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 lower 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.

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 include necrosis of the caudal region, skin lesions, eroded fin tips, and loss of appetite. *F. psychrophilum* has a more pronounced effect on young fry, a condition referred to as rainbow trout fry syndrome. Rainbow trout fry syndrome is responsible for acute losses in trout farms worldwide, as the associated mortality rate is reported to be greater than 50% (12). BCWD is becoming an increasingly difficult disease to treat, as *F. psychrophilum* strains

69 have developed resistance to several commonly used antibiotics (13–15), and there is
70 currently no commercially available licensed vaccine.
71 The National Center for Cool and Cold Water Aquaculture (NCCCWA) utilized family-
72 based selective breeding to develop two distinctive genetic lines of rainbow trout that
73 confer enhanced resistance (ARS-Fp-R), or susceptibility (ARS-Fp-S) to the pathogen *F.*
74 *psychrophilum* (16). Enhanced resistance to *F. psychrophilum*-induced mortality in the
75 ARS-Fp-R line has been described, both in the laboratory setting and on trout farms
76 (17,18). Previous studies have investigated possible host mechanisms that attribute to
77 enhanced resistance. For instance, a strong correlation between resistance to *F.*
78 *psychrophilum* and increased spleen size has been described, although this relation does
79 not appear to translate to other common fish pathogens, such as *Yersinia ruckeri* (19).
80 Additionally, whole-body transcriptome analysis has identified numerous acute phase
81 proteins and inflammatory cytokines that are differentially expressed in each line
82 following challenge with *F. psychrophilum* (20). Further work is needed to better
83 characterize the mechanism(s) by which enhanced resistance is achieved in the ARS-Fp-
84 R line.
85 In this paper, we investigate the hypothesis that the higher innate disease resistance of the
86 ARS-Fp-R line is due to differences in the microbial composition at host mucosal
87 surfaces. Using 16S rDNA amplicon sequencing, we determine and compare the mid-gut
88 and gill microbiomes of rainbow trout lines selectively bred for *F. psychrophilum*
89 resistance and susceptibility.
90

91 **Materials and Methods**

92 **Animals and sampling**

93 The Institutional Animal Care and Use Committee (Leetown, WV) reviewed and
94 approved all animal husbandry practices and disease challenge protocols per standards set
95 forth in the USDA, ARS Policies and Procedures 130.4.v.3 titled 'Institutional Animal
96 Care and Use 84 Committee'. Fish used in these experiments were from the 2017 Year
97 Class and maintained as specific pathogen free as determined by biannual testing as
98 previously described (17). A total of 33 single sire-dam matings contributed to the pool
99 of ARS-Fp-R line fish and 31 matings contributed to the pool of ARS-Fp-S line. The
100 disease resistant phenotype of each genetic line was evaluated at two time-points, 75 and
101 276 days post-hatch. Fish were challenged with *F. psychrophilum* strain CSF259-93 and
102 survival recorded over 21 days as previously described (19). Mean fish body weight at
103 the first evaluation was 1.9 g and a total of 120 ARS-Fp-R and 119 ARS-Fp-S fish (n=3
104 tanks per line) were challenged by intraperitoneal injection with a dose of 1.4E+07 CFU
105 g^{-1} in a total volume of 50 µL using a 26g needle fitted onto an Eppendorf repeating
106 pipette. Mean body weight at the second evaluation was 194 g and a total of 70 ARS-Fp-
107 R and 70 ARS-Fp-S fish (n=2 tanks per line) were challenged by intramuscular injection
108 with a dose of 3.5E+06 CFU g^{-1} body weight in a total volume of 50 µL using a 26g
109 needle fitted onto an Eppendorf repeating pipette. The fish utilized in the second
110 experiment were part of a larger study evaluating experimental vaccination and these fish
111 had been sham vaccinated with PBS 35 days prior to challenge. In both challenges, *F.*
112 *psychrophilum* was isolated from mortalities and confirmed by PCR genotyping.
113 At the time of microbiome sampling, fish were reared under two different tank conditions
114 as described in Supplemental data file 1. Ten fish from each tank system per genetic line

were euthanized using 200 mg L^{-1} MS222 for 5 minutes. Each fish was photographed, weighted, gill tissue sampled, intestinal mid-gut sampled, and spleen weighed within 30 minutes of euthanasia. Sample were placed in SLB (21) on ice and then moved to storage at -80˚ C. Instruments were cleaned between each fish and gloves changed between tank groups. Three control tubes containing SLB alone were included as negative controls.

DNA Extraction, 16S rDNA PCR Amplification, and Sequencing

Whole genomic DNA was extracted from skin and gill samples by first lysing tissue samples using sterile 3 mm tungsten beads (Qiagen) and Qiagen TissueLyser II. Next, using the cetyltrimethylammonium bromide method as previously described (21), DNA was isolated and suspended in 50 µL RNase and DNase free molecular biology grade water. DNA concentration and purity was then assessed using a Nanodrop ND 1000 (Thermo Scientific).

Bacterial DNA was then replicated by PCR using Illumina adapter fused primers targeting the V1-V3 region of the prokaryotic 16S rDNA gene. The primer sequences were as follows: 28F 5'-GAGTTTGATCNTGGCTCAG-3' and 519R 5'GTNTTACNGCGGCKGCTG-3' (where N = any nucleotide, and K = T or G). DNA samples were diluted 1:10 or 1:100, and Quantabio 5PRIME HotMasterMix was used. 16S amplicons were generated using the following conditions: 94° C for 90s; 33 cycles of 94° C for 30s, 52° C for 30s, 72° C for 90s; and a final extension of 72° C for 7 min A positive control of a verified 16S V1-V3 amplicon, and a negative control of molecular biology grade water was included in every PCR reaction. In addition, we included a mock community positive control with each sequencing run, which consisted of equal DNA

amounts of 7 different bacterial isolates previously cloned and a negative control that consisted of SLB handled in the same way as the rest of the tubes during sampling to which no tissue was added. Amplicons were purified using the Axygen AxyPrep Mag PCR Clean-up Kit (Thermo Scientific), and eluted into 30 μL molecular biology grade water. Unique oligonucleotide barcodes were ligated to the 5' and 3' ends of each sample, as well as the Nextera adaptor sequences, using the Nextera XT Index Kit v2 set A (Illumina). DNA concentrations were quantified using a Qubit, and normalized to a concentration of 200 ng/uL for DNA library pooling. Pooled samples were cleaned once more using the Axygen PCR clean-up kit before being sent off for sequencing. Sequencing was performed on the Illumina Miseq platform using the MiSeq Reagent Kit v3 (600 cycle) at the Clinical and Translational Sciences Center at the University of New Mexico Health Sciences Center.

Data Analysis and Statistics

Differences in survival between genetic lines were determined using the product limit method of Kaplan and Meier and calculations were performed using GraphPad v4.0 software. Log-rank (Mantel-Cox) test was used to compare survival curves. Initial sequence data was analyzed using the latest version of Quantitative Insights Into Microbial Ecology 2 (Qiime2 v2018.6) (22). Demultiplexed sequence reads were quality filtered using DADA2 (23). Samples were then rarefied to a sampling depth of 12,603 sequences per sample for mid-gut reads, and 2020 sequences per sample for gill reads. Amplicon sequence variants (ASVs) were picked by aligning to the latest SILVA 16S database (version 132). Core diversity metrics were analyzed, including number of ASVs and Shannon's diversity index for alpha diversity, and PERMANOVA for beta diversity.

Nonmetric multidimensional scaling and generation of heat maps were performed in RStudio (24) using the phyloseq package (25). Random forest modeling was performed in Qiime2. Differential abundance testing was performed in Qiiime2 using ANCOM (26), as well as in RStudio using the DESeq2 package (27). For all statistical analyses, fish were split into groups based on 'Treatment' (ARS-Fp-R, ARS-Fp-S) or 'Tank Treatment'.

