads remained, with a mean of 35,328 reads per sample. Samples were rarified to a
191 sample depth of 12,031, which excluded two ARS-Fp-R samples and two ARS-Fp-S
192 samples. The sample size after rarefaction was n=18 for both the ARS-Fp-R and ARS-
193 Fp-S lines.

194 Gill sample sequencing produced a total of 4,646,971 raw reads. Quality filtering in
195 DADA2 and removal of non-specific reads retained 333,281 reads, with a mean of
196 13,331 reads per sample. Samples were rarified to 2010 reads. The sample size after
197 rarefaction was n=11 for ARS-Fp-R and n=14 for ARS-Fp-S.

198 **3.3. Resistant and susceptible lines display significant differences in alpha**
199 **diversity in the mid-gut, but not the gills**

200 Comparison of alpha diversity metrics obtained from the mid-gut showed
201 significantly lower measures of gut microbial community richness (Observed ASVs),
202 as well as Shannon's diversity index in the ARS-Fp-R line compared to the ARS-Fp-S
203 line (Fig 1A and 1B). There were a total of 15 ASVs reported in the mid-gut of the
204 resistant line, and 29 in the mid-gut of the susceptible line. In the gills, there were no

205 significant differences in alpha diversity between lines with a total of 57 ASVs found
206 in the gills of the resistant line, and 50 in the susceptible line (Fig 1C and 1D).

207 **Fig 1. Comparison of alpha diversity metrics for the mid-gut and gill microbiome of ARS-Fp-R**
208 **and ARS-Fp-S trout.**

209 (A) Total number of observed ASVs in the mid-gut. (B) Shannon's diversity index in the mid-gut. (C)
210 Total number of observed ASVs in the gills. (D) Shannon's diversity index in the gills. ** indicates
211 statistically significant differences $p<0.01$.

212 3.4. Beta diversity analysis suggests possible tank effect on the gut 213 microbiome

214 We assessed the microbial diversity between different treatments, as well as
215 between tanks by performing Nonmetric Multidimensional Scaling (NMDS) using
216 the Bray Curtis distance metric. This ordination showed a discrete grouping of the
217 two tanks containing the ARS-Fp-S line, while the two tanks containing the ARS-Fp-
218 R line were more tightly clustered (Fig 2A). PERMANOVA analysis [28] identified
219 "Treatment" (P value = 0.02) and "Tank" (P value = 0.048 for ARS-Fp-R, P value =
220 0.001 for ARS-Fp-S) as significant determinants of the mid-gut microbial community
221 composition. In the gills, NMDS ordination showed a similar pattern to that found in
222 the gut, where fish from tank 26 of the susceptible line clustered tightly together
223 while fish from tank 12 showed greater variability. Meanwhile, there was no clear
224 separation between individuals held in separate tanks of the resistant line (Fig 2B).
225 PERMANOVA identified only tank housing within the ARS-Fp-S line (P value =
226 0.004) as a significant factor in determining gill microbial communities.

227 **Fig 2. NMDS ordination plots.**

228 NMDS ordination performed using Bray Curtis distance matrix of the (A) mid-gut and (B) gill
229 microbiome of ARS-Fp-R and ARS-Fp-S trout.

230 **3.5. Gut microbial community composition**

231 A total of eight different phyla were identified in the mid-gut across both lines,
232 although only four of these were represented over 1%, including Tenericutes,
233 Spirochaetes, Proteobacteria, and Firmicutes. Tenericutes composed the vast
234 majority of the mid-gut microbiome of both lines, constituting 81% of the total
235 microbial diversity in the susceptible line and 89% in the resistant line (Fig 3A).
236 At the genus level, all Tenericutes reads were identified as *Mycoplasma sp.* The
237 genus *Brevinema sp.* showed greater abundance in the ARS-Fp-S (8.5%) compared
238 to the ARS-Fp-R line (4.8%). Similarly, members of the family Enterobacteriaceae
239 had greater abundance in the ARS-Fp-S line than the ARS-Fp-R line (2.2% and 0%,
240 respectively); however, these differences were not significant. Differential
241 abundance testing with ANCOM revealed three genera that were differentially
242 abundant in the mid-gut of both trout lines; including *Hydrotalea sp., Paenibacillus*
243 *sp.,* and *Variovorax sp.* This was replicated by differential abundance testing using
244 DESeq2, an R package originally developed for differential expression analysis in
245 RNA-seq data, but has been proposed to be used in microbiome studies as well
246 [29,30].
247 The relative distribution of ASVs at the genus level was notably different between
248 tanks of the same line (Fig 3B). Potential opportunistic taxa such as *Brevinema sp.*
249 and Ambiguous Enterobacteriaceae were present in tanks 11 and 12, but were not
250 identified in either tank 25 or 26. This trend is further shown in a heatmap

251 representing the top 25 ASVs observed in each sample (Fig 4). Two of the tanks that
252 were in close proximity to one another displayed similar microbial profiles, despite
253 holding different lines. This was not the case for tanks 25 and 26, as tank 26
254 displayed a microbial profile different from all other tanks since it contained ASVs
255 representative of Enterobacterieaceae, *Hydrotalea sp., Paenibacillus sp,* and
256 *Variovorax sp.*

257 **Fig 3. Relative microbial composition in the gut of each line.**

258 (A) Relative abundance of ASVs at the phylum level for each line. (B) Relative abundance of ASVs for
259 each tank the two lines were divided into.

260 **Fig 4. Heatmap representing the top 25 ASVs present in the mid-gut of ARS-Fp-R and ARS-Fp-S**
261 **trout.**

262 Each column represents one individual. Each row represents one ASV.

263 **3.6. Gill microbial community composition**

264 A total of nine different phyla were present in the trout gills across both lines. Five
265 of these were represented at abundance greater than 1%, including Proteobacteria,
266 Tenericutes, Spirochaetes, Bacteriodetes, and Firmicutes. Proteobacteria was the
267 most abundant phylum in both groups, representing 85% of all bacterial diversity in
268 the ARS-Fp-S line and 95% in the ARS-Fp-R line (Fig 5A). At the genus level, most
269 Proteobacteria reads were identified as *Candidatus Branchiomonas*. This taxon
270 constituted 74% of all diversity in the gills of the susceptible line and 85% in the
271 resistant line. We identified trace amounts of *Flavobacterium sp.* in both lines, as this
272 taxon constituted 0.16 % of the ARS-Fp-S line and 1.1% of the ARS-Fp-R line. No
273 ASVs were differentially abundant in the gill microbial community of both groups
274 through ANCOM or DEseq2 analyses.

