Distinct Gut and Vaginal Microbiota Profile in Women with Recurrent Implantation Failure and Unexplained Infertility

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Abbreviations

AMH, Anti-Müllerian hormone; ASV, Amplicon sequence variants; BMI, Body mass index; ET, Embryo transfer; (F/B) ratio, *Firmicutes* to *Bacteroidetes* ratio; ICSI, Intracytoplasmic Sperm Injection; IL-6, Interleukin 6, IL-10 , Interleukin 10, IVF, *In vitro* fertilization; *L. delbrueckii, Lactobacillus delbrueckii; L. equicursoris*, *Lactobacillus equicursoris*; *L. fermentum, Lactobacillus fermentum*; *L. gasseri, Lactobacillus gasseri; L. Iners*, *Lactobacillus iners*; *L. jensenii, Lactobacillus jensenii; L. reuteri, Lactobacillus reuteri; L. ruminis, Lactobacillus ruminis; L. salivarius; Lactobacillus salivarius; L. vaginalis; Lactobacillus vaginalis;* LDA, Linear discriminant analysis; LEfSe, linear discriminant analysis effect size; LPS, lipopolysaccharides; MAMPs, Microbe-associated molecular patterns; OTU, operational taxonomic units; PERMANOVA, Permutation multivariate analysis of variance; PCoA, Principle Coordinate Analysis; RIF, Recurrent implantation failure; SCFA, short-chain fatty acids; 16S rna, 16S ribosomal RNA; TLRs, toll-like receptors, TMAO Trimethylamine N-oxide; TNF-α, Tumor Necrosis Factor-α; UE, Unexplained infertility.

Abstract

Background: Implantation failure limits the success rate of natural and *in vitro* fertilization (IVF)-assisted conceptions. Evidence suggests dysbiosis in the female reproductive tract impacts implantation failure. However, whether gut dysbiosis influences implantation failure and whether it accompanies reproductive tract dysbiosis remains unexplored.

Method: We recruited 11fertile women as the controls, and a cohort of 20 women diagnosed with implantation-failure associated infertility, which included 10 women diagnosed with recurrent implantation failure (RIF), and 10 women diagnosed with unexplained infertility (UE). Using next-generation amplicon sequencing, we compared the diversity, structure, and composition of fecal and vaginal bacteria of the controls with that of the infertile cohort. While we sequenced fecal samples of all the participants (n=31), we could only sequence 8 vaginal samples in each group (n=24).

Results: Compared with the controls, α-diversity of the gut bacteria, analysed by Chao 1 and Shannon indices, among the infertile groups declined ($p < 0.05$). β-diversity between the controls and infertile cohort, measured by both Bray-Curtis and Jaccard distances, differed significantly ($p < 0.05$). Taxa analysis of the gut bacteria revealed enrichment of Gram-positive bacteria, mainly of the

phylum *Firmicutes,* in the RIF group. In contrast, Gram-negative bacteria were relatively more abundant in the UE group. Additionally, mucus-producing bacteria genera such as *Prevotella* and *Sutterella* declined in the infertile cohort ($p < 0.05$). Intriguingly, significant enrichment ($p < 0.05$) of the genus *Hungatella,* associated with trimethylamine N-oxide (TMAO) production, occurred in the infertile cohort. Vaginal microbiota was dominated by *L. iners* across the groups, with the UE group showing the highest levels. Of the three groups, the RIF group had the least diverse vaginal microbiota. Taxa analysis showed higher levels of anaerobic bacteria such as *Leptotrichia, Snethia,* and *Prevotella* in the controls.

Conclusion: We posit that in the setting of the compromised gut mucosal barrier, the phyla *Firmicutes* generates TNF-α-driven systemic inflammation, leading to RIF, whereas an overload of Gram-negative bacteria induces IL-6-driven systemic inflammation, leading to UE. Additionally, *Hungatella*-induced elevation of TMAO levels causes platelet hypercoagulability, synergistically contributing to implantation failure. Finally, vaginal dysbiosis does not appear to co-occur with gut dysbiosis.

Key Words: Gut microbiota, Vaginal microbiota, Implantation failure, Infertility, Endometrium

1. Introduction

Infertility, defined as a failure to conceive after 1 year of appropriately timed unprotected intercourse, is a distressing and costly reproductive disorder that affects up to 15% of couples globally (Kamel 2010). Some known causes of infertility include pelvic diseases, peritoneal factors, cervical factors, ovulatory disorders, reproductive aging of women, and male factors (Lindsay and Vitrikas 2015).

Despite this knowledge, some couples are diagnosed as having unexplained infertility (UE) because the underlying mechanism(s) remain undefined even after assessment of ovulatory function, tubal patency, and sperm parameters (Bellver, Soares et al. 2008). Frustratingly, many of the infertile couples undergo multiple unsuccessful assisted reproduction technology (ART) cycles (i.e., IVF and/or intracytoplasmic sperm injection (ICSI)) and are thus diagnosed as having repeated implantation failure (RIF) (Bellver, Soares et al. 2008, Simon and Laufer 2012, Polanski, Baumgarten et al. 2014). Yet another subgroup of infertile couples, diagnosed as recurrent pregnancy loss (RPL), exists that conceive several times ($\geq$3), but miscarriage occurs each time before gestational week 28, although controversies exist on its definition (Christiansen, Nielsen et al. 2006, El Hachem, Crepaux et al. 2017). It has been argued that RIF and RPL represent the same condition spectrum (Christiansen, Nielsen et al. 2006).