Data Availability

Sequencing data was deposited in NCBI BioProject # PRJNA488363.

Results

Phenotype Confirmation of Disease Resistance/Susceptibility

The relative phenotype of the two genetic lines was evaluated at time-points either before or after microbiome sampling. At both time points, the survival of the ARS-Fp-R genetic line was significantly higher ($P<0.001$) than the ARS-Fp-S line and consistent with estimated mid-point breeding values. In the first evaluation, a total of 3/120 (3%) ARS-Fp-R line fish died compared to 82/119 (69%) ARS-Fp-S line fish. In the second evaluation, 2/70 (3%) ARS-Fp-R fish died, while 58/70 (83%) ARS-Fp-S line fish died within the 21-day challenge period.

High throughput sequencing analysis

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

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

Resistant and susceptible lines display significant differences in alpha diversity in the mid-gut, but not the gills

Comparison of alpha diversity metrics obtained from the mid-gut showed significantly lower measures of gut microbial community richness (Observed ASVs), as well as Shannon's diversity index in the ARS-Fp-R line compared to the ARS-Fp-S line (Fig 1A and 1B). There was a total of 15 ASVs in the mid-gut of the resistant line, and 29 in the mid-gut of the susceptible line. In the gills, there were no significant differences in alpha diversity between lines with a total of 57 ASVs found in the gills of the resistant line, and 50 in the susceptible line (Fig 1C and 1D).

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

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

Beta diversity analysis suggests possible tank effect on the gut microbiome

We assessed the microbial diversity between different treatments, as well as between tanks by performing Nonmetric Multidimensional Scaling (NMDS) using the Bray Curtis distance metric. This ordination showed a discrete grouping of the two tanks containing the ARS-Fp-S line, while the two tanks containing the ARS-Fp-R line were more tightly clustered (Fig 2A). PERMANOVA analysis (28) identified “Treatment” (P value = 0.02)

and "Tank" (P value = 0.048 for ARS-Fp-R, P value = 0.001 for ARS-Fp-S) as significant determinants of the mid-gut microbial community composition. In the gills, NMDS ordination showed a similar pattern to that found in the gut, where fish from tank 26 of the susceptible line clustered tightly together while fish from tank 12 showed greater variability. Meanwhile, there was no clear separation between individuals held in separate tanks of the resistant line (Fig 2B). PERMANOVA identified only tank housing within the ARS-Fp-S line (P value = 0.004) as a significant factor in determining gill microbial communities.

Fig 2. NMDS ordination plots.

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

Gut microbial community composition

A total of eight different phyla were identified in the mid-gut across both lines, although only four of these were represented over 1%, including Tenericutes, Spirochaetes, Proteobacteria, and Firmicutes. Tenericutes composed the vast majority of the mid-gut microbiome of both lines, constituting 81% of the total microbial diversity in the susceptible line and 89% in the resistant line (Fig 3A).

At the genus level, all Tenericutes reads were identified as *Mycoplasma sp.* Additionally, the genus *Brevinema sp.* was also abundant and showed greater abundance in the ARS-Fp-S (8.5%) compared to the ARS-Fp-R line (4.8%). Similarly, members of the family Enterobacteriaceae had greater abundance in the ARS-Fp-S line than the ARS-Fp-R line (2.2% and 0%, respectively); however, these differences were not significant. Differential abundance testing with ANCOM revealed three genera that were differentially abundant in the mid-gut of both trout lines; including *Hydrotalea sp., Paenibacillus sp.,* and

Variovorax sp. This was replicated by differential abundance testing using DESeq2, an R package originally developed for differential expression analysis in RNA-seq data also used in microbiome studies (29,30).

The relative distribution of ASVs at the genus level was notably different between tanks of the same line (Fig 3B). Potential opportunistic taxa such as *Brevinema sp.* and Ambiguous Enterobacteriaceae were present in tanks 11 and 12, but were not identified in either tank 25 or 26. This trend is further shown in a heatmap representing the top 25 ASVs observed in each sample (Fig 4). Two of the tanks that were in close proximity to one another displayed similar microbial profiles, despite holding different lines. This was not the case for tanks 25 and 26, as tank 26 displayed a microbial profile different from all other tanks since it contained ASVs representative of Enterobacterieaceae, *Hydrotalea sp., Paenibacillus sp,* and *Variovorax sp.*

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

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

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

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

Gill microbial community composition

A total of nine different phyla were present in the trout gills across both lines. Five of these were represented at abundance greater than 1%, including Proteobacteria, Tenericutes, Spirochaetes, Bacteriodetes, and Firmicutes. Proteobacteria was the most abundant phylum in both groups, representing 85% of all bacterial diversity in the ARS-Fp-S line and 95% in the ARS-Fp-R line (Fig 5A). At the genus level, most

Proteobacteria reads were identified as *Candidatus Branchiomonas*. This taxon constituted 74% of all diversity in the gills of the susceptible line and 85% in the resistant line. We identified trace amounts of *Flavobacterium sp.* in both lines, as this taxon constituted 0.16 % of the ARS-Fp-S line and 1.1% of the ARS-Fp-R line. No ASVs were differentially abundant in the gill microbial community of both groups through ANCOM or DEseq2 analyses.

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

Fig 5. Microbial community composition of the gills of ARS-Fp-R and ARS-Fp-S trout.

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

Fig 6. Heatmap representing the top 30 ASVs represented in the gill of ARS-Fp-R and ARS-Fp-S trout.

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

Random Forest Modeling

Random forest modeling (31) was performed in order to see if a machine learning module could accurately predict treatment group as well as spleen index based on microbial community composition. In the mid-gut, this model was able to accurately predict 100%

of FPR samples, but only 50% of FPS samples (Fig 7). Table 1 shows features at the genus level that were rendered as being important in classifying treatment group. Due to the lower sampling size in the gills, we did not include random forest analyses of the gill samples. Finally, we also performed a random forest regressor to attempt to predict spleen index based on gut microbial composition, and found that there was no correlation between these two variables.

Table 1: Features at the genus level that were identified by random forest modeling as being 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
Streptococcus	0.034762947
Staphylococcus	0.027645825
Ambiguous Gammaproteobacteria	0.024251868
Shewanella	0.023781255
Ambiguous Ruminococcaceae	0.014827708
Exiguobacterium	0.002446602
Ambiguous Moraxellaceae	0.000952381

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

This heatmap represents how frequently samples within each line were correctly classified. The correct label is represented on the y-axis and the predicted label is represented on the x-axis, with the prediction frequency shown as a gradient.

Discussion

Commensal microbes have co-evolved with their eukaryotic counterparts, forming an intricate relationship that benefits both parties involved. Several studies have revealed that host genetics influences gut microbiota composition in a variety of species, including humans and rodents (4,5), chickens (32), and *Drosophila* (33). However, other factors such as host diet and environmental conditions are also deeply intertwined and clearly shape host microbial communities (7–9).

Teleost fish live in symbiosis with complex microbial communities that inhabit every mucosal barrier (gut, gills, skin and nose) (34). Fish microbial community composition is influenced by age (35), tissue site (36), diet (37–39), stress (40) and pathogen infection (41). However, few studies have investigated the impact host genetics has on shaping teleost microbiomes. A study on brook charr identified three quantitative trait loci associated with abundance of commensal strains in the skin (42). Another study in Atlantic salmon found significant differences in the skin and gut microbial composition amongst distinct wild populations that were not likely attributed to environmental conditions alone (43). These observations suggest that host genetics play an important role in teleost microbiome assembly, although more work is needed to better understand this relation.