275 We observed discernable differences in the microbial community composition of
276 fish of the same line housed in different tanks (Fig 5B). For example, *Candidatus*
277 *Branchiomonas* was present at levels above 95% in tanks 25 (ARS-Fp-R) and 26
278 (ARS-Fp-S), whereas it constituted 71% of tank 11 (ARS-Fp-R) and 20% of tank 12
279 (ARS-Fp-S). The aforementioned potential opportunistic pathogens *Brevinema sp.*
280 was only identified in tank 12. A heatmap of the top 30 ASVs in each sample (Fig 6)
281 shows signatures of *Brevinema sp.* in fish from tank 12, as well as a reduced
282 abundance of *Candidatus Branchiomonas* in this tank compared to all others.

283 **Fig 5. Microbial community composition of the gills of ARS-Fp-R and ARS-Fp-S trout.**
284 (A) Relative abundance of ASVs at the phylum level for each line. (B) Relative abundance of ASVs for
285 tank the two lines were divided into.

286 **Fig 6. Heatmap representing the top 30 ASVs represented in the gill of ARS-Fp-R and ARS-Fp-S**
287 **trout.**
288 Each column represents one individual fish. Each row represents one ASV.

289 **3.7. Random Forest Modeling**

290 Random forest modeling [31] was performed in order to see if a machine learning
291 module could accurately predict treatment group as well as spleen index based on
292 microbial community composition. In the mid-gut, this model was able to accurately
293 predict 100% of FPR samples, but only 50% of FPS samples (Fig 7). Table 1 shows
294 features at the genus level that were rendered as being important in classifying
295 treatment group. Due to the lower sampling size in the gills, we did not include
296 random forest analyses of the gill samples. Finally, we also performed a random
297 forest regressor to attempt to predict spleen index based on gut microbial
298 composition, and found that there was no correlation between these two variables.

299 **Table 1: Features at the genus level that were identified by random forest modeling as being**

300 **important in the classification of treatment group**

Feature	Importance
Ralstonia	0.194781898
Mycoplasma	0.190003235
Variovorax	0.129277426
Brevinema	0.098010406
Paenibacillus	0.093643145
Hydrotalea	0.084976654
Ambiguous Desulfovibrionaceae	0.08063865
Streprococcus	0.034762947
Staphylococcus	0.027645825
Ambiguous Gammaproteobacteria	0.024251868
Shewanella	0.023781255
Ambiguous Ruminococcaceae	0.014827708
Exiguobacterium	0.002446602
Ambiguous Moraxellaceae	0.000952381

301

302 **Fig 7. Confusion matrix for random forest modeling in the gut.**

303 This heatmap represents how frequently samples within each line were correctly classified. The

304 correct label is represented on the y-axis and the predicted label is represented on the x-axis, with

305 the prediction frequency shown as a gradient.

306

307 ## 4. Discussion

308 Commensal microbes have co-evolved with their eukaryotic counterparts, forming
309 an intricate relationship that benefits both parties involved. Several studies have
310 revealed that host genetics influences gut microbiota composition in a variety of
311 species, including humans and rodents [4,5], chickens [32], and *Drosophila* [33].
312 However, other factors such as host diet and environmental factors are also deeply
313 intertwined and deeply shape host microbial communities [7–9].
314 Teleost fish live in symbiosis with complex microbial communities that inhabit
315 every mucosal barrier (gut, gills, skin and nose) [34]. Fish microbial community
316 composition is influenced by age [35], tissue site [36], diet [37–39], stress [40] and
317 pathogen infection [41]. However, few studies have investigated the impact host
318 genetics have on shaping teleost microbiomes. A study on brook charr identified
319 three quantitative trait loci associated with abundance of commensal strains in the
320 skin [42]. Another study in Atlantic salmon found significant differences in the skin
321 and gut microbial composition amongst distinct wild populations that are not likely
322 attributed to environmental conditions alone [43]. These observations suggest that
323 host genetics play an important role in teleost microbiome assembly, although more
324 work is needed to better understand this relation.
325 Farmed fish are susceptible to many pathogens that threaten the sustainability of
326 the finfish farming industry. Among the most prominent bacterial diseases, BCWD is
327 particularly problematic in rainbow trout. The development of two genetic trout
328 lines with different susceptibilities to BCWD agent, *F. psychrophilum*, offers an
329 excellent platform to understand how host genetics may shape fish microbial
330 communities. The current study suggests that host genetics influence the microbial

331 community composition in the mid-gut, since the mid-gut bacterial community of
332 the susceptible line was significantly more diverse than that of the resistant line. In
333 agreement with previous studies, the mid-gut communities of both lines were
334 dominated by *Mycoplasma sp.* This taxon appears to be highly abundant in the
335 gastrointestinal microbiome of all salmonid species studied, including Atlantic
336 salmon [44–46], rainbow trout [36,47] and Chinook salmon [48]. Interestingly, we
337 identified a lower abundance of *Mycoplasma sp*. in the susceptible line, suggesting
338 that disease susceptibility may be associated with decreased *Mycoplasma sp.* levels
339 in the gut. In support, a recent study investigated the intestinal microbiome of
340 offshore farmed Chinook salmon [48], finding that abundance of potential
341 pathogenic *Vibrio sp.* appeared to be inversely correlated with the presence of
342 *Mycoplasma sp. Mycoplasma sp.* are characterized by their uniquely small genome
343 and lack of a cell wall, which makes culture-based approaches to studying this
344 bacterium difficult to achieve. Considering the widespread presence and abundance
345 of *Mycoplasma sp*. in salmonid gastrointestinal tract across a wide range of
346 geographical locations, including both lab-reared fish and wild-caught, it appears as
347 if there have been strong evolutionary forces that have enabled *Mycoplasma sp.* to
348 thrive in this microenvironment. Future studies are needed to determine the nature
349 of this relationship and the ability of *Mycoplasma sp.* to prevent pathogen
350 colonization in the gastrointestinal tract of salmonids.
351 Potential opportunistic pathogens were observed within the mid-gut of the ARS-Fp-
352 S line at a greater abundance than in the ARS-Fp-R line. Expansions of opportunistic
353 taxa have been previously described in other fish microbiome studies including in

