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Gut microbiota features associated with *Clostridioides difficile* colonization in dairy calves

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Abstract

Diarrheal disease, a major cause of morbidity and mortality in dairy calves, is strongly associated with the health and composition of the gut microbiome. *Clostridioides difficile* is an opportunistic pathogen that proliferates and can produce enterotoxins when the host experiences gut dysbiosis. However, even asymptomatic colonization with *C. difficile* can be associated with differing degrees of microbiome disruption in a range of species, including people, swine, and dogs. Little is known about the interaction between *C. difficile* and the gut microbiome in dairy calves. In this study, we sought to define microbial features associated with *C. difficile* colonization in pre-weaned dairy calves less than 2 weeks of age. We characterized the fecal microbiota of 80 calves from 23 different farms using 16S rRNA sequencing and compared the microbiota of *C. difficile*-positive (n=24) and *C. difficile*-negative calves (n=56). Farm appeared to be the greatest source of variability in the gut microbiota. When controlling for calf age, diet, and farm location, there was no significant difference in Shannon alpha diversity (*P*= 0.50) or in weighted UniFrac beta diversity (P=0.19) between *C. difficile*-positive and –negative calves. However, there was a significant difference in beta diversity as assessed using Bray-Curtiss diversity (*P*=0.0077), and *C. difficile*-positive calves had significantly increased levels of *Ruminococcus (gnavus group)* (*Adj. P*=0.052), *Lachnoclostridium* (*Adj. P*=0.060), *Butyricicoccus* (*Adj. P*=0.060), and *Clostridium sensu stricto 2* compared to *C. difficile*-negative calves. Additionally, *C. difficile*-positive calves had fewer microbial co-occurrences than *C. difficile*–negative calves, indicating reduced bacterial synergies. Thus, while *C. difficile* colonization alone is not associated with dysbiosis and is therefore unlikely to result in an increased likelihood of diarrhea in dairy calves, it may be associated with a more disrupted microbiota.

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Introduction

Infectious diarrheal disease is one of the main causes of mortality in dairy calves (1, 2), and calves less than 30 days of age are at highest risk of developing diarrhea (3, 4). Studies have shown that gut microbial composition is associated with gut health and the likelihood of diarrhea: reductions in microbial diversity are associated with an increased incidence of diarrhea (5), and the colonization of the calf gut with beneficial bacteria along with the decreased colonization of potential pathogens decreases the likelihood of calf diarrhea (6).

Clostridioides difficile is a spore-forming anaerobic, gram-positive bacillus that is a significant enteric pathogen in many species of animals. Colonization with *C. difficile* has been shown to be associated with reduced gut microbial diversity and increased colonization of pathogenic bacteria in people (7, 8), and we recently demonstrated a similar association in puppies (9). Dairy calves, like the neonates of other species, are colonized with *C. difficile* at high rates, with reported prevalences ranging from 28-56% (10, 11). While there is some evidence that infection with *C. difficile* can result in diarrhea in calves (12), the effect of the asymptomatic colonization of calves on the gut microbiome is unknown. Given the crucial role of the gut microbiome in providing colonization resistance against pathogens that cause diarrhea (13, 14), a better understanding of the effect of pathogens such as *C. difficile* on the calf gut microbiome is needed. The goal of this study was thus to define the gut microbiota features associated with *C. difficile* colonization in dairy calves and to define the effects of calf age, diet, and farm on the risk of colonization.

Methods

Sample collection: Fecal samples were manually collected from up to five randomly selected healthy calves less than two weeks of age from each of 23 dairy farms in Pennsylvania, Maryland and Delaware.

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This study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Detection of *C. difficile*:

Individual fecal samples were tested for *C. difficile* using the Xpert *C. difficile* assay (Xpert CD assay; Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions. This assay detects the cytotoxin gene (*tcdB*) and binary toxin genes (*cdtA* and *cdtB*). Additionally, the assay has a callout for ribotype NAP1/B1/027.

To rule out the possibility of colonization with non-toxigenic *C. difficile*, pooled fecal samples from each farm were also submitted for anaerobic culture. Briefly, 0.5 g of formed fecal sample was mixed with 0.5 ml of 100% ethanol. The mixture remained for 60 minutes at room temperature before being inoculated on Cycloserine-cefoxitin fructose modified agar (CCFA) (Remel™) or *Clostridium difficile* Selective Agar (BBL™) and Columbia CNA agar (Thermo Fisher Scientific Remel Products). Inoculated plates and broth were incubated in BD Gas-Pak™ EZ container systems with BD BBL™ CO2 generators and BD BBL™ Gas Pak™ anaerobic CO2 indicators (Franklin Lakes, NJ) at 36˚C ± 2˚C under anaerobic growth conditions for seven days and checked for growth every other day. Suspect colonies were identified and isolated. Isolates were confirmed to be *C. difficile* by Maldi-TOF identification and/or RapID ANA II System (Thermo Fisher Scientific Remel Products).

16S rRNA sequencing

DNA was extracted from fecal samples using Qiagen PowerSoil DNA extraction kit. 16S rRNA sequencing was performed as described previously (9, 15). Briefly, the V4 region of the 16S rRNA gene was amplified using PCR, which was performed using Accuprime Pfx Supermix and custom primers for 30 cycles (15). PicoGreen quantification was used to normalize post-PCR products and AMPureXP beads were used to

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clean the combined pools. Libraries were quantified and sized using a Qubit 2.0 and Tapestation 4200, respectively. 250bp paired-end sequencing was performed using an Illumina MiSeq.

Sequence data processing using QIIME2

The QIIME2 pipeline (16) was used to process and analyze 16S sequencing data. Samples were demultiplexed using q2-demux and denoised using Dada2 (17). Sequences were aligned using maaft (18) and phylogenetic trees were reconstructed using fasttree (19). Shannon alpha diversity, weighted UniFrac and Bray-Curtis beta diversity metrics were estimated using q2-core-metrics-diversity after samples were rarefied to 1941 reads per sample, and p-values were adjusted for multiple hypothesis testing using Benjamini-Hochberg (B-H) false discovery rate (FDR) corrections (20). Taxonomy was assigned to sequences using q2-feature-classifier classify-sklearn (21) against the Silva reference database (22). Taxa were collapsed to the genus level, when possible. OTUs with less than 1% average relative abundance across all samples were removed.

Correlation analysis and differential feature selection

The correlation between *C. difficile* culture status and Shannon alpha diversity was determined using a linear mixed effects model as implemented in the lme4 package (23) in R where age was controlled for as a fixed effect and with farm and diet as random effects. The correlation between *C. difficile* culture status on gut microbiota beta diversity was determined using PERMANOVA as implemented in the vegan package (24) in R controlling for age, farm, and diet. Principal coordinate analyses were performed using the phyloseq package in R (25). Differentially-abundant taxa were determined using LDA Effect Size (LEfSe) (26) and Analysis of Composition of microbiomes (ANCOM), and p-values were adjusted for multiple hypothesis testing using B-H FDR corrections in R. The Dice index (27)

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was used to determine the co-occurrence of bacterial genera. Boxplots and LEfSe plots were visualized using ggplot2 (28) and ggthemes.

Results:

Subject characteristics and *C. difficile* status

Fecal samples were collected from a total of 92 Holstein calves from 23 farms. All calves appeared systemically healthy at the time of sampling and none had received antimicrobial therapy. The mean (SD) age of the calves was 7.0 (5.0) days. Thirty-six (35.6%) calves were fed waste milk, while the remaining calves were fed either colostrum or whole milk.

C. difficile was detected by qPCR in 28 calves (30.4%, 95% CI 21.2-40.9%) (**Fig. 1**). Of the 28 samples that were positive for *C. difficile* on qPCR, 1 (3.6%) was positive for Toxin B only, 14 (50%) were positive for binary toxin only, and 13 (46.4%) were positive for both Toxin B and the binary toxin. None of the organisms were identified as the NAP1/B1/027 ribotype. On 14 farms, there were both *C. difficile*-positive and *C. difficile*-negative calves, whereas on the remaining farms, all of the calves were *C. difficile*-negative. There were no farms where all samples were qPCR-negative but the pooled sample was culture-positive. Neither calf age nor feeding of waste milk were significantly associated with the likelihood of detecting *C. difficile* among the calves (OR=1.01, p=0.805 and OR=0.71, p=0.493, respectively) (**Fig. 1**).

Effect of *C. difficile* status on microbiota diversity

Microbiota community structure of 87 calf fecal samples was assessed by sequencing and analyzing the V4 region of the 16S rRNA gene. Three samples were dropped from subsequent analyses because of low coverage and four additional samples were dropped because there was not enough sample for qPCR

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analysis. Among the 80 remaining samples, 24 were positive for *C. difficile* by qPCR and 56 were negative (**Fig 1**).