312 Farmed fish are susceptible to many pathogens that threaten the sustainability of the
313 finfish farming industry. Among the most prominent bacterial diseases, BCWD is
314 particularly problematic in rainbow trout. The development of two genetic trout lines
315 with different susceptibilities to BCWD agent, *F. psychrophilum*, offers an excellent
316 platform to understand how host genetics may shape fish microbial communities. The
317 current study suggests that host genetics influence the microbial community composition
318 in the mid-gut, since the mid-gut bacterial community of the susceptible line was
319 significantly more diverse than that of the resistant line. In agreement with previous
320 studies, the mid-gut communities of both lines were dominated by *Mycoplasma sp.* This
321 taxon appears to be highly abundant in the gastrointestinal microbiome of all salmonid
322 species studied, including Atlantic salmon (44–46), rainbow trout (36,47) and Chinook
323 salmon (48). Interestingly, we identified a lower abundance of *Mycoplasma sp*. in the
324 susceptible line, suggesting that disease susceptibility may be associated with decreased
325 *Mycoplasma sp.* levels in the gut. In support, a recent study investigated the intestinal
326 microbiome of offshore farmed Chinook salmon (48), finding that abundance of potential
327 pathogenic *Vibrio sp.* appeared to be inversely correlated with the presence of
328 *Mycoplasma sp. Mycoplasma sp.* are characterized by their uniquely small genome and
329 lack of a cell wall, which makes culture-based approaches to studying this bacterium
330 difficult to achieve. Considering the widespread presence and abundance of *Mycoplasma*
331 *sp*. in salmonid gastrointestinal tract across a wide range of geographical locations,
332 including both lab-reared and wild-caught fish, it appears that strong evolutionary forces
333 have enabled *Mycoplasma sp.* to thrive in this microenvironment. Future studies are

334 needed to determine the nature of this relationship and the ability of *Mycoplasma sp.* to
335 prevent pathogen colonization in the gastrointestinal tract of salmonids.
336 Potential opportunistic pathogens were observed within the mid-gut of the ARS-Fp-S line
337 at a greater abundance than in the ARS-Fp-R line. Expansions of opportunistic taxa have
338 been previously described in other fish microbiome studies including in the skin of
339 Atlantic salmon experimentally infected with salmonid alphavirus (37) and in Atlantic
340 salmon experimentally infected with the parasite *Lepeophtheirus salmonis* (41).
341 Specifically, we found *Brevinema sp.* and ambiguous members of the family
342 Enterobacteriaceae in the mid-gut of the susceptible trout line. *Brevinema sp.* was further
343 identified as *Brevinema andersonii* at the species level in the SILVA database, which is a
344 pathogenic taxon at least in rodents (49). However, upon sequence search using BLAST,
345 it was most closely identified as an uncultured Spirochaeta sequence derived from an
346 aquatic environment. Spirochaetes members have been described to cause disease in
347 other marine species (50), and there are multiple well-studied members that are human
348 pathogens (51). Deeper taxonomic characterization would be necessary to determine if
349 this ASV is in fact a pathogen in rainbow trout. Meanwhile, Enterobacteriaceae reads
350 were most closely identified as *Hafnia paralvei,* which is associated with infections in a
351 variety of animals, including fish (52). Although the relative abundance of these taxa was
352 small, these results suggest that ARS-Fp-R trout may possess a more resilient mid-gut
353 microbiome, as compared to ARS-Fp-S. It is important to note that these fish were
354 reared on spring water and sampled only at one timepoint. Additional studies are needed
355 to examine whether these trends are observed in other environments, stages of
356 development or upon introduction of a perturbance.

357 Differences in the microbial community composition between both trout lines were less
358 pronounced in the gills compared to the gut, although it should be noted that the sample
359 size was smaller in this tissue due to difficulties amplifying the 16S rDNA region in
360 certain samples. Nonetheless, alpha diversity metrics were similar in each line.
361 Interestingly, *Candidatus Branchiomonas* was the dominant taxon in both lines. This is a
362 known pathogen that has been shown to cause gill epitheliocystis in Atlantic salmon (53),
363 although it has not been previously described in rainbow trout. Fish from both lines were
364 visually healthy, suggesting that *Candidatus Branchiomonas* may be a common member
365 of the trout gill microbiome in certain environments. Further studies should evaluate
366 which factors favor the colonization of *Candidatus Branchiomonas* in trout gills.
367 *Flavobacterium sp.* was identified in the gills of both lines, although sequence search
368 using BLAST did not yield species level taxonomic resolution. Although this taxon was
369 present at low abundance in both lines, it was surprising that we detected higher
370 *Flavobacterium sp.* abundance in the gill microbiome of the resistant line compared to
371 the susceptible line. Considering that the resistant and susceptible lines maintained the
372 survival phenotype following the challenge experiment, our results indicate that
373 susceptibility to *F. psychrophilum* infection is not due to increased abundance of this
374 pathogen as a member of the indigenous microbial community. Disease susceptibility
375 may be instead due to the greater ability of this pathogen to displace the microbial
376 communities in the susceptible line compared to the resistant line.
377 One of the interesting aspects of the present study was the identification of differences in
378 the microbial composition between tanks of the same trout genetic lines. All tanks were
379 supplied water from the same source, and proper water quality was maintained

380 throughout the experiment. This tank effect complicates the ability to discern between
381 genetic lines, although there still appears to be notable differences when comparing each
382 line, particularly in the gut as discussed earlier. There were similarities between two of
383 the neighboring tanks that housed different lines, in tanks 11 and 12. However, this did
384 not occur in the other two neighboring tanks, as the susceptible trout from tank 26
385 displayed an expansion of pathogenic taxa compared to tank 25, which housed resistant
386 trout. Work in zebrafish has shown that interhost dispersal can actually outweigh genetic
387 factors that contribute to microbiome assembly (54). In order to assess whether host
388 genetics contribute to host microbiome assembly, all external factors that potentially
389 contribute to this network must also be accounted for. In the present study, diet was
390 consistent across fish of all tanks, and environmental factors such as water quality and
391 temperature were also consistently maintained. Still, differences amongst tanks were
392 present that add noise to the between-group comparisons. This finding highlights the
393 importance of adequate experimental design in fish microbiome studies as well as the fact
394 that different factors differentially shape microbial assembly at different tissue sites (i.e.
395 gut versus gills).
396

397 **Conclusions**

398 In conclusion, the present study reveals differences in the microbial composition of the
399 gut but not the gills of two rainbow trout lines with differential susceptibility to *F.*
400 *psychrophilum* infection. Disease susceptibility was associated with a more diverse gut
401 microbiome and the presence of potentially pathogenic taxa although important tank
402 effects were also detected. Thus, selective breeding programs may not only select for host