354 the skin of Atlantic salmon experimentally infected with salmonid alphavirus (Reid,
355 2017) and in Atlantic salmon experimentally infected with the parasite
356 *Lepeophtheirus salmonis* [41]. Specifically, we found *Brevinema sp.* and ambiguous
357 members of the family Enterobacteriaceae in the susceptible line. *Brevinema sp.* was
358 further identified as *Brevinema andersonii* at the species level in the Silva database,
359 which is a pathogenic taxa that has been isolated in rodents [49]. However, upon
360 sequence search using BLAST, they were most closely identified as an uncultured
361 Spirochaeta sequence derived from an aquatic environment. Spirochaetes members
362 have been described to cause disease in other marine species [50], and there are
363 multiple well-studied members that are human pathogens [51]. Deeper taxonomic
364 characterization would be necessary to determine if this ASV is in fact a pathogen in
365 rainbow trout. Meanwhile, Enterobacteriaceae reads were most closely identified as
366 *Hafnia paralvei,* which is associated with infections in a variety of animals, including
367 fish [52]. Although the relative abundance of these taxa was small, these results
368 suggest that ARS-Fp-R trout may possess a more resilient mid-gut microbiome, as
369 compared to ARS-Fp-S. It is important to note that these fish were reared on spring
370 water and sampled only at one timepoint. Additional studies are needed to examine
371 whether these trends are observed in other environments and stages of
372 development.
373 Differences in the microbial community composition between both trout lines were
374 less pronounced in the gills compared to the gut, although it should be noted that
375 the sample size was smaller in this tissue due to difficulties amplifying the 16S rDNA
376 region in certain samples. Nonetheless, alpha diversity metrics were similar in each

377 line. Interestingly, *Candidatus Branchiomonas* was the dominant taxon in both lines.
378 This is a known pathogen that has been shown to cause gill epitheliocystis in
379 Atlantic salmon [53], although it has not been previously described in rainbow
380 trout. Fish from both lines were visually healthy, suggesting that *Candidatus*
381 *Branchiomonas* may be a common member of the trout gill microbiome in certain
382 environments. Further studies should evaluate which factors favor the colonization
383 of *Candidatus Branchiomonas* in trout gills.
384 *Flavobacterium sp.* was identified in the gills of both lines, although sequence search
385 using BLAST did not yield species level taxonomic resolution. Although this taxon
386 was present at low abundance in both lines, it was surprising that we detected
387 higher *Flavobacterium sp.* abundance in the gill microbiome of the resistant line
388 compared to the susceptible line. Considering that the resistant and susceptible
389 lines maintained the survival phenotype following the challenge experiment, our
390 results indicate that susceptibility to *F. psychrophilum* infection is not due to
391 increased abundance of this pathogen as a member of the indigenous microbial
392 community. Disease susceptibility may be instead due to the greater ability of this
393 pathogen to displace the microbial communities in the susceptible line compared to
394 the resistant line.
395 One of the interesting aspects of the present study was the identification of
396 differences in the microbial composition between tanks of the same trout genetic
397 lines. All tanks were supplied water from the same source, and proper water quality
398 was maintained throughout the experiment. This tank effect complicates the ability
399 to discern between genetic lines, although there still appears to be notable

400 differences when comparing each line, particularly in the gut as discussed earlier.
401 There were similarities between two of the neighboring tanks that housed different
402 lines, in tanks 11 and 12. However, this did not occur in the other two neighboring
403 tanks, as the susceptible trout from tank 26 displayed an expansion of pathogenic
404 taxa compared to tank 25, which housed resistant trout.
405 Work in zebrafish has shown that interhost dispersal can actually outweigh genetic
406 factors that contribute to microbiome assembly [54]. In order to assess whether
407 host genetics contribute to host microbiome assembly, all external factors that
408 potentially contribute to this network must also be accounted for. In the present
409 study, diet was consistent across fish of all tanks, and environmental factors such as
410 water quality and temperature were also consistently maintained. Still, differences
411 amongst tanks were present that add noise to the between-group comparisons. This
412 finding highlights the importance of adequate experimental design in fish
413 microbiome studies as well as the fact that different factors differentially shape
414 microbial assembly at different tissue sites (i.e. gut versus gills).
415

416 **5. Conclusions**

417 In conclusion, the present study reveals differences in the microbial composition of
418 the gut but not the gills of two rainbow trout lines with differential susceptibility to
419 *F. psychrophilum* infection. Disease susceptibility was associated with a more
420 diverse gut microbiome and the presence of potentially pathogenic taxa although
421 important tank effects were also detected. Thus, selective breeding programs may
422 not only select for host genetic factors but also, as a consequence, unique microbial