The relationship between *C. difficile* infection and microbial diversity of the gut microbiota was assessed. Since calves ranged in age, diet, and farm location, a linear mixed effects model was performed to assess the relationship between *C. difficile* infection and alpha diversity by setting age as a fixed variable and farm and feeding type as random-effect variables. The association between *C. difficile* status and Shannon alpha diversity was not significant (P= 0.50) as determined by ANOVA when controlling for age, diet, and farm location (**Fig. 2**). PERMANOVA was then used to test associations between *C. difficile* infection status and beta diversity of the gut microbiome. Farm location alone explained most of the variation in gut microbiota composition across samples using both Bray-Curtis (P=1e-4; R2=0.43) and weighted UniFrac (P=1e-4; R2=0.46) beta diversity metrics (**Fig. 3, Fig. 4**). Age and diet were not significantly associated with gut microbiota composition after controlling for farm (P>0.1). After controlling for farm, age, and diet, *C. difficile* status was significantly associated with Bray-Curtis beta diversity (P=0.0077; R2=0.023), explaining 2.3% of the variation in gut microbiota composition. *C. difficile* status was not significantly associated with weighted UniFrac beta diversity (P= 0.1934; R2=0.013) after controlling for farm, age, and diet (**Fig. 3**). Some clustering by farm and by *C. difficile* status within farms was apparent on principal coordinate analysis (**Fig. 4**).

Bacterial community composition

Since *C. difficile* status was associated with differences in gut microbiota composition as determined by beta diversity, we next sought to determine the specific bacterial taxa associated with *C. difficile* infection. At the phylum level, there were no significant differences between bacterial communities in *C. difficile*-positive and -negative samples (**Fig. 5**). The Firmicutes phylum predominated

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(57.1% in *C. difficile*-positive samples and 51.4% in *C. difficile*-negative samples), followed by Proteobacteria (17.1% and 24.3%), Bacteroides (16.7% and 11.5%), and Actinobacteria (8.1% and 9.7%).

At the genus level, the only significant difference between *C. difficile*-positive and –negative samples by ANCOM occurred for Clostridioides. When considering LEFse analysis, there were four taxa among the 19 taxa with average relative abundance greater than 1% that were statistically significantly (*Adj. P*<0.1) associated with *C. difficile* status. *Ruminococcus (gnavus group)* (*Adj. P*=0.052), *Lachnoclostridium* (*Adj. P*=0.060), *Butyricicoccus* (*Adj. P*=0.060), and *Clostridium* (*sensu stricto 2*) (*Adj. P*=0.064) were all found in higher abundance among *C. difficile*-positive calves than in *C. difficile*-negative calves (**Fig. 6**). While not statistically significantly different among the two groups, levels of *Lactobacillus, Megasphaera*, and *Streptococcus* were increased in *C. difficile*-positive samples, while levels of *Blautia, Fusobacterium, Tyzzerella, Enterobacteriaceae, Fecalibacterium, Dorea*, and *Collinsella* were decreased.

Because microbes work synergistically in the gut, we sought to determine the associative interactions between bacteria using a co-occurrence analysis based on the Dice index. When considering all levels of abundance, more co-occurrence of bacterial taxa appeared in the *C. difficile*-negative samples, with 1,488 (65.5%) highly (correlation coefficient>0.6) and significantly (p<0.01) correlated genera pairs. Most co-occurrences were among members of the Firmicutes phylum (1295, 55.0%). However, members of Firmicutes also showed high co-occurrence with Actinobacteria and Bacteroidetes. In the *C. difficile*-positive samples, there were fewer highly co-occurring genera, with 830 (73.3%) highly and significantly correlated genera pairs. When only considering taxa with levels of abundance greater than 1%, there were no significant differences in co-occurrence patterns **(Fig. 7)**.

Discussion:

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178 In this study, we characterized microbial features associated with asymptomatic *C. difficile* colonization in
179 dairy calves. While the role of *C. difficile* in calf diarrhea remains equivocal (12), exploring the association
180 between this pathogen and the gut microbiome is important for understanding factors that affect gut
181 health and enteric diseases. While a number of studies have examined the epidemiology of *C. difficile* in
182 animals of veterinary importance, the association between the microbiome and *C. difficile* is only
183 beginning to be explored in dogs (9), horses (29), and pigs (30). Notably, in pigs, the presence of *C.*
184 *difficile* is associated with significantly reduced microbial diversity and increased levels of
185 enteropathogens associated with neonatal diarrhea (30).

186 Unsurprisingly, as in other studies (31-33), we found that the farm was the source of most of the
187 variation in gut microbiota composition. However, even among calves from the same farm, there was
188 variability in both *C. difficile* colonization status and gut microbial diversity, suggesting, as have other
189 studies (32, 34), that the farm environment is only one of many competing influencers of the developing
190 calf gut microbiome. Neither diet nor age were significantly associated with microbiome composition
191 when controlling for farm, but this is almost certainly due to the small sample size within each farm and
192 the lack of within-farm variability in factors such as diet. When controlling for age, diet, and farm, we
193 noted a significant difference in beta diversity between *C. difficile*-positive and *C. difficile*-negative fecal
194 samples when considering the Bray-Curtis metric but not the unweighted UniFrac metric. While both of
195 these metrics are weighted by abundance, the latter metric weighs diversity by phylogenetic relationship.
196 Thus the lack of a significant difference when considering the weighted UniFrac metric suggests that,
197 while there may be a significant difference in the composition of microbial communities, the
198 differentially-abundant microbes might be closely related to one another. Indeed, all four genera
199 identified as differentially-abundant by LEfSe are members of the *Clostridia* class, with two belonging to
200 the *Clostrideaceae* family.

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While the lack of a consistent difference in alpha and beta diversity between *C. difficile*-positive and *C. difficile*-negative samples suggests that the effect of *C. difficile* colonization on the gut microbiome of calves is minimal, other findings suggest that *C. difficile* colonization is associated with a more disrupted – but not dysbiotic – gut microbiome. *C. difficile* colonization was preferentially associated with certain bacterial taxa of the class *Clostridia* that do have associations with dysbiosis. Notably, the overrepresentation of *Ruminococcus gnavus* and *Lachnoclostridia* in *C. difficile*-positive calves point to the possibility of an underlying imbalance in the gut microbiome. *R. gnavus,* a Gram-positive anaerobe that is typically found in the gut of over 90% of healthy people at abundances less than 0.1%, has been robustly associated with inflammatory dysbiotic conditions such as Crohn's disease (35-37), allergic airway disease (38), eczema (39), and spondyloarthritis (40). Dramatic blooms of *R. gnavus* occur in patients experiencing flares of inflammatory bowel disease, with abundance levels that can peak at 69% of the gut microbiota (37). Notably, this association appears to occur across species, as the gut microbiomes of both infants (7) and piglets (30) colonized with *C. difficile* also had increased levels of *Ruminococcus* species, including *R. gnavus*. Additionally, *Ruminococcus* was one of six bacterial genera in the gut microbiome that predicted the occurrence of diarrhea in calves in another study (41). The increased relative abundance of *Clostridium sensu stricto* and *Lachnoclostridia* in *C. difficile*-positive calves also points to the possibility of a less healthy gut environment. An increased relative abundance of *Clostridium sensu stricto*, which was also found in *C. difficile*-positive piglets (30), was associated with food allergies in infants (42) and diarrhea in piglets (43). A tentative association between increased levels of *Lachnoclostridia* and neoplasia of the gastrointestinal tract has been identified in people (44, 45). While no such association has been explored in animals, the overrepresentation of this taxon in *C. difficile*-positive calves may be the result of a more disrupted gut microbiota. However, it is also important to note that the increased relative abundance of these taxa were only detected using LEfSe analysis and not ANCOM, which suggests that the association is likely relatively weak.

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Certain bacterial taxa that predominate in healthy calves were found at lower (but not statistically significantly lower) levels in *C. difficile*-positive calves. Notably, *Fecalibacterium, Dorea, Enterobacteriaceae* and *Collinsella* are among the most abundant genera in healthy pre-weaned calves (46-49), and some of these taxa provide colonization resistance against *C. difficile* (8, 50). Their decreased relative abundance in *C. difficile*-positive calves is thus also reflective of a more disrupted gut microbiome. The decreased co-occurrence of bacterial taxa in *C. difficile*-positive calves compared to *C. difficile*-negative calves when considering all levels of abundance may also corroborate the notion of a slightly more disrupted gut microbiome in colonized calves. However, because the difference occurred only in rare taxa (abundance < 1%), this difference appears unlikely to result in dysbiosis.