Pathologies of all these enigmatic conditions converge on mechanisms by which the embryo fails to implant in the uterus—also called implantation failure (Graham, Seif et al. 1990, Christiansen, Nielsen et al. 2006, Bellver, Soares et al. 2008, Simon and Laufer 2012). It is the main limiting factor for natural and *in vitro* fertilization (IVF)-assisted pregnancies (Bellver, Soares et al. 2008, Hernández-Vargas, Muñoz et al. 2020). Failed implantation involves a triumvirate of a poor quality embryo and an unreceptive endometrium and ill-timed embryo-endometrium interaction, of which unreceptive endometrium appears to be the most critical factor. (Simon and Laufer 2012). In fact, unreceptive endometrium has been implicated in two-thirds of all the failures (Simon and Laufer 2012). While multiple factors that disrupt endometrium receptivity, including steroidal hormonal imbalance, thrombotic abnormalities, hyperhomocysteinemia, and immune dysfunctions, have been identified, much remains recondite (Bellver, Soares et al. 2008, Simon and Laufer 2012, Cho, Kim et al. 2019).

The reproductive tract bacteria influence implantation failure of endometrial origin (Al-Nasiry, Ambrosino et al. 2020). For example, women with non-*Lactobacillus*-dominated microbiota in a receptive endometrium had decreased implantation rates than women with a *Lactobacillus*-dominated (>90%) microbiota (Moreno, Codoñer et al. 2016). Chlamydia species in the endocervix of women undergoing IVF-ET were correlated with implantation failure (Witkin, Sultan et al. 1994).

Increasingly researchers are exploring how the local microbiota influences physiology at distal sites, especially how the gut bacteria, the densest and most diverse bacterial communities of the body, impact distal organs, including the brain and lungs, leading to the notions of the gut-brain axis and gut–lung microbiota axis (Ravel and Brotman 2016, Budden, Gellatly et al. 2017). However, hitherto, gut bacteria's role in implantation failure of endometrial origin remains unexplored, let alone the gut-reproductive tract microbiota axis, even though a compelling rationale exists. Indeed, the gut bacteria impact the immune system, hormonal homeostasis, and the coagulation system—all of which mediate implantation success (Alexander, Targan et al. 2014, Baker, Al-Nakkash et al. 2017, Vinchi 2019). The comorbidity of gut disorders (e.g., celiac disease) with infertility disorders, including recurrent pregnancy loss, allude to a role of 'gut-reproductive tract axis' in implantation failure (Tersigni, D'Ippolito et al. 2018).

Hence we investigated whether gut dysbiosis occurs in women with implantation failure, and if so, whether it accompanies vaginal dysbiosis, which usually involves a decline in either the levels of *Lactobacillus* or protective species of *Lactobacillus* and a simultaneous rise in the diversity and density of other bacteria (Madhivanan, Alleyn et al. 2015, Amabebe and Anumba 2018, Wang, Fan et al. 2020). To this end, by using 16S rRNA gene sequencing, we compared the diversity, structure

144 and taxonomic composition of the fecal and vaginal microbiota of fertile women
145 with that of infertile women with a history of RIF and UE.
146

2. Materials and Methods

2.1 Study participants

For this retrospective, single-center cohort study, fertile and infertile women, referred to Akanksha Hospital and Research Institute between September 2018 and February 2019, were recruited and divided into three groups: the control, RIF, and UE groups. The RIF group's inclusion criteria were women who could not conceive after ≥2 fresh IVF-embryo transfer cycles/ Intracytoplasmic Sperm Injection (ICSI) or had ≥3 consecutive miscarriages. UE was diagnosed if a cause remains undefined after our routine fertility tests with the following criteria: infertility of more than 1 year, normal male partner, normal menstrual rhythm with regular ovulation, and normal hormonal tests (i.e., thyroid, prolactin, AMH) Exclusion criteria included diabetes, polycystic ovary syndrome and endometriosis, diarrhea, ongoing pregnancy, addiction (e.g., drugs, alcohol, tobacco etc.) and the use of antibiotics within at least two weeks before sample collection.

2.2 Ethical approval and Consent to participate

We performed all the sampling and experiments with the approval of the local Ethics Committee of Sat Kaival Hospital Pvt. Ltd (EC2013/053). Participants gave their oral and written informed consent for fecal and vaginal sample collections

and subsequent microbiological analysis. We recorded and compared participants' characteristics **(Table 1).**

2.3 Sample Collection

The fecal and vaginal samples were freshly and simultaneously collected. Participants collected the fecal samples in a sterile plastic container with a tight closing lid. To collect the vaginal samples, using a sterile swab stick, clinicians thoroughly wiped the posterior fornix of the vagina of the participants. These swabs were stored in sterile vials. Both types of samples were packaged and first placed in a frozen storage at −20°C in the hospital and later, within 24 hours, transported on ice to be stored at −80°C at Gujarat Biotechnology Research Centre (GBRC) for further analysis.

2.4 DNA extraction

DNA extraction was performed from approximately 200 mg of fecal samples and ~1ml of thoroughly vortexed swab sample using QIAamp DNA Stool Mini Kit according to the manufacturer's instructions. Total DNA was eluted in 30 µL of AE buffer. DNA concentration was quantified fluorometrically with a Qubit 2.0 dsDNA HS Assay kit. DNA was stored at-20^0C for further procedures.