423 assemblies, which in turn, may render the host more or less resilient to pathogen
424 invasion or infection.
425
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433 imply recommendation or endorsement by the U.S Department of Agriculture. USDA
434 is an equal opportunity employer.
435
436 **Data Availability**
437 All datasets obtained have been deposited at NCBI BioProject and are publicly
438 available under BioProject ID number PRJNA488363.
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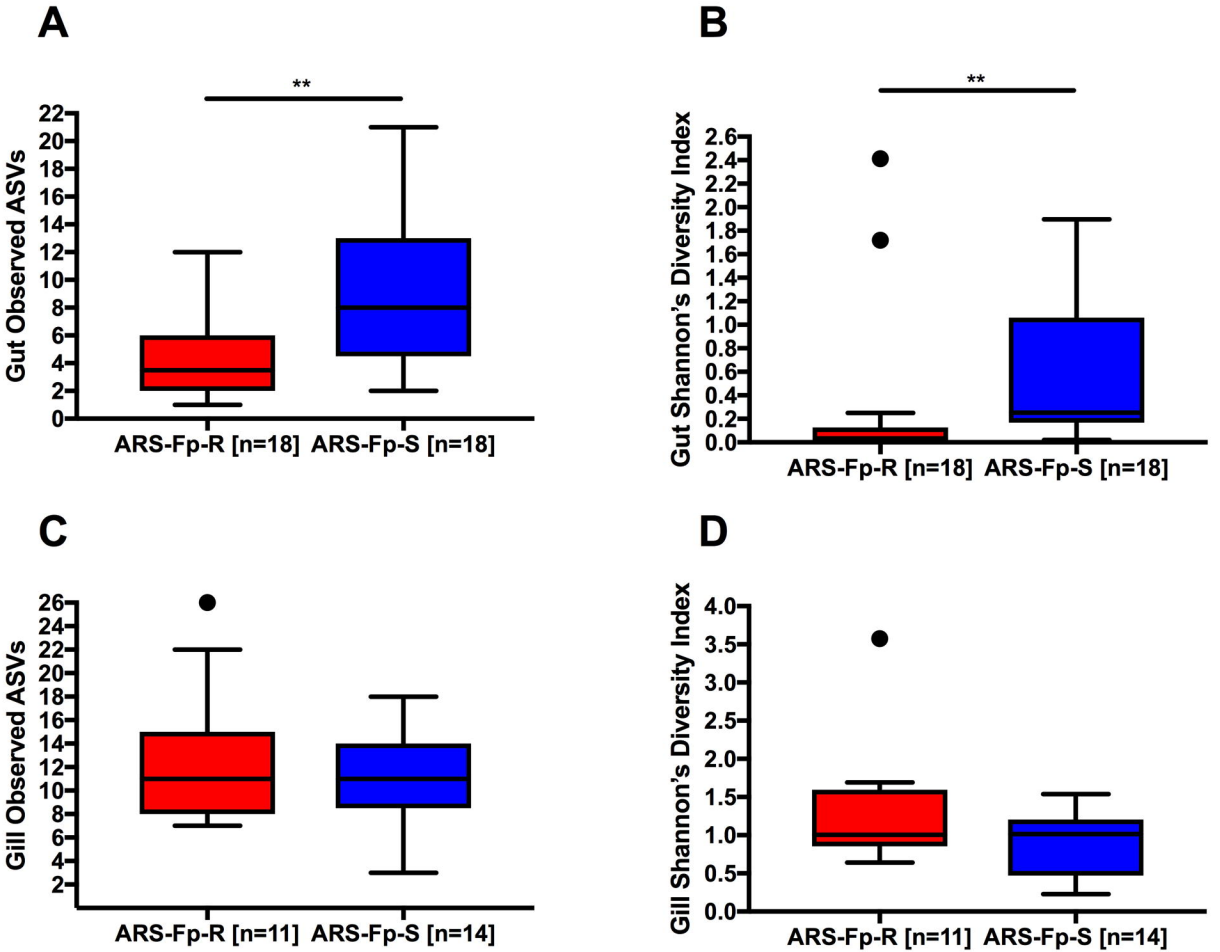
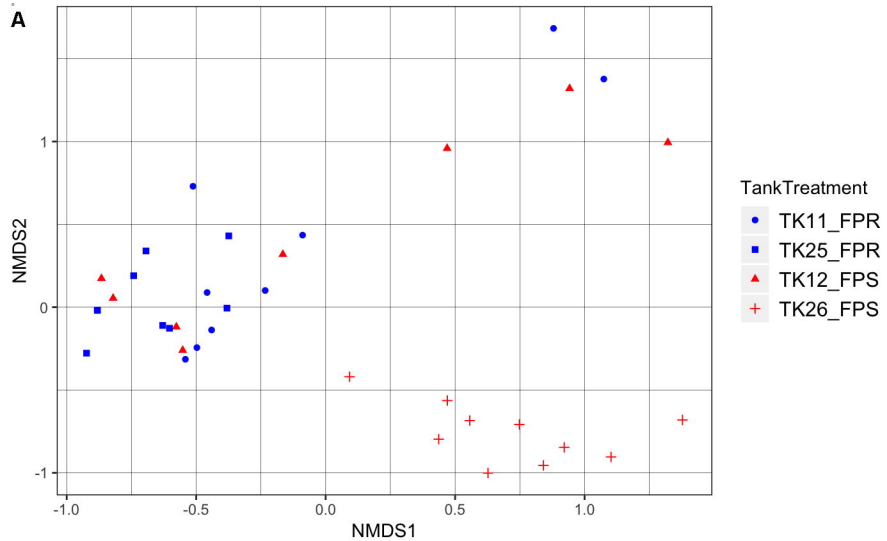
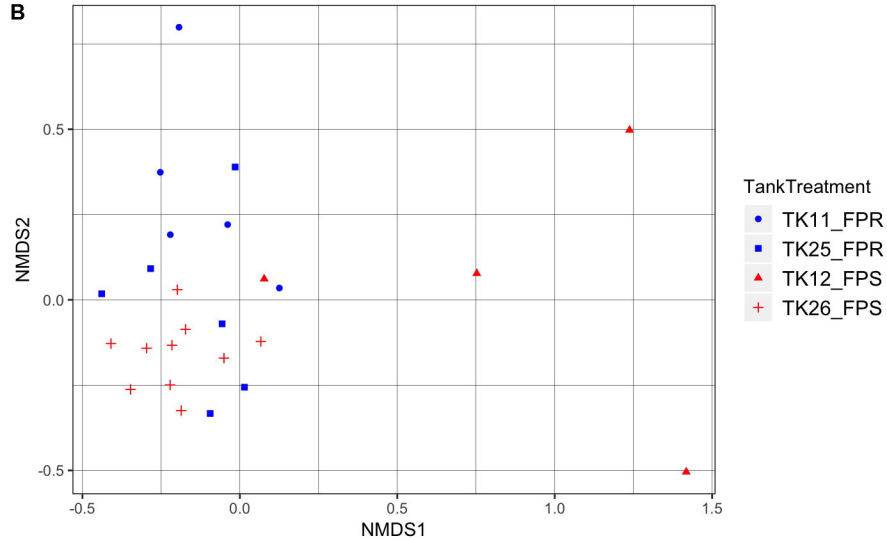
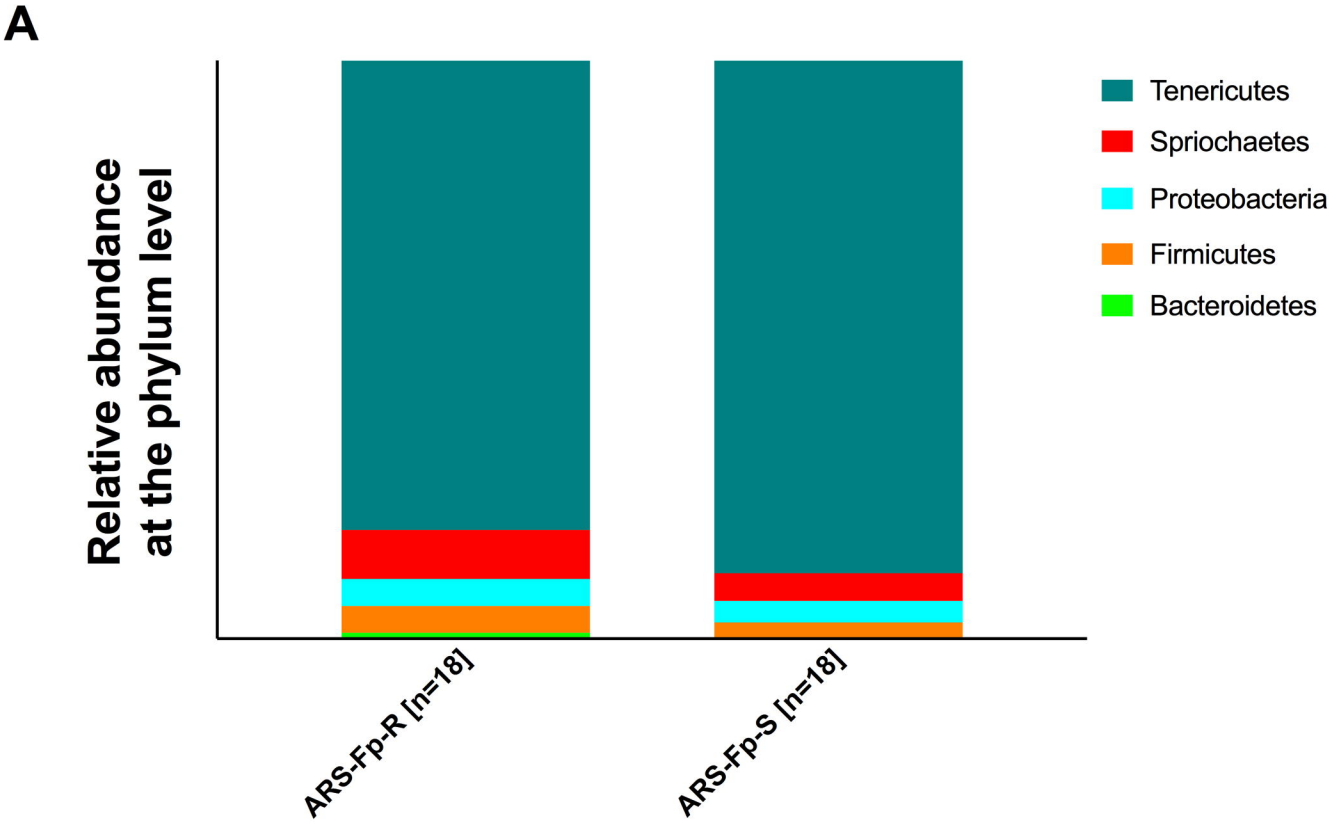
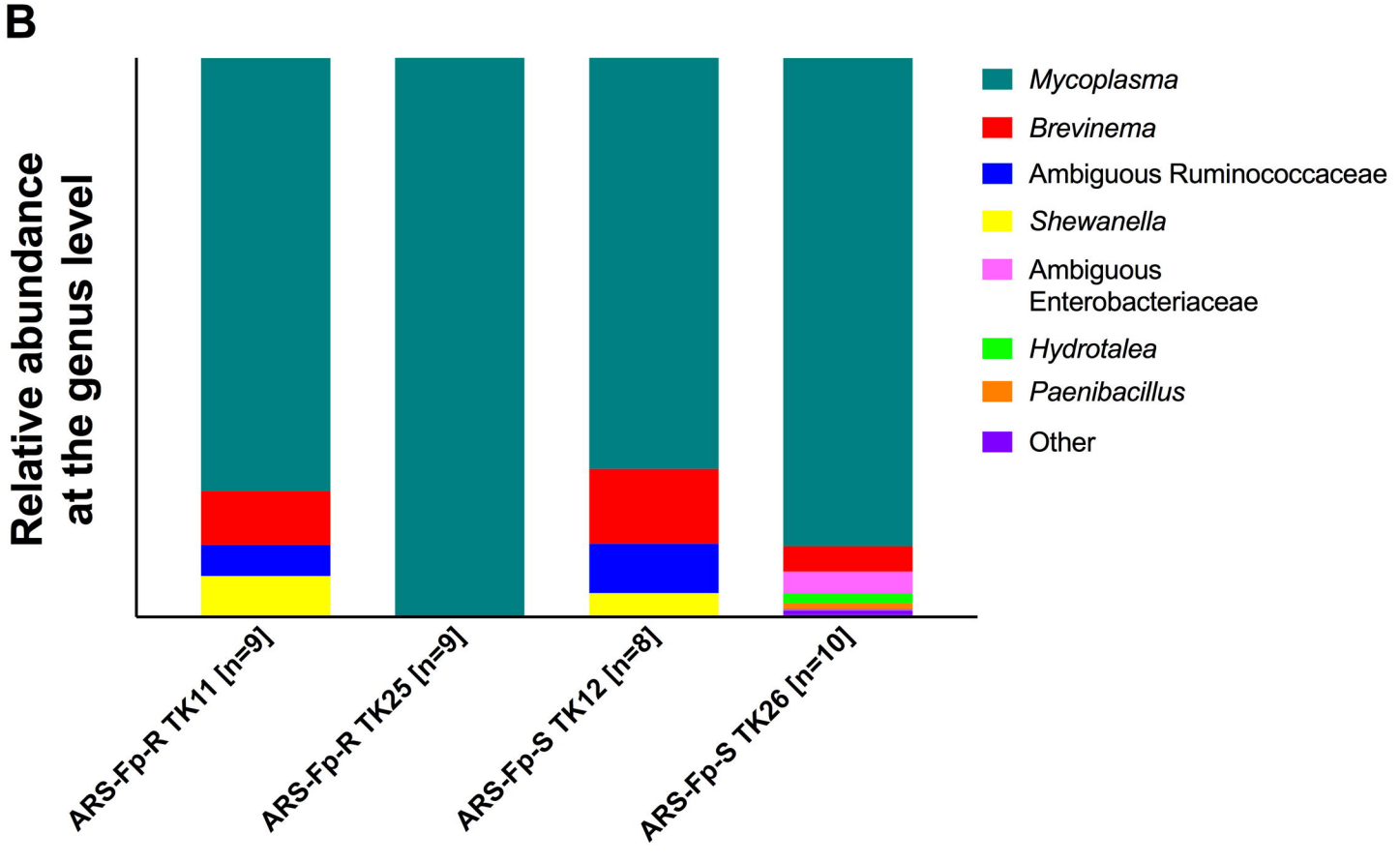
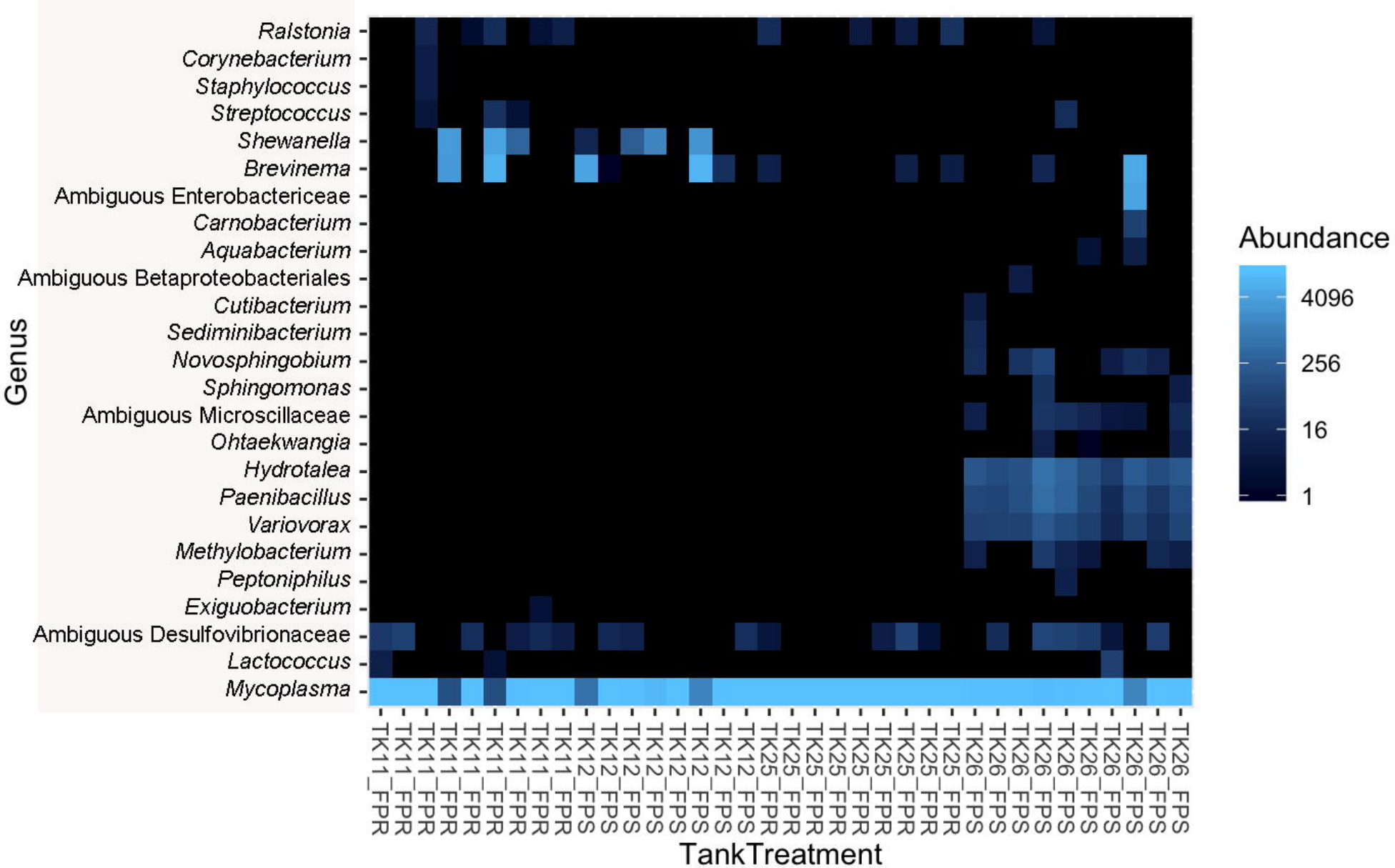