One finding that is in contradiction to the general trend of *C. difficile* colonization being associated with disrupted microbiota is the increased abundance of *Butyricicoccus* in *C. difficile*-positive calves. In people, *Butyricicoccus* species of bacteria are generally found in *lower* levels in people colonized with *C. difficile* (51) or diagnosed with inflammatory bowel disease (52, 53), and at higher levels in healthy dairy calves compared to calves with diarrhea (48, 54). It is unclear why they were found at higher levels in *C. difficile*-positive calves compared to *C. difficile*-negative calves. *Butyricicoccus* bacteria produce butyrate, an important nutrient source for gut colonocytes and a beneficial driver of the immunological maturation of the gut mucosa (55), and account for one of the most abundant genera in dairy calves 7 days after birth (56). The differential levels in calves compared to people with enteric disease may be due to species-specific patterns of development of the neonatal gut. Species-specific differences may also explain why *C. difficile* colonized calves had higher levels of *Clostridial* genuses but colonized puppies had lower levels (9). While rumen development is minimal in pre-weaned calves, they are nevertheless ruminants and thus have fundamentally different enteric physiologies and microbial ecologies compared to true monogastric species.

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Some limitations apply to this study. Heterogeneity in farm location, age, and diet across all of the sampled calves may have obscured features of the microbiome that would otherwise have been associated with *C. difficile* colonization. The cross-sectional nature of the study also precludes the possibility of drawing any conclusions about the duration of colonization and its effect on an already rapidly evolving gut microbiome. Finally, because we used qPCR to detect *C. difficile* in the calves' feces, we were unable to detect non-toxigenic *C. difficile*. It is likely that toxigenic and non-toxigenic *C. difficile* occupy a similar ecological niche and compete for similar resources within the gut microbiota; thus the presence of non-toxigenic *C. difficile* could account for the lack of a significant difference in alpha diversity and microbial composition between *C. difficile*-positive and *C. difficile*-negative calves. However, we believe this possibility to be unlikely, as there were no samples that were negative on qPCR but came from a farm where the pooled sample was positive for *C. difficile* on anaerobic culture.

Conclusion

The greatest source of variability in the calf microbiome was the farm, and there were few or no statistically significant differences in alpha or beta diversity between *C. difficile*-positive and *C. difficile*-negative calves. *C. difficile* colonization thus does not appear to be associated with dysbiosis or with increased levels of enteropathogens that cause calf diarrhea. However, microbial community signatures – including increased relative abundance of bacterial taxa that that have been associated with dysbiotic states in other species and in people - suggest that the microbiota of *C. difficile*-colonized calves is more disrupted than that of non-colonized calves.

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Figure legends

Figure 1: Distribution of age and *C. difficile* colonization status in 92 pre-weaned Holstein dairy calves

Figure 2: Alpha diversity of the gut microbiome in 86 pre-weaned Holstein dairy calves by *C. difficile* colonization status

Figure 3: Beta diversity of the gut microbiome in 86 pre-weaned Holstein dairy calves by *C. difficile* colonization status. A. Bray-Curtis beta diversity. B. Weighted UniFrac.

Figure 4: Bray-Curtis principal coordinate analysis (PCoA) of fecal samples from 86 pre-weaned dairy calves by *C. difficile* colonization status and by farm

Figure 5: Distribution of bacterial phyla by *C. difficile* status in fecal samples from 86 pre-weaned dairy calves. The nine most abundant phyla are displayed.

Figure 6: Distribution of bacterial taxa that were found at higher levels in *C. difficile*-positive calves by *C. difficile* colonization status in 86 pre-weaned Holstein dairy calves. A. *Butyricicoccus*. B. *Clostridium sensu stricto 2*. C. *Ruminococcus gnavus*. D. *Lachnoclostridium*.

Figure 7. Analysis of co-occurrence among microbial lineages scored using the Dice index by *C. difficile*-colonization status (positive and negative). Dice indexes are shown as a heat map for all genera present at a level of abundance greater than 1% and with statistically significant ($p<0.01$) co-occurrence are shown as a heatmap. The degree of co-occurrence is shown by the color code at the bottom.

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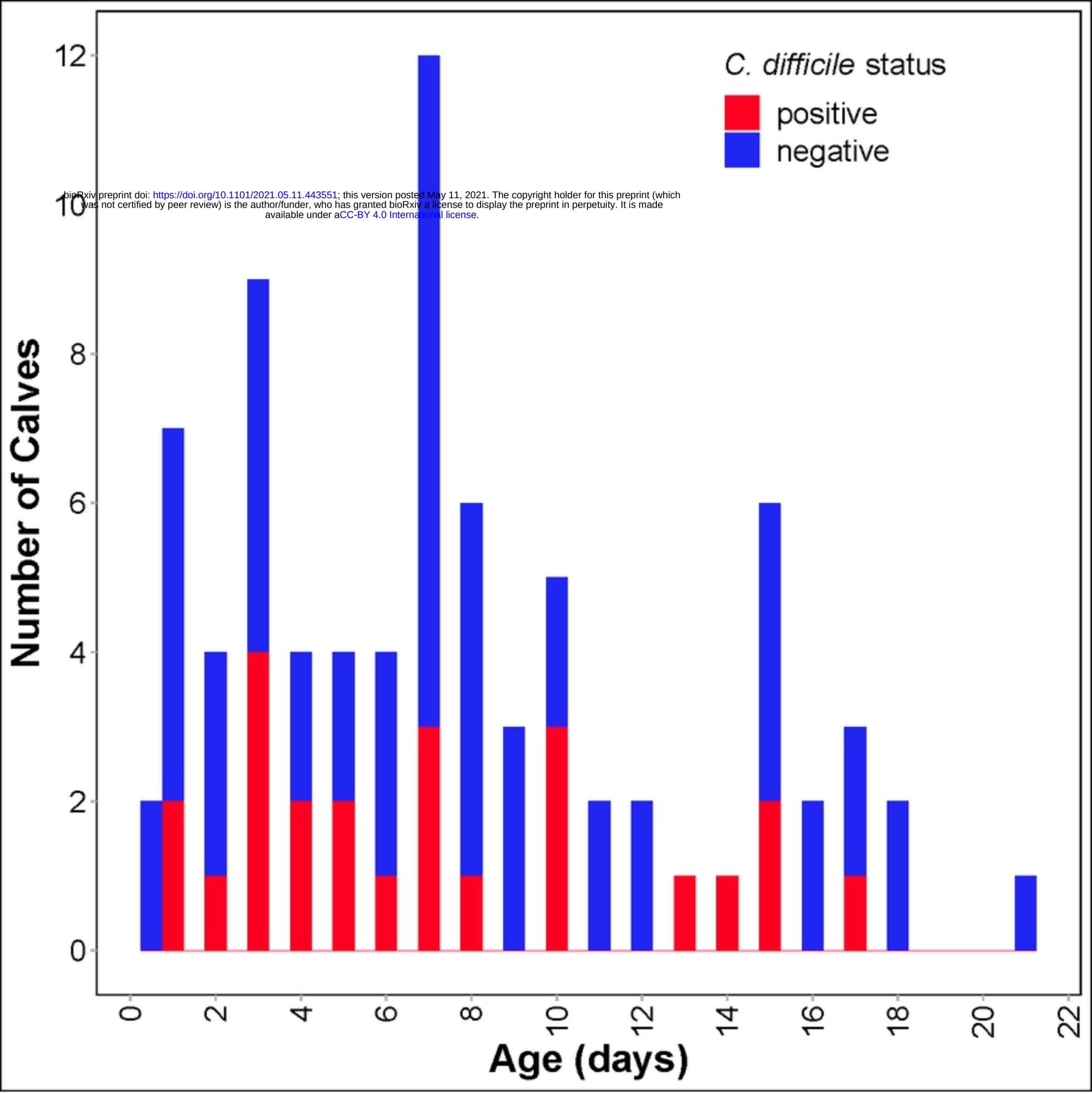