2.5 Library preparation and 16s rRNA Sequencing

The V2-V3 hypervariable regions of the 16S rRNA gene were amplified using fusion primers, 101 F5′ACTGGCGGACGGGTGAGTAA 3′ and 518 R 5′CGTATTACCGCGGCTGCTGG 3′. Amplicon libraries were purified using the Agencourt AMPure XP (Beckman Coulter). For quality control, we used Bioanalyzer with a DNA-HS assay kit. All the libraries were quantified using Qubit fluorimeter v4.0 and were pooled into equimolar concentrations. Clonal amplification (Emulsion PCR) sequencing was performed on the Ion S5 system using the Ion 520/530 kit OT2 (ThermoFisher Scientific) with Ion One Touch 2 system, which uses a 530 chip on Ion S5 plus sequencer according to the manufacturer's instructions.

2.6 Bioinformatics and Statistical analysis

Diversity

Microbial community richness and diversity was evaluated by α-diversity that included Chao1 and Shannon indices. The Kruskal–Wallis test used to determine statistical differences between the three groups. The Mann–Whitney U test was used to determine the influence of diet on α-diversity. β-diversity, differences in microbial community structures between the three groups, was analysed using Principle Coordinate Analysis (PCoA) based on Bray-Curtis and Jaccard distances,

and the statistical difference between the groups was calculated using non-parametric permutational multivariate analysis of variance (PERMANOVA).

QIIME2 software was used to calculate alpha and beta diversity indices. The demultiplexed sequences were uploaded to QIIME2 environment, and denoising was carried out using DADA2. Amplicon sequence variants (ASVs) were predicted at a minimum sampling depth of 25000 for Gut datasets, and 9000 for the vaginal datasets. The predicted ASVs were taxonomically classified using the pre-trained classifier of the full 16S rRNA gene sequence of the SILVA database.

Taxonomic structure

We analysed the gut microbiota at the community level to determine differences in the microbial composition between groups and to identify taxa with significantly different abundance (relative abundance > 0.001 and $P < 0.05$). Additionally, linear discriminant analysis effect size (LEfSe) method was employed to identify species with significant differences in abundance between the three groups ($|LDA| > 3$ and $P < 0.05$). Kruskal–Wallis and Mann–Whitney U tests were performed for computing statistical differences between the groups at taxonomic levels in the study viz., three major groups and the infertile cohort versus controls using STAMP v2.1 software (Parks et al., 2014).

Continuous data are presented as mean ± standard deviation (SD) or frequencies (number and percentages), calculated using GraphPad Prism statistical software 6.0. To determine statistical differences in subjects' characteristics between groups, we performed one-way ANOVA followed by post-hoc Tukey testing.

3. Results

3.1 Clinical Characteristics among the Control, RIF, and UE Groups

We included 31 study subjects, comprising 11 fertile women, enrolled as the control group, 10 women with a history of RIF, and 10 women with a history of UE **(Table 1)**. Comparisons of the mean age between the three groups showed that the controls were statistically significantly younger than the RIF group ($F=6.8$, $p<0.01$), while the UE group was older than the controls and younger than the RIF group, although both differences were statistically insignificant **(Table 1).** The average BMI was not significantly different between the controls and the UE group and between the RIF and UE groups but was significant between the controls and the RIF group ($F=3.7$, $p<0.05$) **(Table 1)**. Among women with RIF, eight were nullipara (80%), and two were nulligravida (20%), while all women with UE (100%) were nullipara. There were more vegetarian participants in the UE group (9 of 10; 90%) than in the RIF group (4 of 10; 40%) and the controls (4 of11; 36%) **(Table 1)**.

3.2 Metagenomics Findings of Gut and Vaginal bacteria

In total 31 fecal samples and 24 vaginal swab samples, sequencing of the V2-V3 region of the 16S rRNA gene created 235810 sequences, with an average of 1,01, 767 sequences, and 178260 high-quality sequences, with an average of 3,76,012

sequences, per sample for fecal and vaginal samples respectively. Based on the results of the operational taxonomic units (OTUs) analysis, rarefaction curves show that the sequencing depth was adequate to analyse the gut and vaginal bacteria in the three groups **(Figures S1 (a), (b) and Figures S2 (a), (b)).**

3.3 Richness and diversity of Gut bacteria

We performed bacterial diversity analyses by comparing the richness using Chao 1 and evenness using the Shannon index for the fecal microbial communities between the three groups. We found that richness differed significantly between the three groups (Chao 1 index (Kruskal–Wallis test, p= 0.049)) **(Figure 1(a)).** Precisely, the controls had a significantly higher richness than the RIF group (pChao 1 = 0.04) and UE group (pChao 1 = 0.03). Richness was similar between RIF and UE groups (pChao 1 = 0.75). We discovered that evenness differed significantly between the three groups: Shannon index (Kruskal–Wallis test, p = 0.003) **(Figure 1(b)).** Specifically, the controls had more evenness than the RIF (pShannon = 0.006) and the UE groups (pShannon = 0.002). In contrast, evenness was similar between the RIF and the UE groups (pShannon = 0.65). Interestingly, the diet did not affect alpha diversity (pShannon = 0.165) **(Figure 3S).**

Regarding the bacterial community structure differences, the PCoA plot of the Bray-Curtis and Jaccard dissimilarity showed that bacteria of the RIF and UE

groups overlapped and that both groups differed markedly from the controls (**Figures 2(a), (b)**). In the PCoA plot based on Bray-Curtis distances, the first and second axes of the PCoA explained 21.5% of the total variance with a significant difference (PERMANOVA, $P < 0.05$, $R2 = 0.12$; **Figures 2(a)**). Showing the similar clustering pattern, in the Jaccard based PCoA plot, the first and second axes explained 23.4% of the total variance with a significant difference (PERMANOVA, $P<0.05$, $R2 = 0.10$; **Figures 2(b)**).