403 genetic factors but also, as a consequence, unique microbial assemblies, which in turn,
404 may render the host more or less resilient to pathogen invasion or infection.
405
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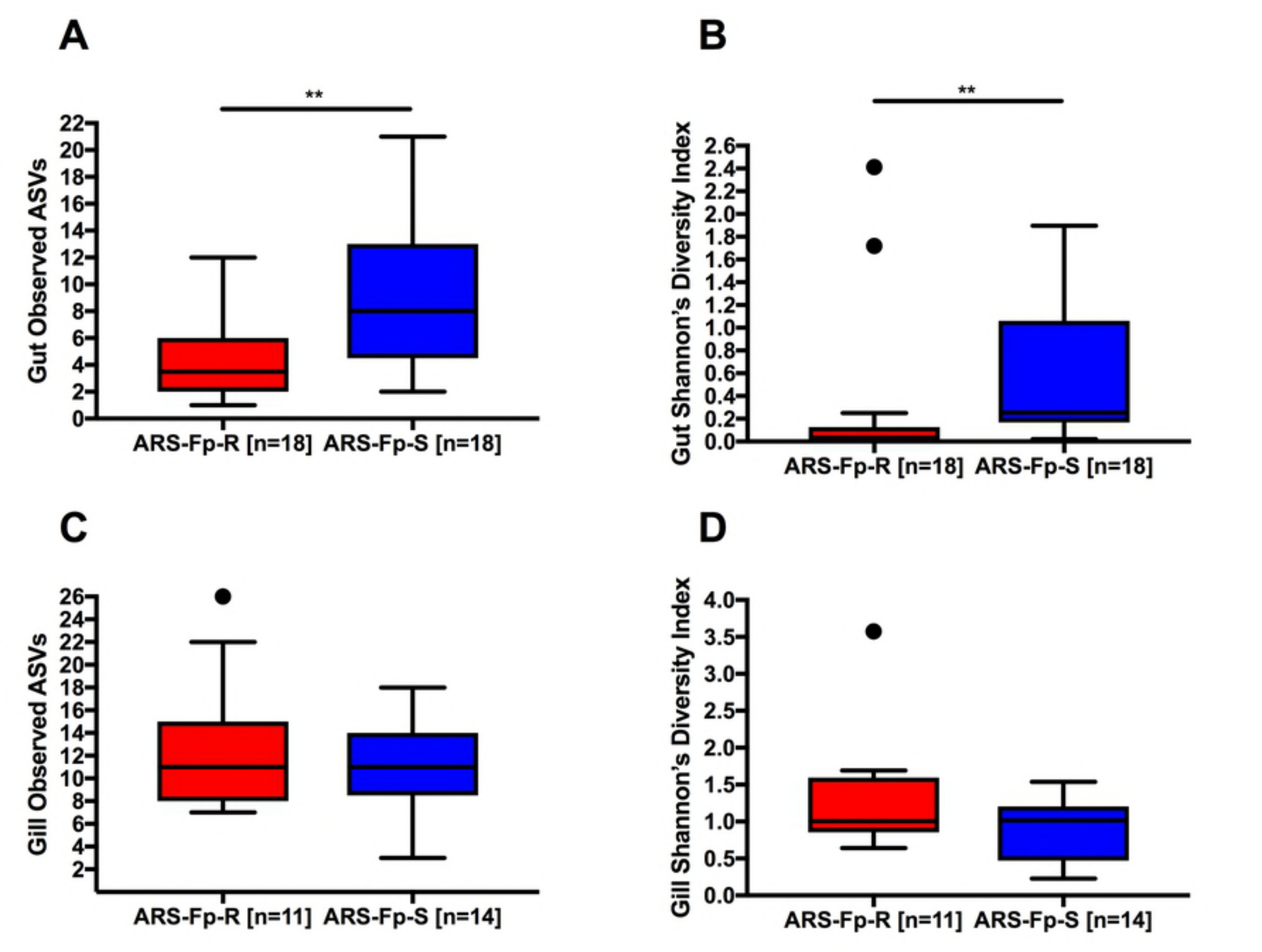
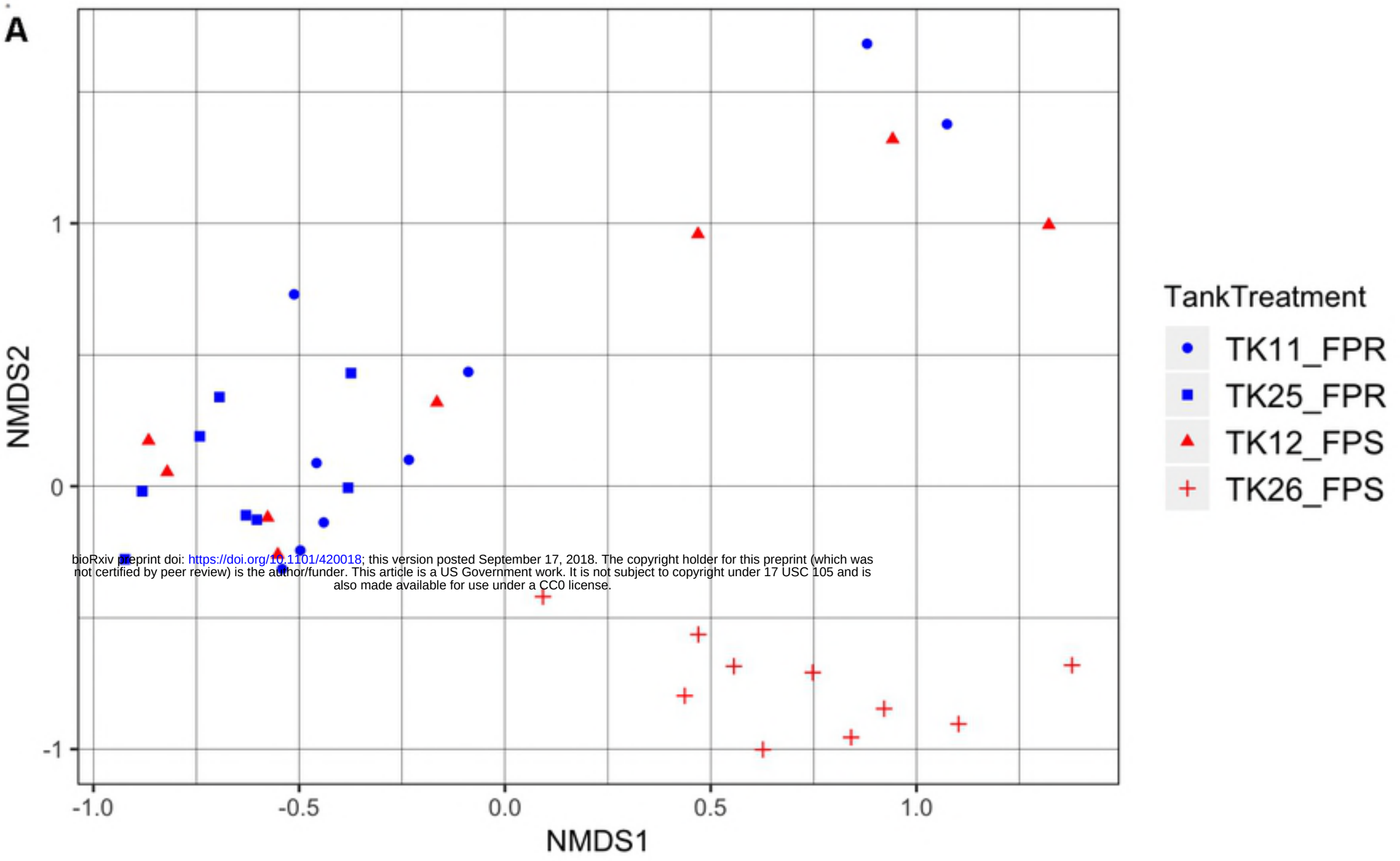

A
**
Gut Observed ASVs
0
2
4
6
8
10
12
14
16
18
20
22
ARS-Fp-R [n=18]
ARS-Fp-S [n=18]
B
**
Gut Shannon's Diversity Index
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
ARS-Fp-R [n=18]
ARS-Fp-S [n=18]
C
Gill Observed ASVs
2
4
6
8
10
12
14
16
18
20
22
24
26
ARS-Fp-R [n=11]
ARS-Fp-S [n=14]
D
Gill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