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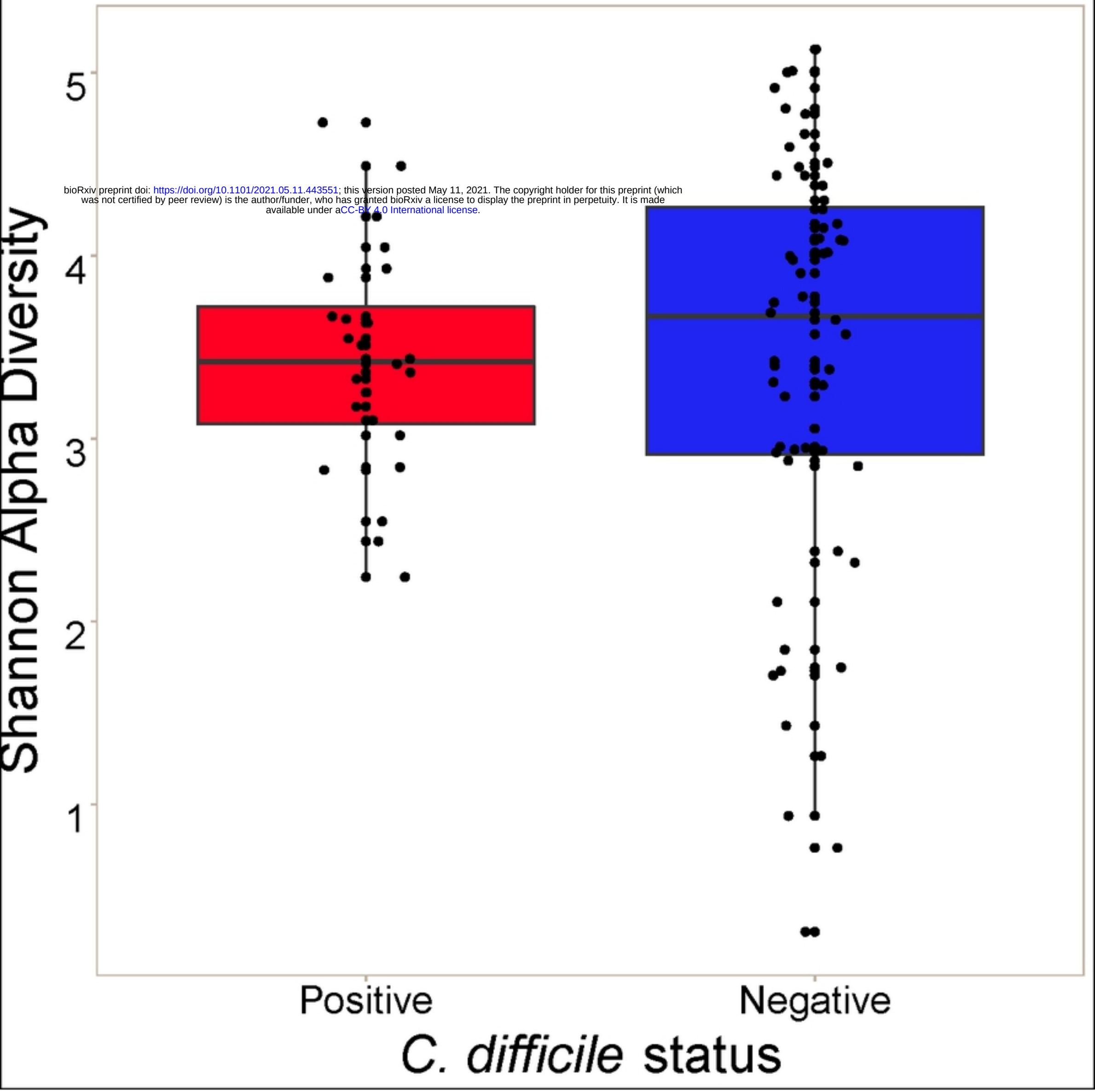

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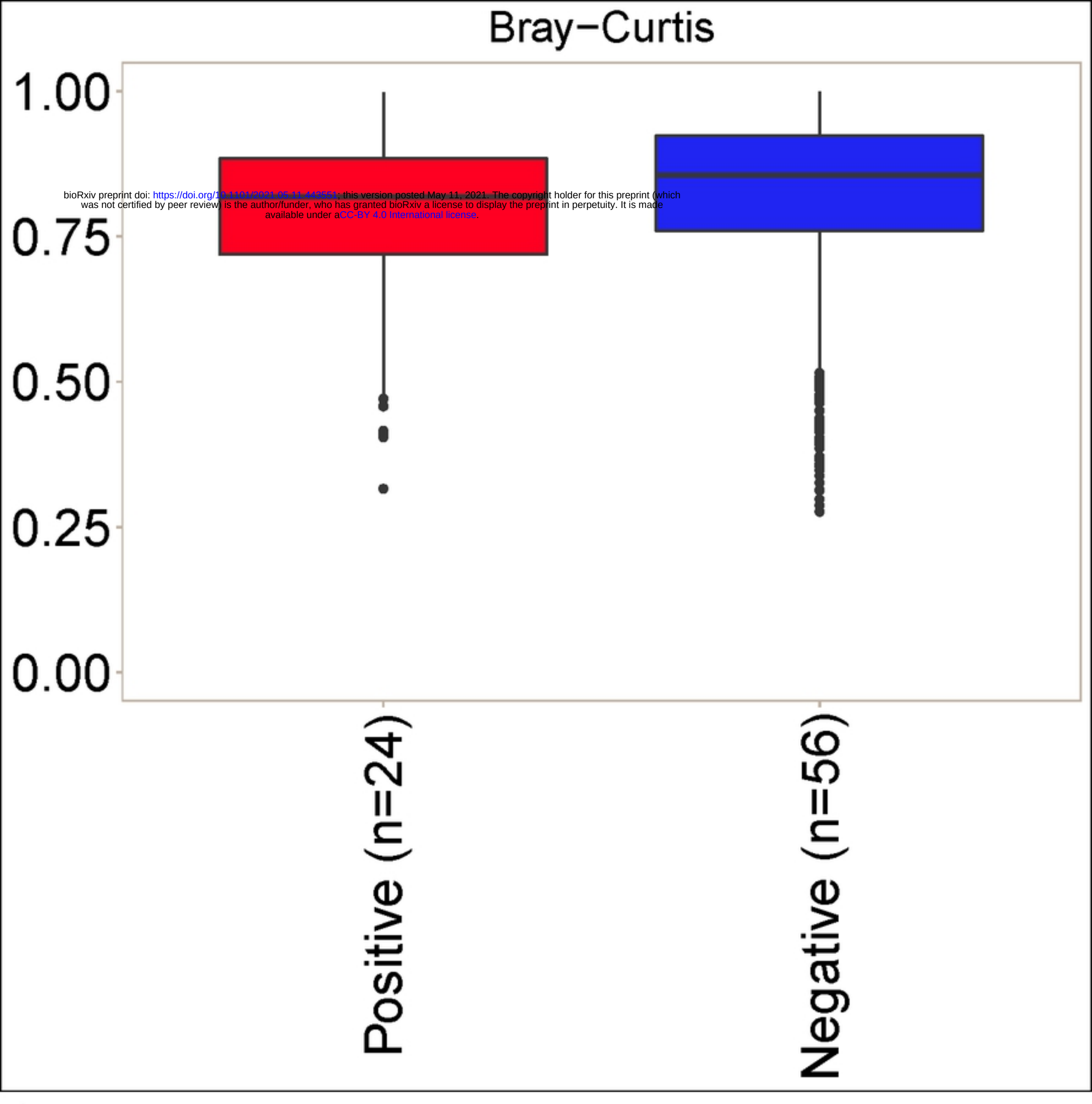