A
**
Gut Observed ASVs
22
20
18
16
14
12
10
8
6
4
2
0
ARS-Fp-R [n=18]
ARS-Fp-S [n=18]
B
**
Gut Shannon's Diversity Index
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ARS-Fp-R [n=18]
ARS-Fp-S [n=18]
C
Gill Observed ASVs
26
24
22
20
18
16
14
12
10
8
6
4
2
ARS-Fp-R [n=11]
ARS-Fp-S [n=14]
D
Gill Shannon's Diversity Index
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ARS-Fp-R [n=11]
ARS-Fp-S [n=14]


A
NMDS2
NMDS1
1
0
-1
-1.0
-0.5
0.0
0.5
1.0
TankTreatment
TK11_FPR
TK25_FPR
TK12_FPS
TK26_FPS


B
NMDS2
NMDS1
0.5
0.0
-0.5
-0.5
0.0
0.5
1.0
1.5
TankTreatment
TK11_FPR
TK25_FPR
TK12_FPS
TK26_FPS


A
Relative abundance at the phylum level
Tenericutes
Spriochaetes
Proteobacteria
Firmicutes
Bacteroidetes
ARS-Fp-R [n=18]
ARS-Fp-S [n=18]


B
Relative abundance at the genus level
Mycoplasma
Brevinema
Ambiguous Ruminococcaceae
Shewanella
Ambiguous Enterobacteriaceae
Hydrotalea
Paenibacillus
Other
ARS-Fp-R TK11 [n=9]
ARS-Fp-R TK25 [n=9]
ARS-Fp-S TK12 [n=8]
ARS-Fp-S TK26 [n=10]