A



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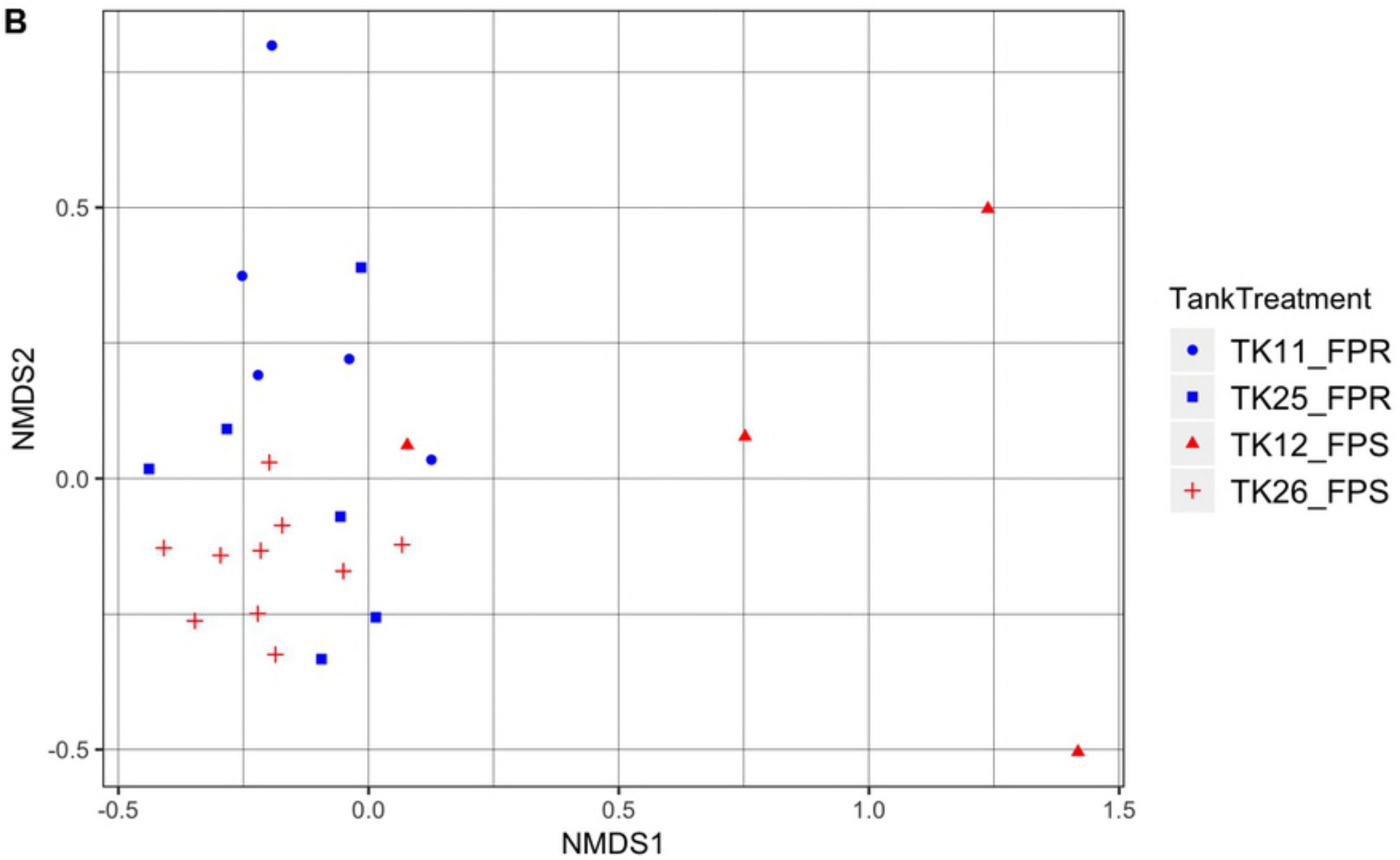

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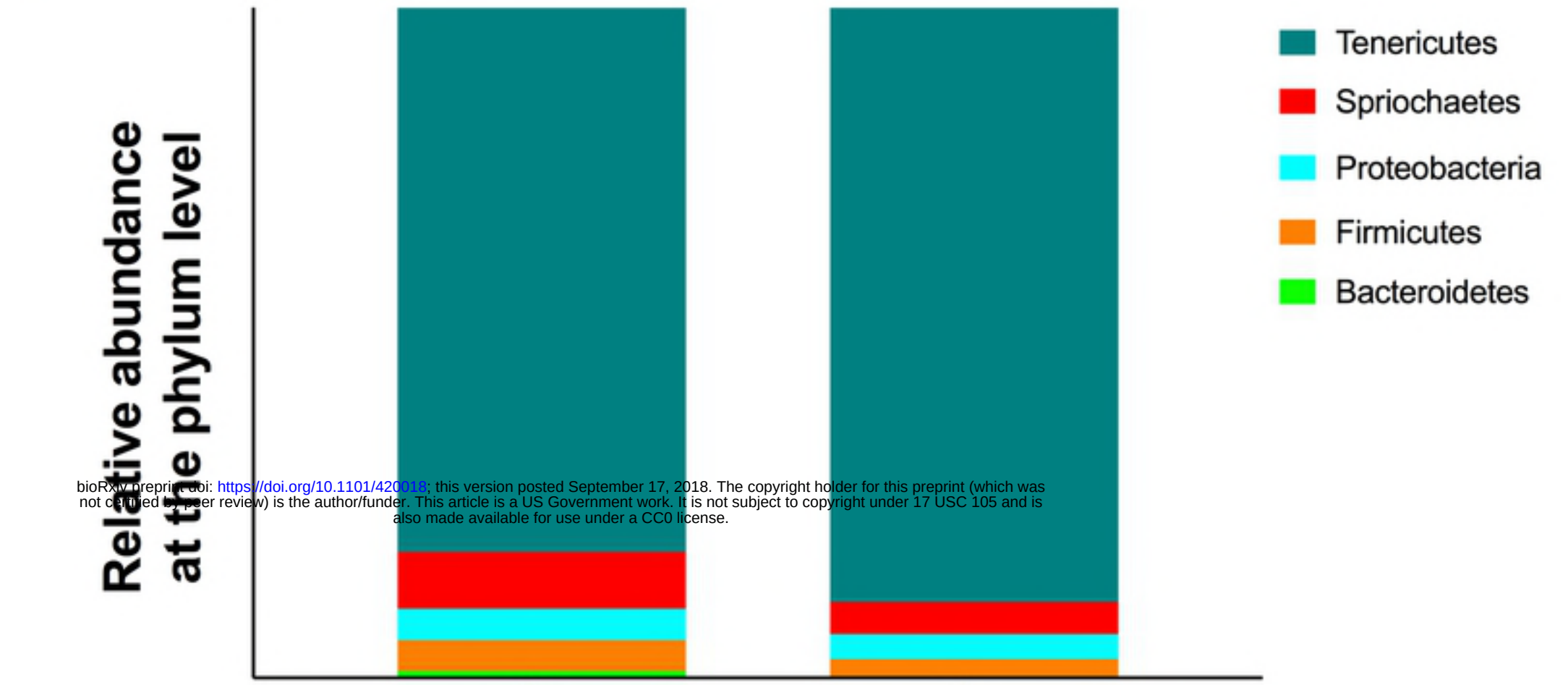