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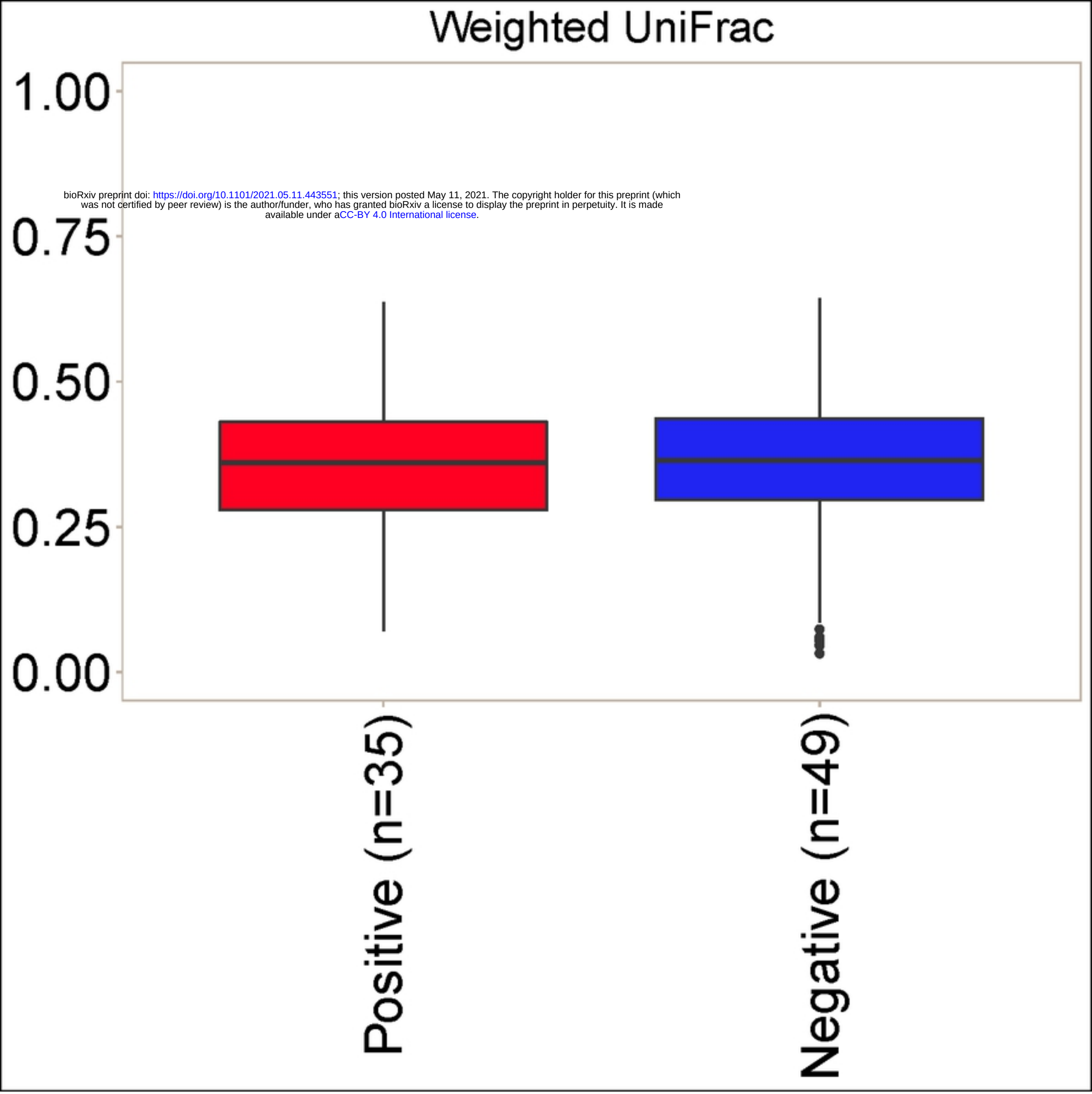
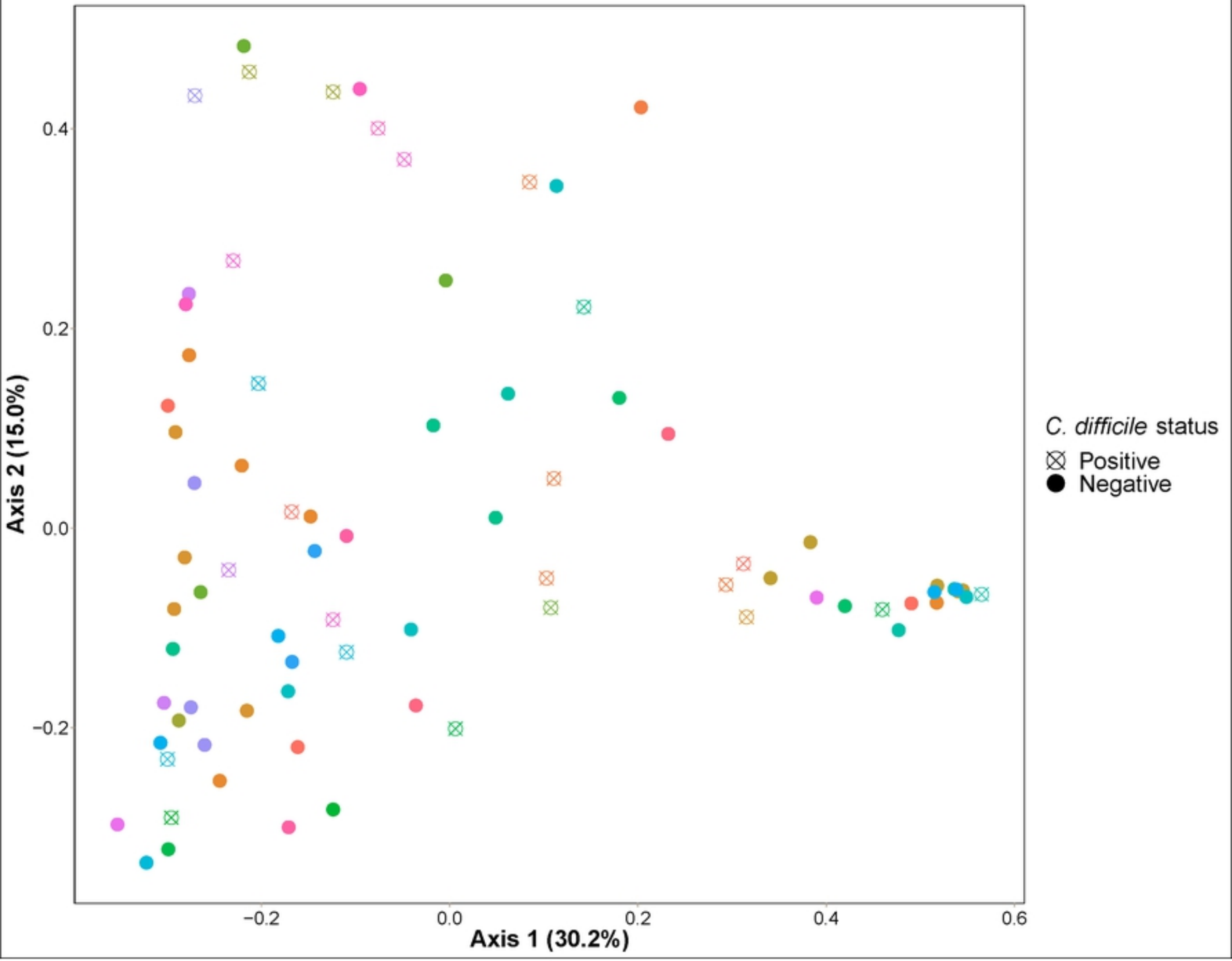
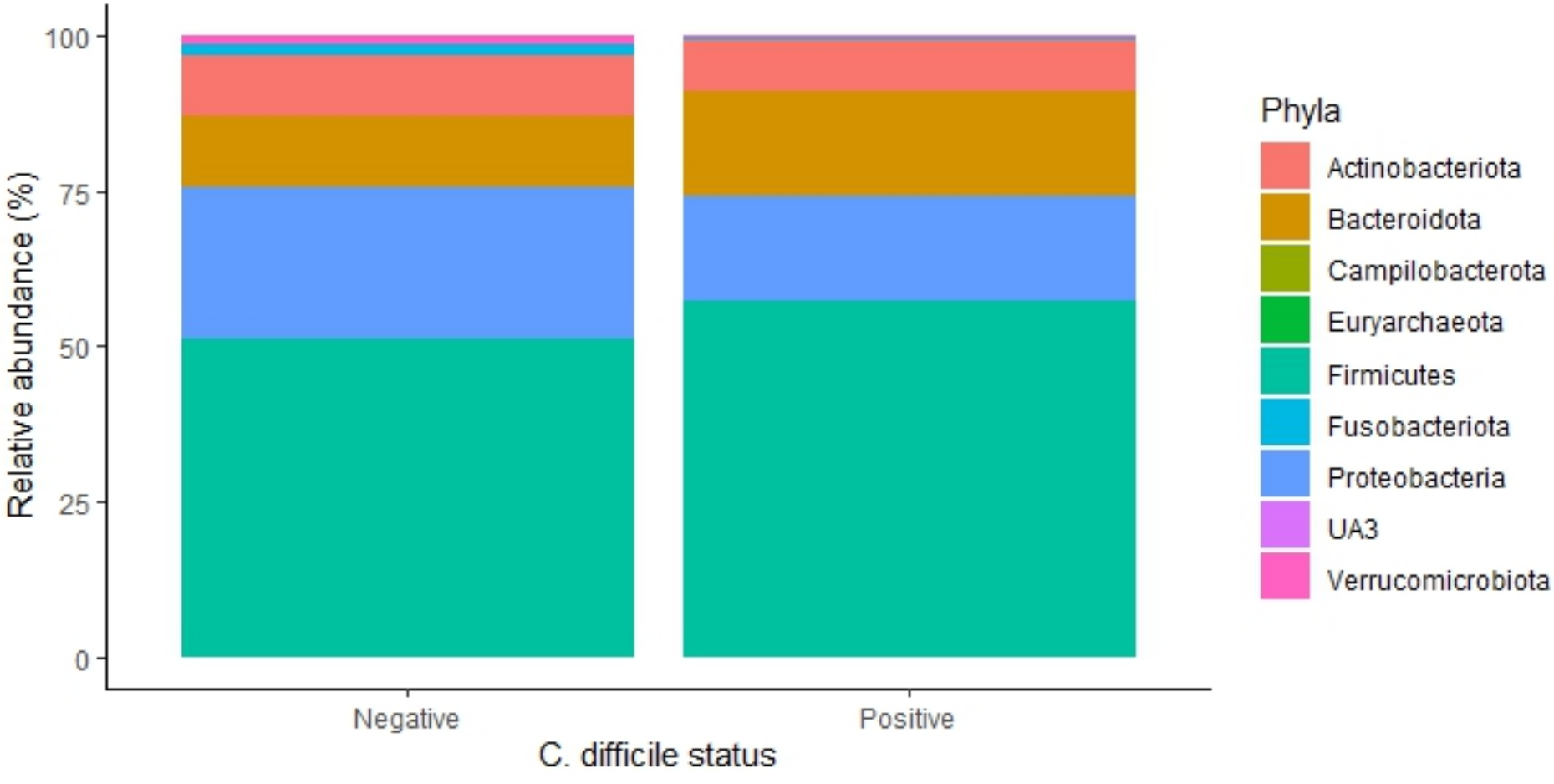