3.4 Taxonomic analysis of Gut bacteria

After excluding the sequences that were present in less than 3%, we clustered the high-quality sequences into OTUs and assigned taxonomic identities. Consequently, we found 550 OTUs pertaining to 481 genera, 265 families, 156 orders, 68 classes, 715 species and 28 phyla. To evaluate the contribution of different taxa to diversity and composition, we calculated the relative abundance of taxa at the phylum, family and genus levels. Except at the genus level, we could not find statistically significant alteration of particular taxa at any other levels. Though, noticeable trends from a clinical point of view were still apparent in the data.

Across all 31 participants, the phyla level composition, represented by read percentages, showed usual human microbiota structures. Overall, among the detected 28 phyla, the four most abundant microbes were *Firmicutes* (85.10%), *Bacteroidetes* (7.70%), *Proteobacteria* (4.75%), and *Actinobacteria* (1.8%) **(Figure 3(a)).** With a relative abundance of less than 1%, the remaining bacterial population belonged to four phyla, including *Verrucomicrobia, Tenericutes*, *Cyanobacteria, and Chloroflexi.*

Firmicutes were abundant (7-9 %) in the RIF group than both the control and UE groups. *Firmicutes* were only slightly more abundant (2%) in the UE group than the controls. *Bacteroidetes* were depleted (50% less) in the RIF group than the other two groups. In contrast, the control and UE groups had the same levels of *Bacteroidetes*. *Proteobacteria* were relatively less abundant (4-7% less) in the RIF group than the control and UE groups **(Figure 3 (a)).** Among the three groups, the UE group had the highest levels of *Proteobacteria*, almost 2 fold higher than the controls. *Actinobacteria* were depleted in the UE group (~4 fold less) compared to the other two groups, with the RIF group showing the highest abundance amongst all the groups, with 2 fold more abundance than the control group **(Figure 3(a)).**

The dominant bacterial families for all the subjects in the descending order of abundance were *Lachnospiraceae*, *Ruminococcaceae*, *Bifidobacteriaceae*,

Erysipelotrichaceae, *Lactobacillaceae*, *Prevotellaacae*, *Vellinollacaea* and *Enterobacteriaceae* **(Figure 3(b)).** The levels of *Lachnospiraceae* and *Ruminococcaceae* were similar between the three groups. Notably, the levels of *Lactobacillaceae* and *Prevotellaacae* families were highest in the controls as compared to the other two infertile groups. *Bacteroidaceae*, *Vellinollacaea* and *Enterobacteriaceae* were highest in the UE group compared to the RIF and control groups, while *Bifidobacteriaceae* and *Erysipelotrichaceae* families were highest in the RIF group as compared to the control and UE groups **(Figure 3(b)).**

We further determined statistical differences in the specific bacterial genera of the three groups. Among 481 genera, the 6 were statistically significantly ($p < 0.05$) different: *Bacteroides, Prevotella 9*, *Hungatella*, *Ruminococcaceae UCG-004, Ruminococcaceae UCG-010,* and *Sutterella.* Aside from *Bacteroides and Prevotella 9*, the abundance of the other 5 genera, while statistically significant ($p < 0.05$), occurred in much lower proportions (<1%). Notably, *Bacteroides* and *Hungatella* were abundant in the infertile cohort, especially in the UE group, than the controls **(Figure 3(c)).**

Bacteroides and Prevotella 9, the two most highly abundant genera, varied significantly between the three groups. Compared to the controls, the relative abundance of *Prevotella 9* plunged noticeably in the RIF group (7 fold), and the

UE group (8 fold), and the relative abundance of *Bacteroides* rose in the RIF group (1.5 fold) and UE group (3.2 fold) **(Figure 3(c)).** When we compared the infertile group against the controls, we found that in the infertile cohort *Prevotella 9, Ruminococcaceae UCG-004, Ruminococcaceae UCG-010* ($p < 0.05$) declined, whereas *Bacteroides, Dorea, oral clone FR58* and *Peptoniphilus* increased ($p < 0.05$) **(Figure 4S).**

3.5 LEfSe analysis

LEfSe analysis was performed to assess the differentially abundant communities in the three groups **(Figure 4 (a), (b)).** In the controls, we observed diverse microbial communities with a high LDA score (Log10), with *Clostridia* showing the highest LDA score> 9 ($p<0.05$). In the RIF group, only *Eubacterium halli* showed the highest dominance with an LDA score>7 ($p<0.05$). In the UE group, unconventional *Firmicutes* such as *Veillonellaceae*, *Selenomonadales*, of the class *Negativicutes,* showed the highest preponderance with an LDA score > 7 ($p<0.05$).

3.6 Metagenomics of vaginal bacteria

Across the 24 vaginal samples, we found 384 distinct species belonging to 301 different genera classified in 135 different families, distributed into 10 phyla. We compared taxa between the three groups. Given the small sample size, we could

not detect a significant statistical difference between them. Nonetheless, interesting trends in the data were apparent from a clinical point of view.