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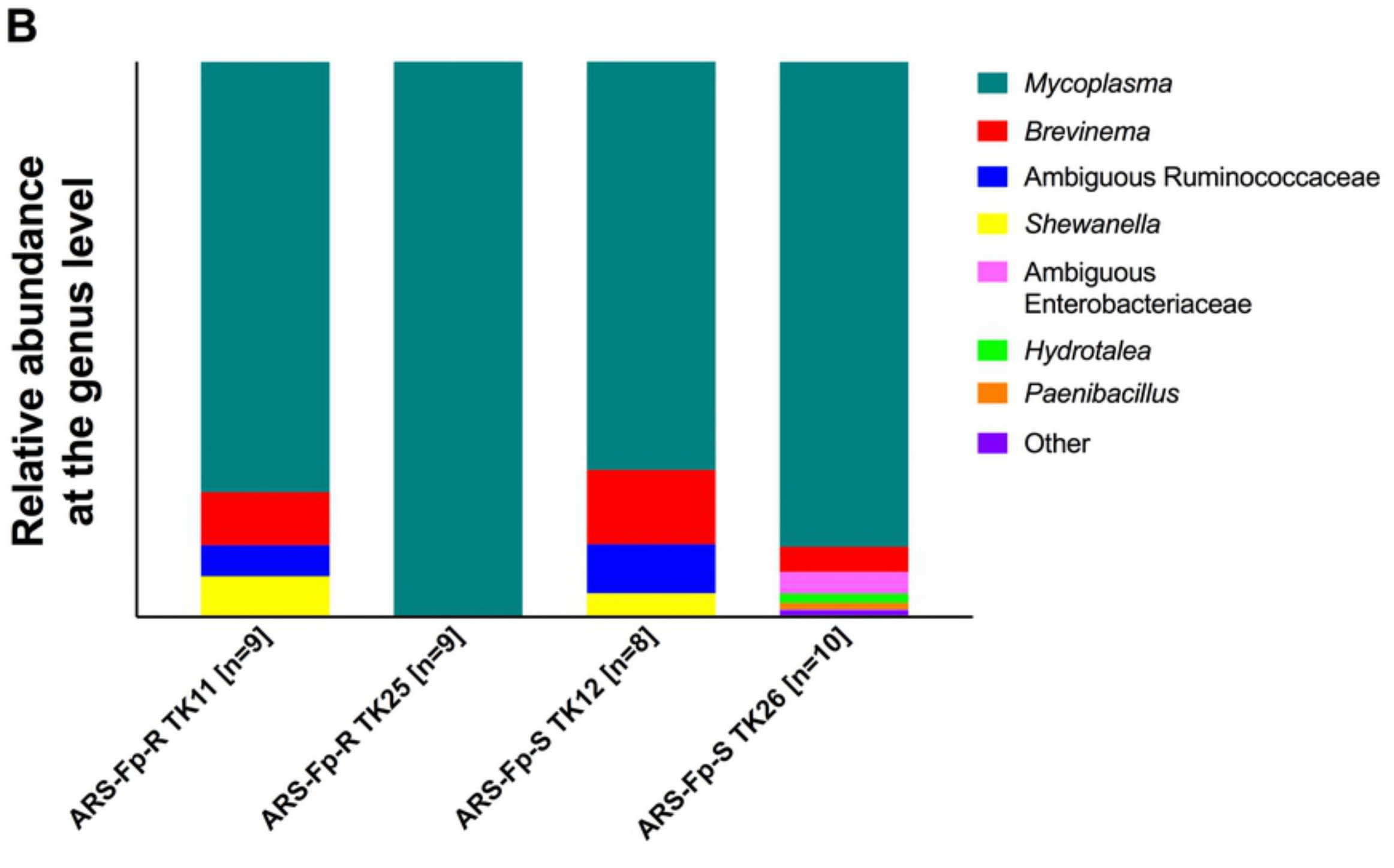

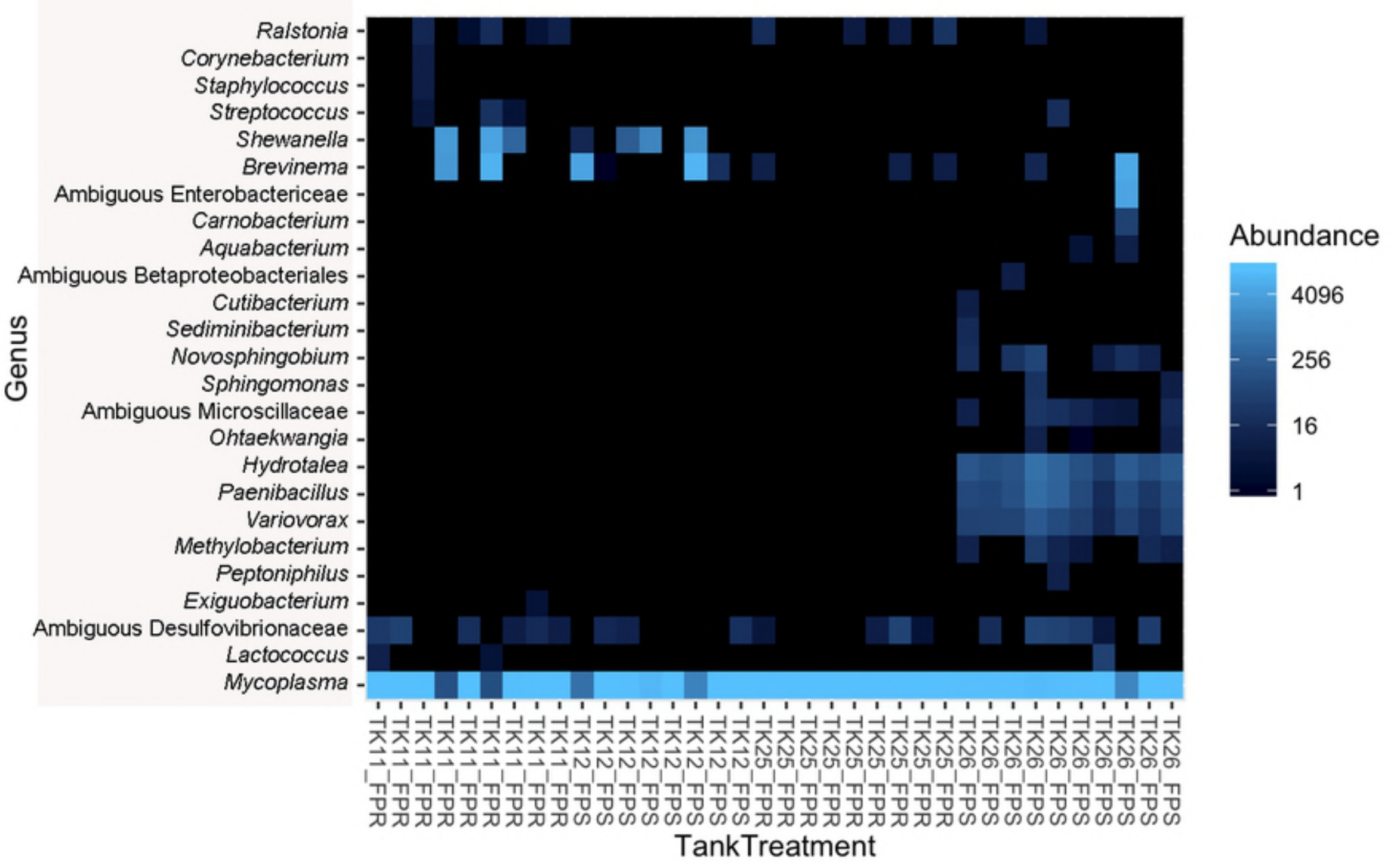
Genus
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Corynebacterium
Staphylococcus
Streptococcus
Shewanella
Brevinema
Ambiguous Enterobactericeae
Carnobacterium
Aquabacterium
Ambiguous Betaproteobacteriales
Cutibacterium
Sediminibacterium
Novosphingobium
Sphingomonas
Ambiguous Microscillaceae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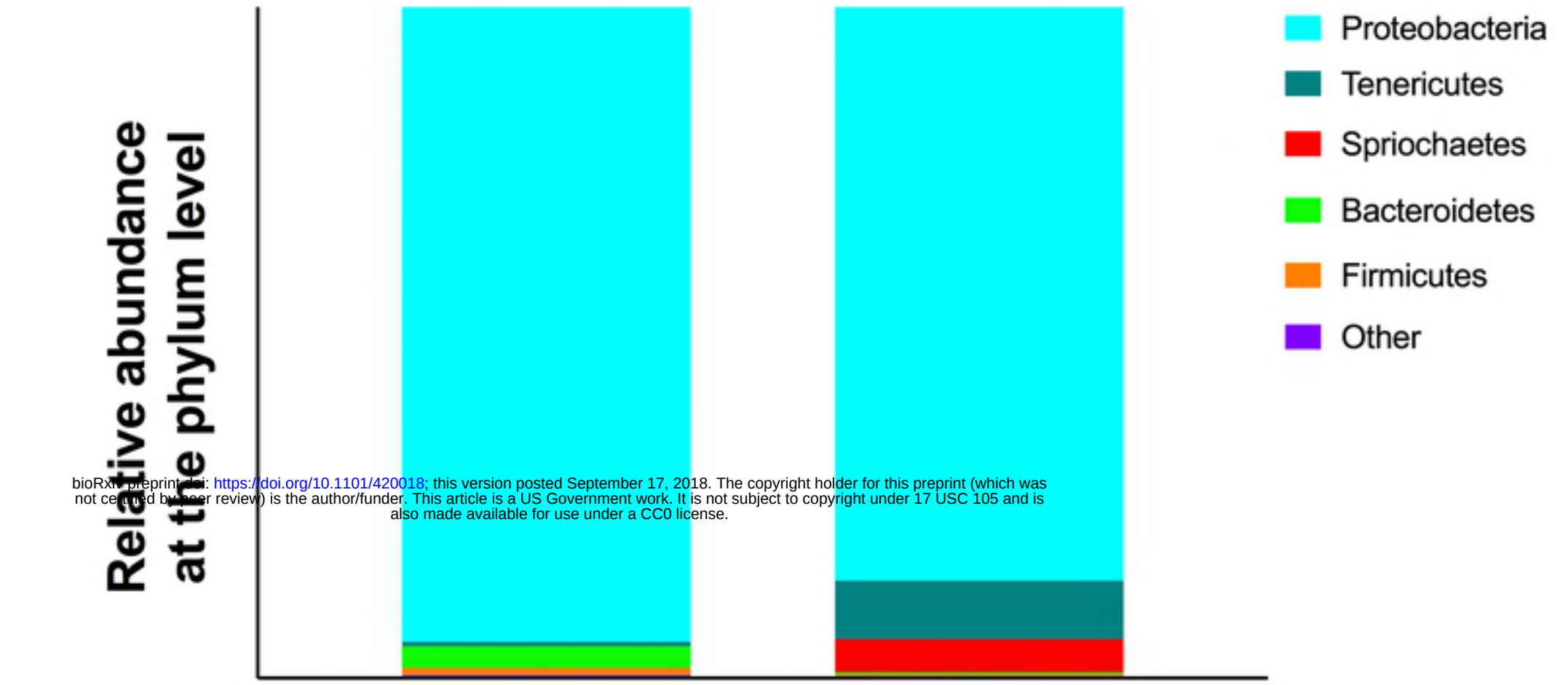
TankTreatment
Abundance
4096
256
16
1