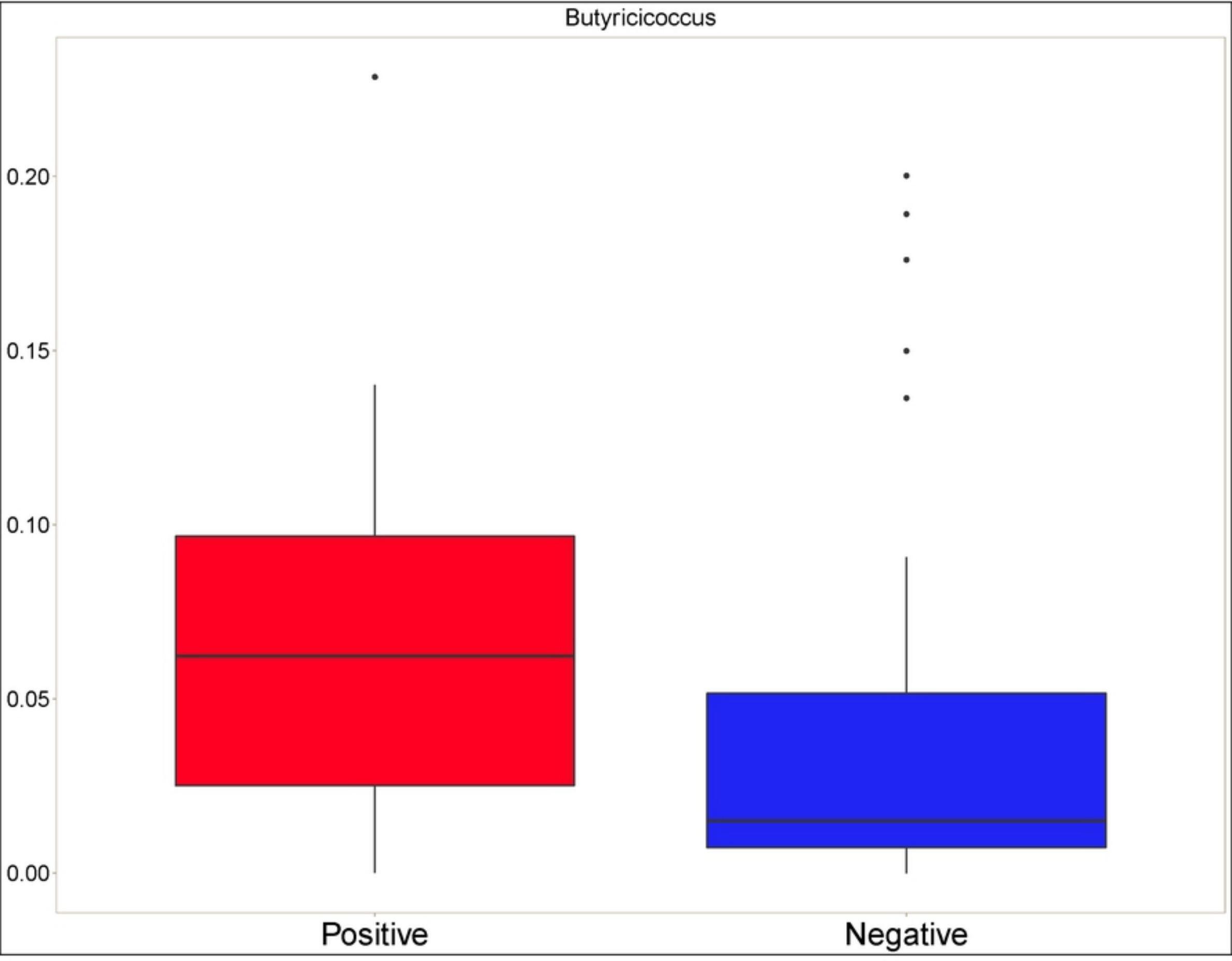
Figure


0.4
0.2
0.0
−0.2
Axis 2 (15.0%)
−0.2
0.0
0.2
0.4
0.6
Axis 1 (30.2%)
C. difficile status
Positive
Negative