3.7 Taxonomic Analysis of Vaginal Bacteria

In the descending order, the dominant phyla, among the 10 detected phyla, included *Firmicutes, Fusobacteria*, *Proteobacteria, Actinobacteria, Bacteroidetes,* and *Patescibacteria* **(Figure 5(a))**. Of these, *Firmicutes* accounted for the vast majority of the vaginal bacteria in all the groups, with both the RIF (69%) and UE (69.71%) groups showing similar relative abundance, which was higher than the controls (53%). *Fusobacteria* (18% Vs. 0.07 Vs. 0.14) and *Bacteriodetes* (4.1% Vs0.17 Vs. 0.92) were relatively abundant in the controls than the RIF and UE groups. *Proteobacteria* were marginally more abundant in both RIF (15%Vs.11%) and UE (19% Vs. 11%) groups compared to the controls **(Figure 5(a)).**

The dominant families for all the groups in the descending order of abundance were *Lactobacillaceae, Bifidobacteriaceae, Leptotrichiaceae,* and *Prevotellaceae* **(Figure 5(b)).** *Lactobacillaceae* and *Bifidobacteriaceae* were present in all the groups. Reflecting this trend at the phylum level, levels of *Lactobacillaceae* were higher in all the groups, with UE (65.3%) and RIF (58.41%) women showing the highest levels compared with the controls (47.2%).

360 At the genus level, 5 genera were detected, of which three were present in all the
361 groups **(Figure 5(c)).** *Lactobacillus* was the most dominant genus among them,
362 followed by *Gardnerella* and *Parvimonas*. *Gardnerella, Prevotella*, *Parvimonas,*
363 and *Snaethia* were relatively more abundant in the controls compared to the
364 infertile groups. Compared to the controls, *Lactobacillus* was relatively more
365 abundant in the RIF and the UE groups (2-fold).

366 At the species levels, *Sneathia ammni* (0.36%) was detected only in the control
367 groups. In contrast, *Lactobacillus iners AB-1*werepresent in all the groups, with
368 descending order of relatively high abundance in the following manner: the UE
369 group (62%), the controls (16%), and the RIF group (11.02%) **(Figure 5(d)).**

370 **3.8 LEfSe analysis**

371 We performed LEfSe analysis to assess the differentially abundant vaginal
372 bacterial communities in the three groups. We could only find significant
373 differences in the controls, with *Leptotrichia,* of the phylum *Fusobacteria,*
374 showing an LDA (Log10) score >3 ($p<0.05$) **(Figure 6).**

375 **3.9 Alterations of the genus Lactobacillus Species**

376 Within the genus of *Lactobacillus*, 9 species were identified. *L. gasseri, L. ruminis,*
377 *and L. iners AB-1* were found in all the groups, with *Lactobacillus iners AB-1*
378 being the most abundant species **(Figure 7).** Among these, *L. jensenii* and *L.*

379 *vaginalis* were only detected in the UE group, while *L.reuteri* was unique to the
380 RIF group. *L. equicursoris, L. fermentum* and *L. salivarius* were unique to the
381 controls.

382

4. Discussion

We compared, for the first time, the gut-vaginal microbiota axis of fertile women with that of women diagnosed with RIF and UE. The core findings include i) the infertile groups had gut dysbiosis as evident by low α-diversity indices and beta diversity metrics; ii) the gut microbial composition of the RIF and UE groups differed noticeably, with a set of Gram-positive taxa, mainly members of the phylum *Firmicutes*, being dominant in the former group and a set of Gram-negative bacteria, comprising members of the phylum *Proteobacteria* and the class *Negativicutes*, of the phylum *Firmicutes*, being dominant in the latter group; iii) butyrate-producing genera such as *Prevotella* declined in the infertile cohort; iv) elevated levels of the genus *Hungatella* occurred in the infertile cohort, especially in the UE; and v) the infertile cohort, especially the RIF group, had a comparatively healthy vaginal microbiota than the controls.

Gut microbial richness and diversity, defined by α-diversity indices, declined amongst the infertile groups, with the highest decline in the UE group. Studies have suggested that reduced α-diversity, an important indicator of gut microbiome health, indicates low-grade inflammatory disorders such as inflammatory bowel disease, metabolic disorder and obesity (Ott and Schreiber 2006, Le Chatelier, Nielsen et al. 2013, Al-Assal, Martinez et al. 2018). Of note, diet (vegetarian vs. Non-vegetarian) had little influence on α-diversity. Although, a fine-grained

403 analysis showed vegetarian women had a higher proportion of the phyla
404 *Patescibacteria* and the genus *Bacteroides*, likely reflecting its role in the digestion
405 of plant fiber (Matijašić, Obermajer et al. 2014, Jang, Choi et al. 2017). Gut
406 dysbiosis was also reflected in beta diversity indices, suggesting a distinct bacterial
407 composition between the infertile cohort and the controls.

408 Notably, the reduction in the richness of gut bacteria in the RIF group was seen for
409 the phyla *Bacteroidetes* and *Proteobacteria* compared with the other groups.
410 Mirroring this, we observed a lower percentage of *prevotellaceae* (phylum
411 *Bacteroidetes*), *Veillonellaceae* (phylum *Proteobacteria*), *Enterobacteriaceae*
412 (phylum *Proteobacteria*). This was partly reflected at the genus level by a several-
413 fold decrease in the genus *Prevotella* (phylum *Bacteroidetes*) and a mild elevation
414 in *Bacteroidaceae* (phylum *Bacteroidetes*). In contrast, in the UE group, the
415 reduction in richness was seen mainly for the phyla *Actinobacteria* and marginally
416 for *Bacteroidetes.* In keeping with this, reduced levels of the *Bifidobacteriaceae*
417 family (Phylum *Actinobacteria*) were noted, and a marginal decline in the phyla
418 *Bacteroidetes* was reflected by the depletion of the *prevotellaceae* family (phylum
419 *Bacteroidetes*) and a modest rise in *Bacteroidaceae* (phylum *Bacteroidetes*).