A



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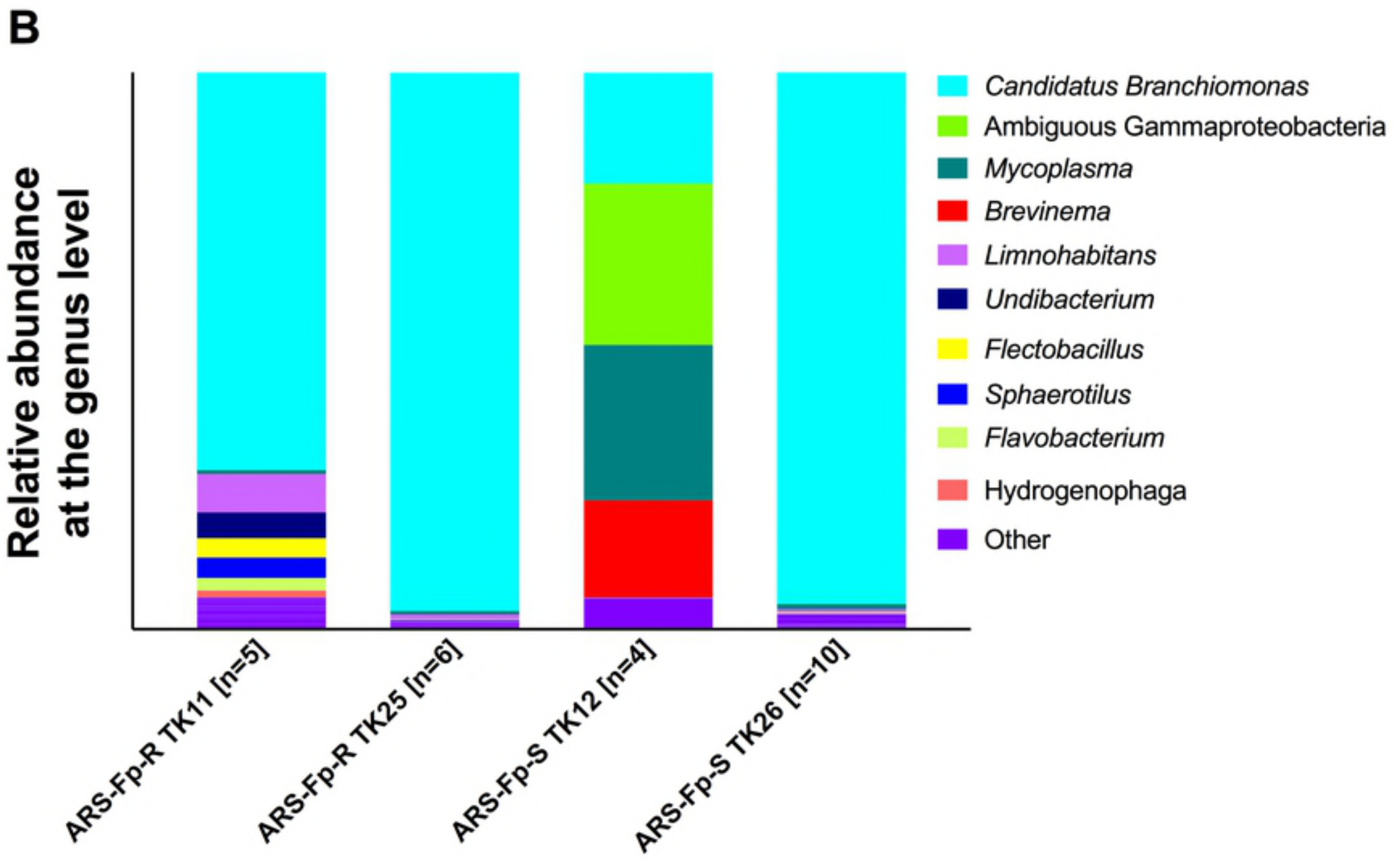