Genus
Ralstonia
Corynebacterium
Staphylococcus
Streptococcus
Shewanella
Brevinema
Ambiguous Enterobactericeae
Carnobacterium
Aquabacterium
Ambiguous Betaproteobacteriales
Cutibacterium
Sediminibacterium
Novosphingobium
Sphingomonas
Ambiguous Microscillaceae
Ohtaekwangia
Hydrotalea
Paenibacillus
Variovorax
Methylobacterium
Peptoniphilus
Exiguobacterium
Ambiguous Desulfovibrionaceae
Lactococcus
Mycoplasma
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK26_FPS
TK26_FPS
TK26_FPS
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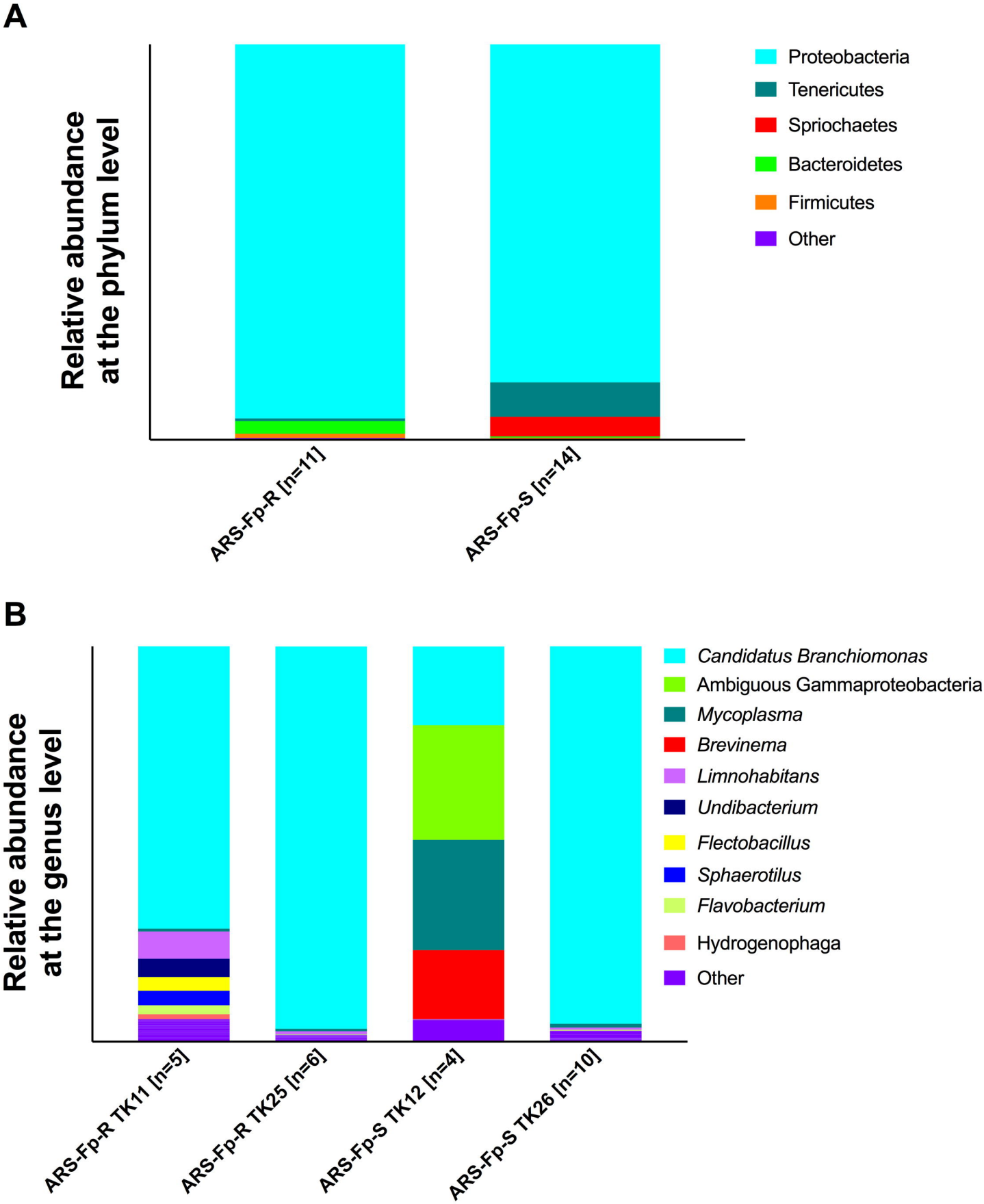
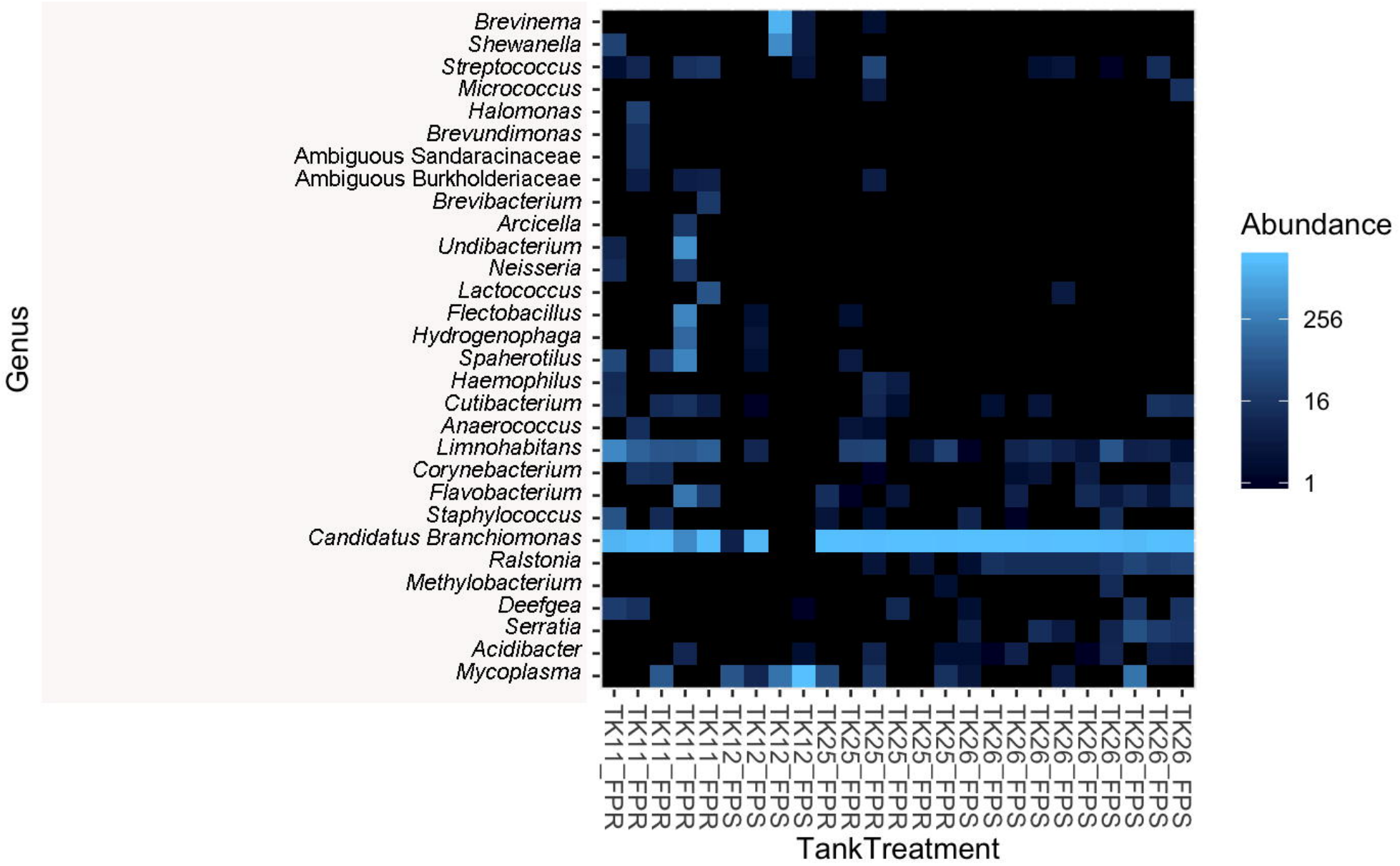
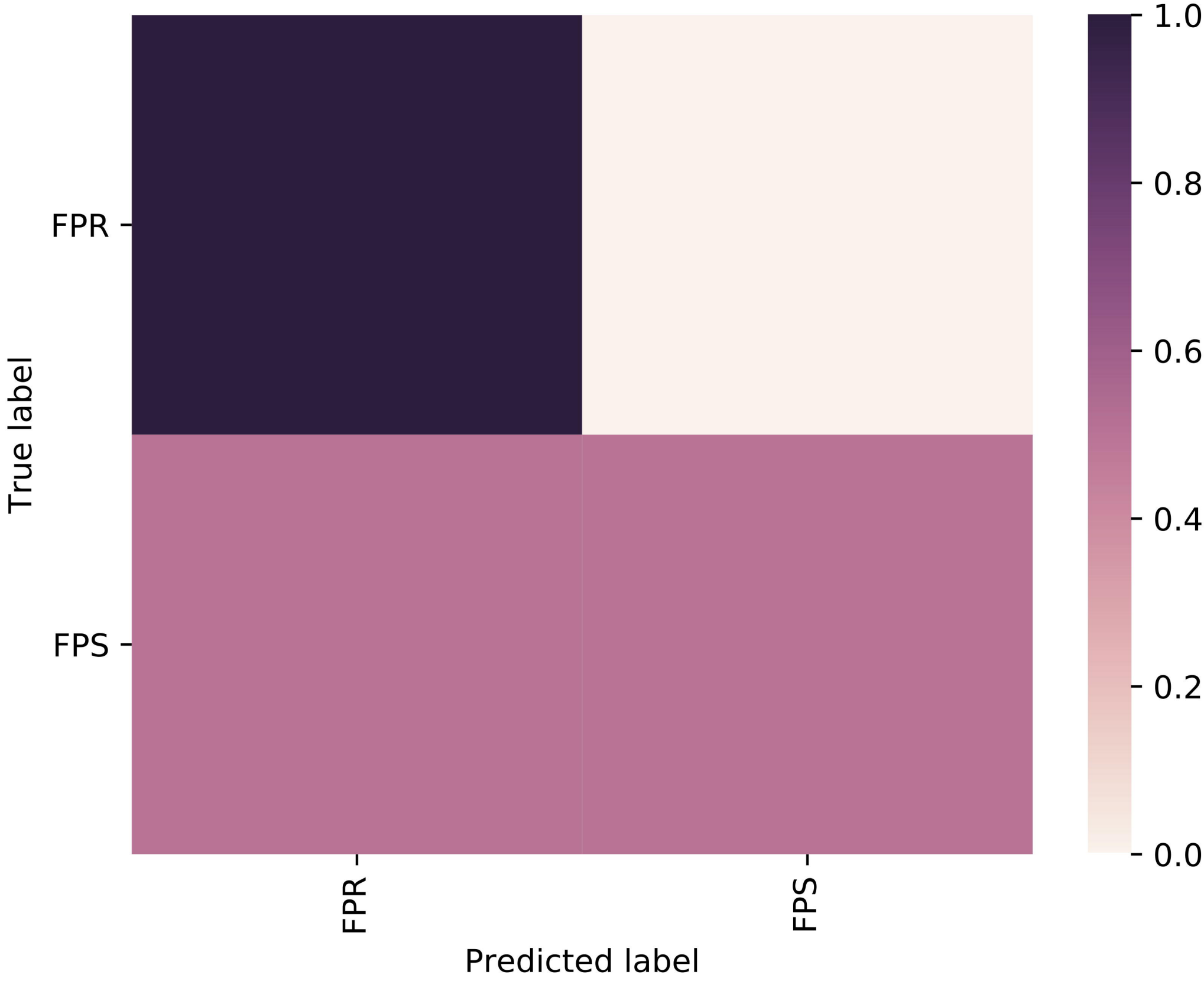
TK26_FPS
TkTreatment
Abundance
4096
256
16
1