420 Altogether, the data clearly indicate that the reduced microbial richness in the RIF
421 and UE groups is characterised by contrasting types of abundance at the taxa level.
422 Nonetheless, data showed a partial overlap at the genus level in that a relative

decline in the abundance of *Prevotella* and an increase in the abundance of *Bacteroides* commonly occurred in both the groups. Since *Prevotella* builds the protective gut mucosal barrier from short-chain fatty acids (SCFAs) such as butyrate and *Bacteroides* impedes mucin synthesis by producing metabolites such as succinate, acetate, and propionate, these findings suggest thinned mucosal protection is the common abnormality in the infertile cohort. (Mejía-León and Barca 2015). Of note, although not highly abundant in the infertile cohort, *Ruminococcaceae UCG-004 and Ruminococcaceae UCG-010,* of the *Ruminococcaceae* family and the genus *Sutterella* declined as well. These are also butyrate- producing genera (Vital, Karch et al. 2017, Jennings, Koch et al. 2019, Wang, Wichienchot et al. 2019).

When the mucus barrier is thinned, the gut bacteria and other microbe-associated molecular patterns (MAMPs) come in direct contact with toll-like receptors (TLRs), located in the gut epithelial cells such as the Paneth cells (Uchida, Oyanagi et al. 2014, Okumura and Takeda 2017, Steimle, Michaelis et al. 2019). TLRs recognise microbes and MAMPs, and subsequently elicit an immune response, leading to localised and systemic inflammation, which can cause implantation failure (Uchida, Oyanagi et al. 2014, Mejía-León and Barca 2015, Okumura and Takeda 2017, Steimle, Michaelis et al. 2019).

442 We proffer the following hypotheses to explain how the gut dysbiosis in the
443 infertile groups causes implantation failure by separate mechanisms that promote
444 systemic inflammation. We posit that gut dysbiosis–induced metabolic
445 dysregulation plays a role in RIF. At the phyla level, abundances of *Bacteroidetes*
446 and *Proteobacteria* were lower. By contrast, *Firmicutes* and *Actinobacteria*'s
447 levels were higher in the RIF group compared with the other two groups--
448 indicating an obesity-associated microbiota profile (Koliada, Syzenko et al. 2017).
449 Obesity has been linked to an increase in *Firmicutes* to *Bacteroidetes* (F/B) ratio
450 (Verdam, Fuentes et al. 2013, Koliada, Syzenko et al. 2017).

451 Conversely, weight loss has been shown to normalise this ratio (Koliada, Syzenko
452 et al. 2017). The F/B ratio was elevated in six out of ten women in the RIF group.
453 In contrast, only two to three subjects had elevated F/B ratio in the other groups.
454 Unsurprisingly, the RIF group's mean BMI, highest among the three groups, was
455 in the obesity range (obesity $\geq$25 kg/m^2 for Asian Indians (Mahajan and Batra
456 2018)), which chimes with the fact that obesity is a risk factor for RIF(Zhang, Liu
457 et al. 2019).

458 Notably, the Clostridium XIVa cluster, of the *Firmicutes* phylum, whose members
459 comprise flagellated bacteria with a tendency to colonise mucus, play a critical role
460 in metabolic dysregulation such as obesity (including visceral
461 adiposity) (Lopetuso, Scaldaferri et al. 2013, Verdam, Fuentes et al. 2013,

462 Jennings, Koch et al. 2019). Indeed, a trend towards an increase in the relative
463 abundance of *Firmicutes* genera in this cluster such as *Lachnoclostridium, Dorea,*
464 *Ruminococcus 2, and Eubacterium* was duly noted in the RIF group (Lopetuso,
465 Scaldaferri et al. 2013). Crucially, LEfSe analysis found that *Eubacteriumhalli*, a
466 member of this cluster, previously found to be elevated in human obesity, is a RIF
467 biomarker (Sanz, Santacruz et al. 2008, Verdam, Fuentes et al. 2013). Strikingly,
468 the RIF group had the highest levels of the *Erysipelotrichaceae* (phylum
469 *Firmicutes*) family. In contrast, it was almost absent in the two groups. Elevated
470 levels of *Erysipelotrichaceae* has been linked to human obesity and has been
471 correlated to elevated levels of Tumor Necrosis Factor-alpha (TNF-α), a pro-
472 inflammatory cytokine involved in obesity-linked insulin resistance (Kaakoush
473 2015). Tellingly, a high relative abundance of *Firmicutes* has been shown to
474 correlate with increased levels of peripheral TNF-α (Orbe-Orihuela, Lagunas-
475 MartÃ-nez et al. 2018). A rodent study found that a high-fat diet first increased the
476 phylum *Firmicutes,* corresponding with the changes of Paneth cell-antimicrobial
477 peptides, which was later followed by the elevations of circulating inflammatory
478 cytokines, including TNF-*α,* thus establishing causality between the phylum
479 *Firmicutes* and TNF-α (Guo, Li et al. 2017).