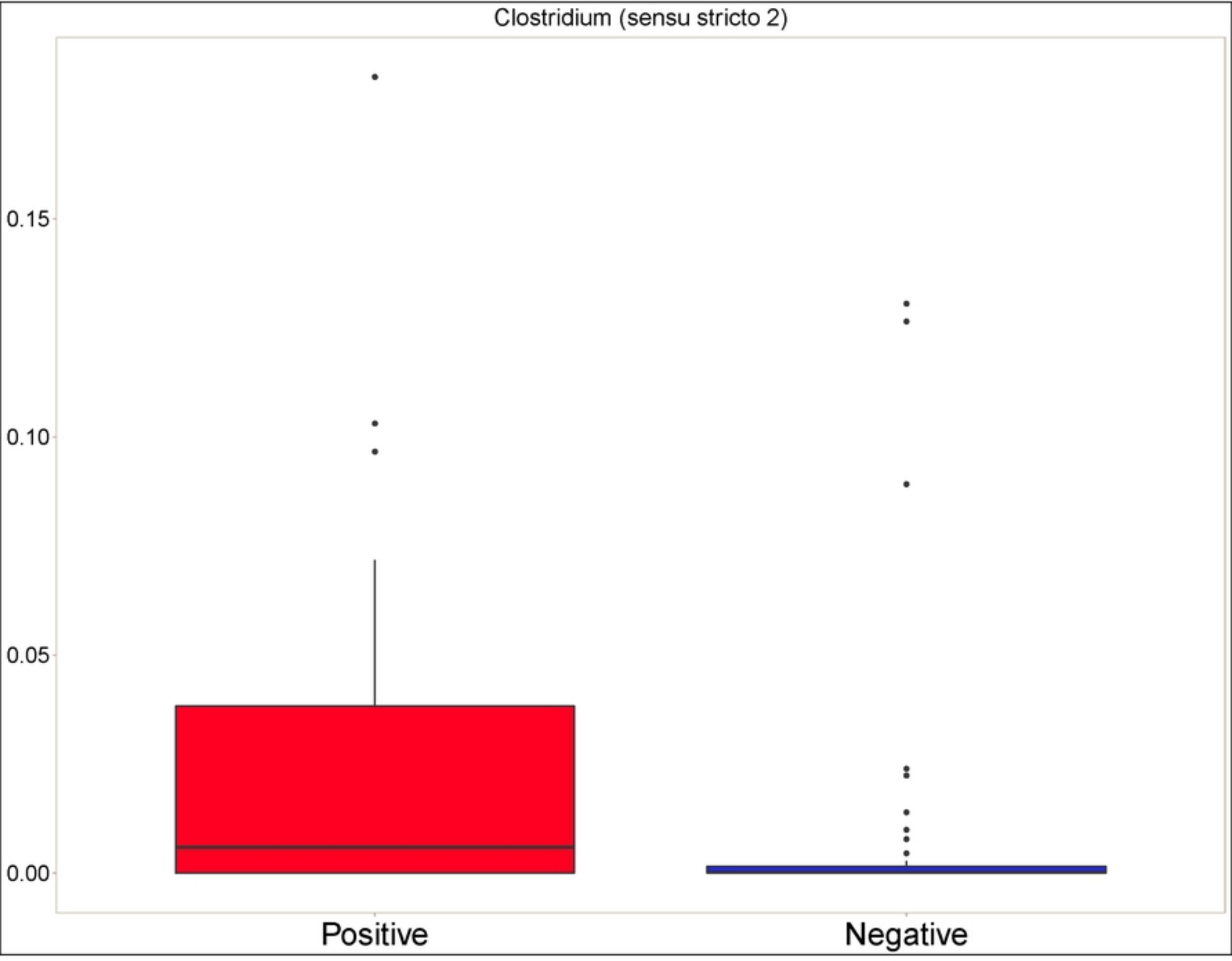
Figure


100
75
50
25
0
Relative abundance (%)
Negative
Positive
C. difficile status
Phyla
Actinobacteriota
Bacteroidota
Campilobacterota
Euryarchaeota
Firmicutes
Fusobacteriota
Proteobacteria
UA3
Verrucomicrobiota