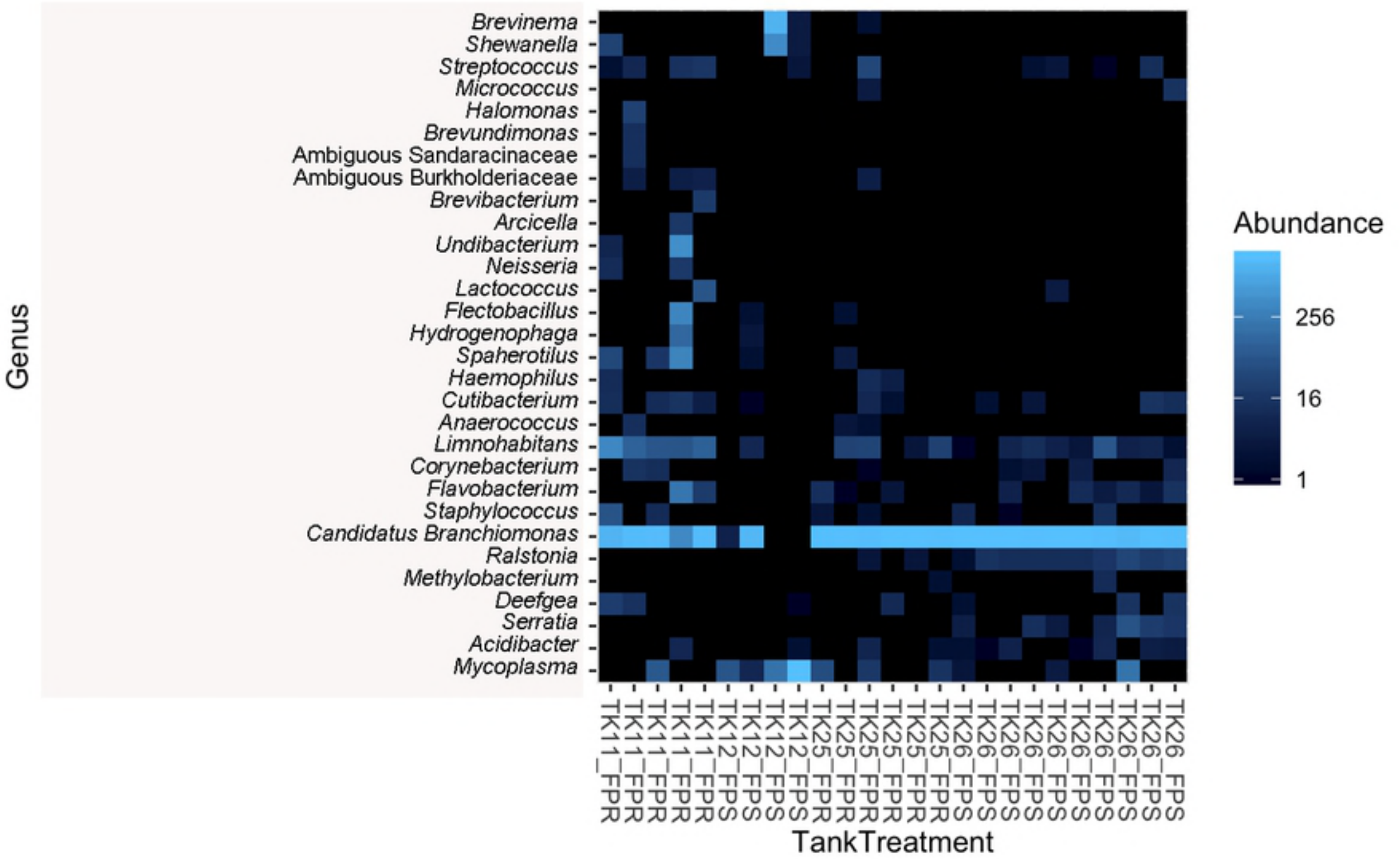
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Shewanella
Streptococcus
Micrococcus
Halomonas
Brevundimonas
Ambiguous Sandaracinaceae
Ambiguous Burkholderiaceae
Brevibacterium
Arcicella
Undibacterium
Neisseria
Lactococcus
Flectobacillus
Hydrogenophaga
Spaherotilus
Haemophilus
Cutibacterium
Anaerococcus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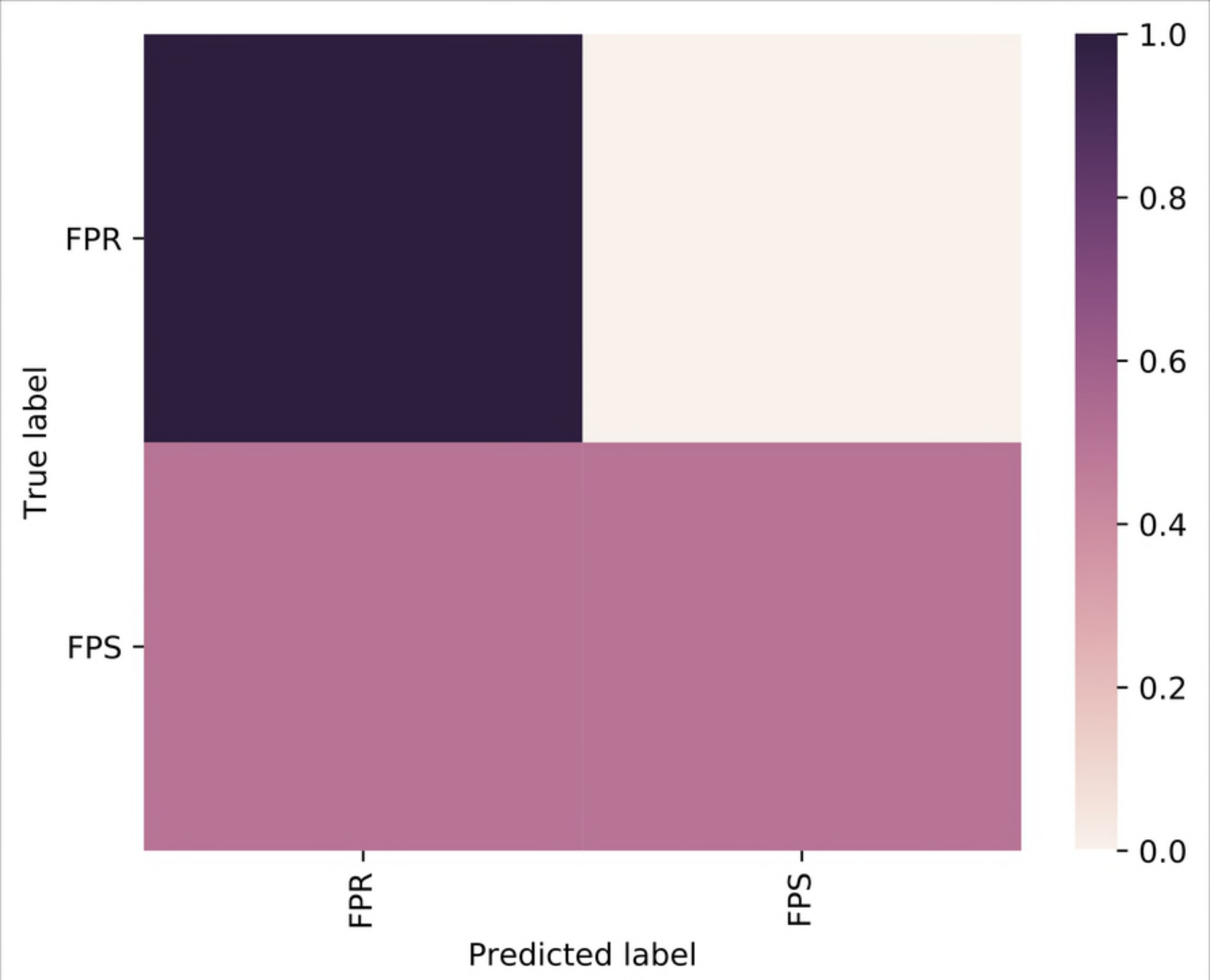
TK26_FPS
TankTreatment
Abundance
256
16
1


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