480 Therefore, precisely, we postulate that the phylum *Firmicutes* generates TNF-α-
481 driven systemic inflammation, and consequent insulin resistance may cause RIF.

Strikingly, investigators showed elevated TNF-α/IL-10 ratio correlates with an increased risk of IVF failure (Winger, Reed et al. 2011). Chan *et al.* found that insulin resistance reduces implantation rate in *in vitro* maturation-*in vitro* fertilization-embryo transfer cycle (Chang, Han et al. 2013). Metformin, known to reduce the F/B ratio, has been shown to increase pregnancy rate in IVF repeaters without polycystic ovary syndrome (Jinno, Watanabe et al. 2010, Wang, Saha et al. 2018). Investigator showed an arginine-rich diet, known to reduce obesity and increase insulin sensitivity, corrects the elevated F/B ratio and increases embryo survival (Dai, Wu et al. 2011).

The most striking phyla level changed in the UE group involved depletion of *Actinobacteria* and abundance of *Proteobacteria*, the pro-inflammatory phylum, comprising common pathogens (e.g., *Escherichia, Salmonella*) (Sekirov, Russell et al. 2010). Compared to the controls, only marginal changes occurred in the levels of phyla *Firmicutes* and *Bacteroidetes* in this group, suggesting a critical role of *Proteobacteria* phyla in UE. This concurs with the fact that *Bifidobacterium*, a genus of the depleted phyla *Actinobacteria,* inhibits gut pathogens (Azad, Sarker et al. 2018). Unsurprisingly, compared to the other two groups, the UE group had the highest enrichment of pathogenic Gram-negative families, whose outer membrane contains a unique component, lipopolysaccharide (LPS) (Brown 2019). These bacterial families included: *Bacteroidaceae* (phylum *Bacteroidetes*),

Veillonellaceae (phylum *Firmicutes*) and *Enterobacteriaceae* (phylum *Bacteroidetes*). Cogently, LEfSe analysis revealed members of the *Negativicutes* class—such as *Veillonellaceae*, *Selenomonadales*, which are atypical gram-negative *Firmicutes,* which possess lipopolysaccharides in the outer membranes—were biomarkers of UE (Vesth, Ozen et al. 2013).

The abundance of *Negativicutes* has been linked with an increase in the systemic levels of IL-6, the pro-inflammatory cytokine (Leite, Rodrigues et al. 2017). Along this line, enrichment of other Gram-negative species has been shown to increase plasma IL-6 levels (de Man, van Kooten et al. 1989, Leite, Rodrigues et al. 2017, Higuchi, Rodrigues et al. 2018). LPS of Gram-negative species, a pro-inflammatory endotoxin, binds to TLR-4 in the gastrointestinal mucosa, triggering an inflammatory cascade that causes localised NF-κB activation, ensuing the systemic secretion of IL-6 (Steimle, Michaelis et al. 2019).

Taken together, we proffer that in the setting of the enfeebled mucosal barrier, the overload of Gram-negative bacteria activates the gut innate immune system, generating IL-6-driven systemic low-grade inflammation, ultimately leading to UE. Indeed, Demir *et al.* found higher serum IL-6 levels, but not TNF-α levels, in women with UE than fertile women (Demir, Guven et al. 2009). Since elevated IL-6 levels impair various aspects of reproductive physiology, including LH secretion, LH-induced ovulation, and FSH-stimulated E2 and progesterone release, the gut

522 bacteria-induced higher IL-6 levels may thus cause UE through these mechanisms
523 (Demir, Guven et al. 2009).

524 In the infertile cohort, fascinatingly, elevated levels of *Hungatella*, producers of
525 trimethylamine N-oxide (TMAO), which enhances thrombotic potential through
526 platelet hyperreactivity, were found than the controls (Zhu, Gregory et al. 2016,
527 Genoni, Christophersen et al. 2019). This data raises the possibility that an
528 overactive coagulation system is a common mechanism of implantation failure.
529 Since levels of *Hungatella* were highest in the UE group, this indicates an
530 important role of thrombosis in UE. Indeed, Azem *et al*. found inherited
531 thrombophilia plays a role in repeated IVF failures, particularly in the subgroup
532 with UE (Azem, Maslovich et al. 2001).

533 Regarding the landscape of the vaginal microbiota, data showed that the vaginal
534 bacterial community was less diverse than in the gut. Rarefaction analysis found
535 that the RIF group had the lowest microbial diversity of the three groups,
536 suggesting a healthy vaginal microbiota in the RIF group (Lokken, Richardson et
537 al. 2019). We posit that through TNF-α-driven systemic insulin resistance, gut
538 dysbiosis in the RIF group causes hyperglycemia and consequently increases
539 glycogen levels in the vaginal epithelium, which is required for the maintenance of
540 healthy microbiota (Carrara, Bazotte et al. 2009, Amabebe and Anumba 2018).
541 Alternatively, adipose tissue-driven rise in peripheral estrogen may increase the

glycogen content of vaginal epithelial cells (Lokken, Richardson et al. 2019). This chimes with the finding that obesity protects against vaginal dysbiosis (Lokken, Richardson et al. 2019). By contrast, the highest microbial diversity in the control group suggests vaginal dysbiosis. LEfSe analysis found *Leptotrichia*, an opportunistic pathogen of the female urogenital tract of the phylum *Fusobacteria*, in this group (Thilesen, Nicolaidis et al. 2007). In line with this, other pathogenic genera such as *Gardnerella, Prevotella*, and *Snaethia* were relatively more abundant in the controls compared to the infertile groups (Thilesen, Nicolaidis et al. 2007, Amabebe and Anumba 2018).