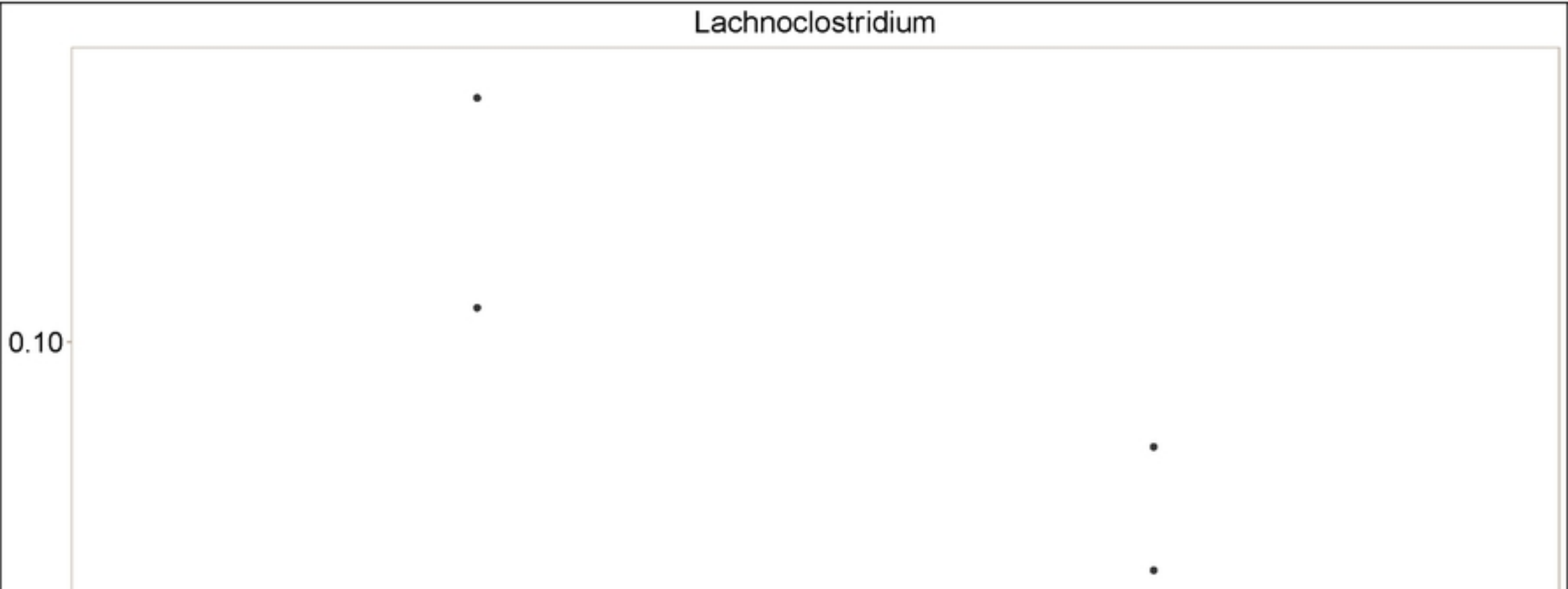
Figure



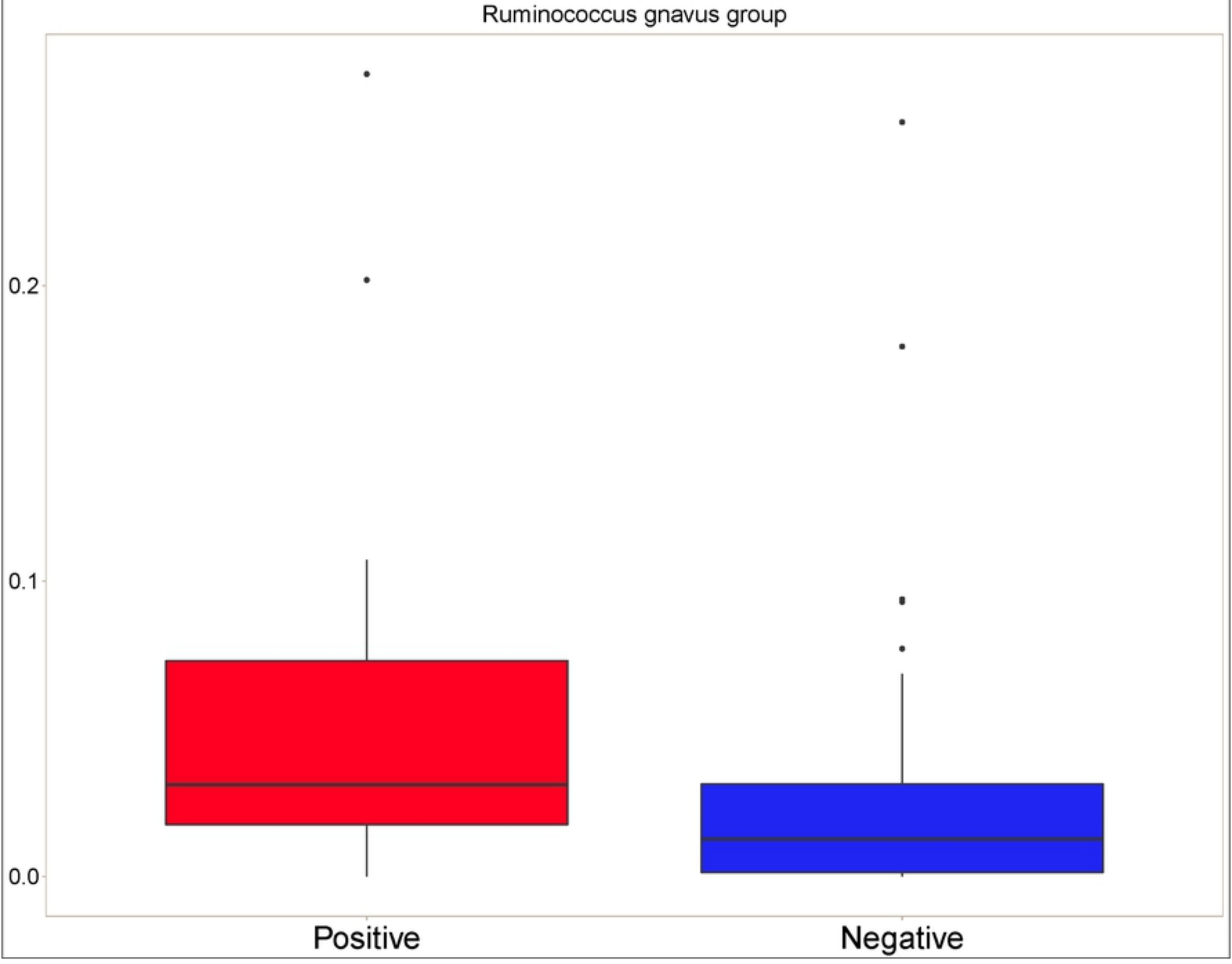
Figure


Clostridium (sensu stricto 2)
0.15
0.10
0.05
0.00
Positive
Negative

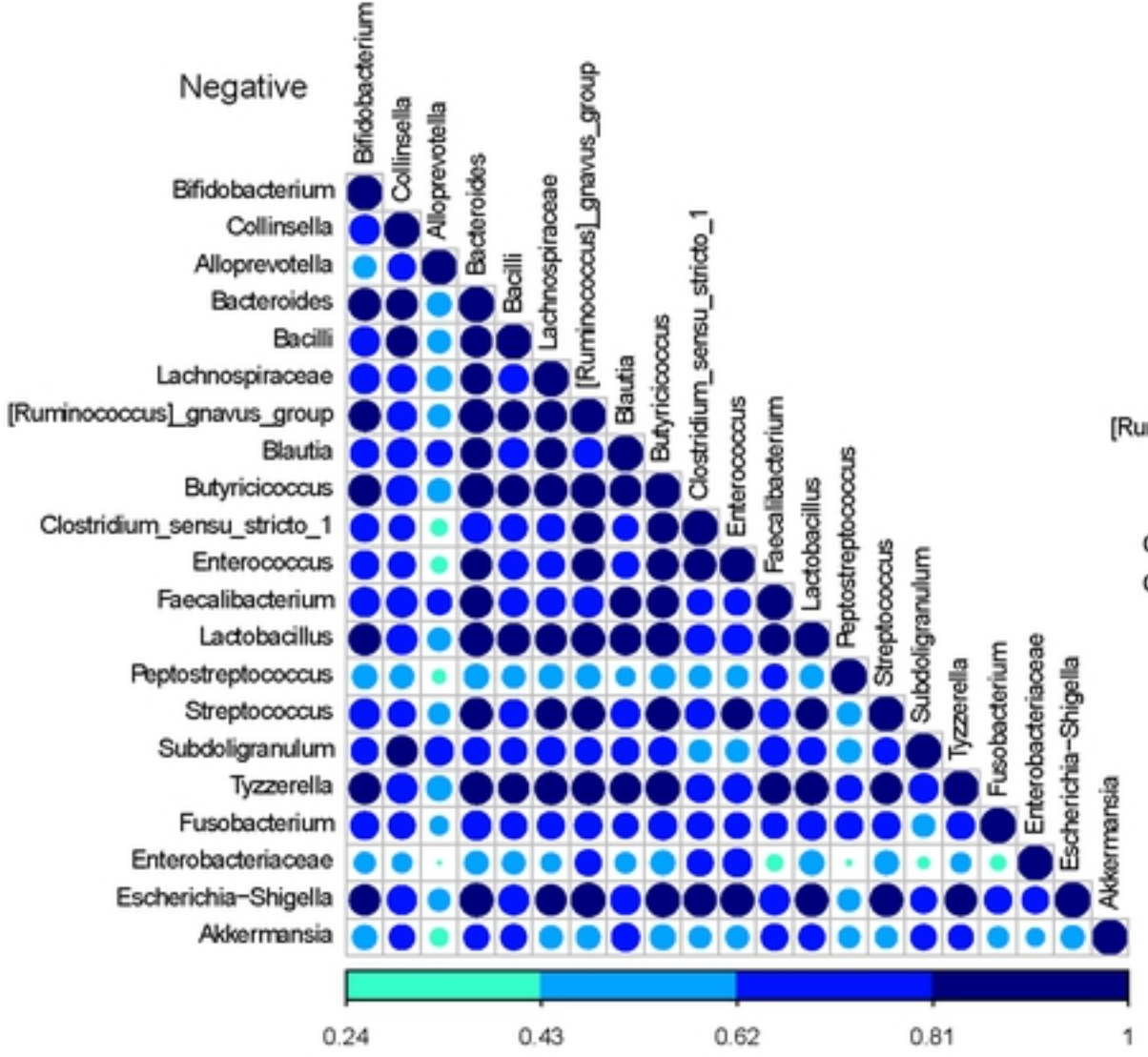
Figure

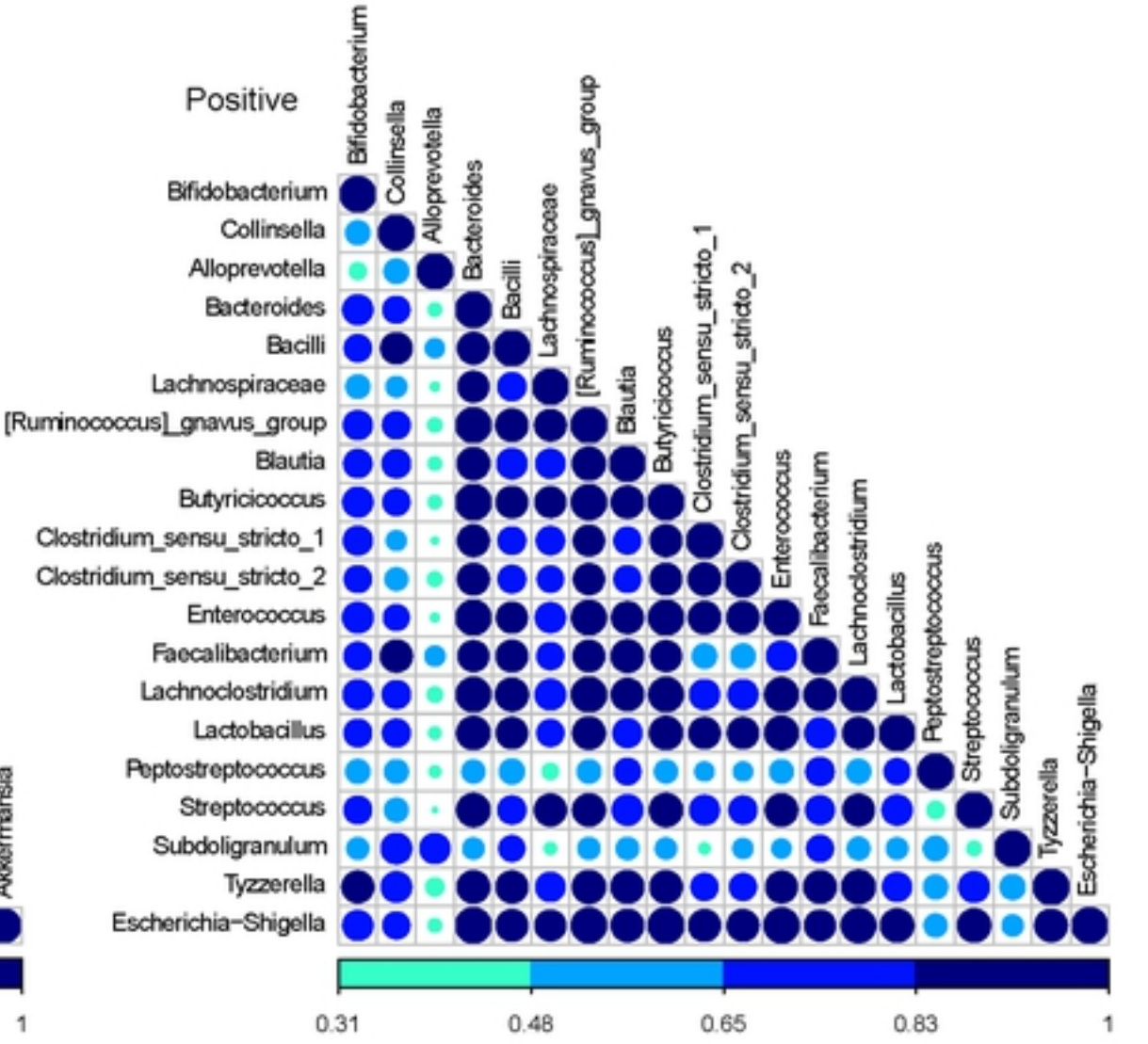


Figure



Figure


Negative
Bifidobacterium
Collinsella
Alloprevotella
Bacteroides
Bacilli
Lachnospiraceae
[Ruminococcus]_gnavus_group
Blautia
Butyricicoccus
Clostridium_sensu_stricto_1
Enterococcus
Faecalibacterium
Lactobacillus
Peptostreptococcus
Streptococcus
Subdoligranulum
Tyzzerella
Fusobacterium
Enterobacteriaceae
Escherichia−Shigella
Akkermansia
0.24
0.43
0.62
0.81
1


Positive
Bifidobacterium
Collinsella
Alloprevotella
Bacteroides
Bacilli
Lachnospiraceae
[Ruminococcus]_gnavus_group
Blautia
Butyricicoccus
Clostridium_sensu_stricto_1
Clostridium_sensu_stricto_2
Enterococcus
Faecalibacterium
Lachnoclostridium
Lactobacillus
Peptostreptococcus
Streptococcus
Subdoligranulum
Tyzzerella
Escherichia−Shigella
0.31
0.48
0.65
0.83
1

Figure