A
Relative abundance at the phylum level
Proteobacteria
Tenericutes
Spriochaetes
Bacteroidetes
Firmicutes
Other
ARS-Fp-R [n=11]
ARS-Fp-S [n=14]
B
Relative abundance at the genus level
Candidatus Branchiomonas
Ambiguous Gammaproteobacteria
Mycoplasma
Brevinema
Limnohabitans
Undibacterium
Flectobacillus
Sphaerotilus
Flavobacterium
Hydrogenophaga
Other
ARS-Fp-R TK11 [n=5]
ARS-Fp-R TK25 [n=6]
ARS-Fp-S TK12 [n=4]
ARS-Fp-S TK26 [n=10]


Genus
Brevinema
Shewanella
Streptococcus
Micrococcus
Halomonas
Brevundimonas
Ambiguous Sandaracinaceae
Ambiguous Burkholderiaceae
Brevibacterium
Arcicella
Undibacterium
Neisseria
Lactococcus
Flectobacillus
Hydrogenophaga
Spaherotilus
Haemophilus
Cutibacterium
Anaerococcus
Limnohabitans
Corynebacterium
Flavobacterium
Staphylococcus
Candidatus Branchiomonas
Ralstonia
Methylobacterium
Deefgea
Serratia
Acidibacter
Mycoplasma
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK11_FPR
TK12_FPS
TK12_FPS
TK12_FPS
TK12_FPS
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK25_FPR
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TK26_FPS
TankTreatment
Abundance
256
16
1


FPR
FPS
True label
FPR
FPS
Predicted label
1.0
0.8
0.6
0.4
0.2
0.0