Consistent with previous research, *Firmicutes*, mainly *Lactobacilli* spp., constituted the bulk of total bacteria of all the groups (Madhivanan, Raphael et al. 2014, Madhivanan, Alleyn et al. 2015). Of the nine detected *Lactobacilli* spp., four belonged to the *L. delbrueckii*, subsp. of the *L. acidophilus* group (*L. iners, L.gasseri, L. jensenii, L. equicursoris*), three belonged to the *L. reuteri* group (*L. fermentum, L. reuteri, L. vaginalis*), and two belonged to the *L. salivarius* group (*L.ruminis, L. salivarius*) (Salvetti, Torriani et al. 2012). Of the three groups, the *L. reuteri* group comprises mostly obligate heterofermentative lactobacilli, consistent with the finding that compared to the women in the USA, Indian women housed a higher proportion of obligate heterofermentative lactobacilli, reflecting a need for metabolic plasticity and an adaptation against high pathogen load (Salvetti,

562 Torriani et al. 2012, Madhivanan, Raphael et al. 2014, Madhivanan, Alleyn et al.
563 2015). Three *Lactobacillus* species dominated the vaginal microbiota across the
564 groups: *L. iners, L.gasseri*, and *L.ruminis*, with *L. iners* being the most abundant,
565 suggesting the existence of community state type 3 (CST 3) of the five human
566 vaginal microbial communities (HVMC) as classified by Ravel et al. (2011)
567 (Ravel, Gajer et al. 2011). To put this in context, *L. crispatus*, *L. jensenii*, *L.*
568 *gasseri*, and *L. iners* occur most frequently in the healthy vagina worldwide,
569 although substantial geographic, socio-economic and ethnic variations exist
570 (Madhivanan, Krupp et al. 2008, Madhivanan, Raphael et al. 2014).
571 By lowering the vaginal PH<4 through lactic acid, generating bacteriocins and
572 hydrogen peroxide (H_2O_2), or acting as a competitive inhibitor, *Lactobacilli* spp.
573 protect the vagina from opportunistic pathogens (Kalia, Singh et al. 2020, Wang,
574 Fan et al. 2020). Notably, though this protection varies according to strains based
575 on their ability to produce D–lactic acid, which keeps PH<4, and antibacterial
576 compounds (Wang, Fan et al. 2020). Most studies have shown that *L. iners,*
577 lacking the ability to produce D-lactic acid*,* is less protective than other strains
578 such as *L. crispatus* and *L. salivarius* (Pino, Bartolo et al. 2019, Kalia, Singh et al.
579 2020, Wang, Fan et al. 2020). Indeed, the highest relative proportions of
580 *Lactobacillus iners AB-1* occurred in the UE group, suggesting a weakened
581 resistance to colonisation by pathogens (Wang, Fan et al. 2020).

4.1 Conclusion

In sum, we illuminated gut and vaginal bacterial communities' landscape, both at broader and finer levels, in implantation failure-associated infertile women and fertile women and offer conjectures to explain the data. We discovered that the infertile cohort had gut dysbiosis. Most importantly, these findings suggest that gut dysbiosis does not necessarily lead to vaginal dysbiosis. We hope that this study has laid the foundation of research on the link between the gut microbiota, the gut-reproductive microbiota axis, and implantation failure, which can lead microbiota-based diagnostic tools and therapeutic strategies.

4.2 Limitations

Our study has a few limitations. First, since it is an underpowered single-center study, multi-center longitudinal studies with a large sample size are needed. Second, although we suggested the mechanistic hypotheses, we did not measure alterations in the immune system, hormones, platelet parameters and bacterial metabolic products such as short-chain fatty acids. Third, owing to the limited resolution of the 16S rRNA-sequencing technique, we could not identify what specific bacterial species or strains were involved. Fourth, we have not controlled for the factors that influence the vaginal microbiota such as menses, vaginal douching, and contraception. Finally, the functional significance of many species such as *peptoniphilus* remains undetermined in our analysis as the literature is

602 scant on these genera. Hence, future investigations should address these
603 shortcomings.

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We presented a portion of the data of this study in the 4th World Congress on Recurrent Pregnancy Loss (WCRPL 2019) in Jerusalem, Israel.

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Availability of data and materials:

The 16S rRNA gene sequencing data for all the gut and vaginal microbiota samples analyzed in this study have been deposited with the National Center for Biotechnology Information (NCBI): reference number PRJNA7020230.

Authors' contributions:

BP conceptualized the idea, contributed to the design, made a few suggestions on the statistical methods, interpreted the data, and wrote the manuscript except for the metagenomics part of the method section. NP contributed to the design, patient identification, sample collection, ethical approval, financially supported the clinical work, presented a part of the work, and critically discussed and corrected the manuscript's clinical aspects. NP collected fecal and vaginal samples, performed DNA isolation, sequencing, assisted in the analysis, drafted a part of the material and method section, and prepared some graphs and tables. SP collected fecal and vaginal samples, performed DNA isolation, library preparation of samples, and contributed to the correction of materials and method section. NN analysed the metagenomics data and contributed to the writing of the metagenomics section of the manuscript. RP sequenced fecal samples and corrected material and method section. CJ, the director of GBRC, provided financial and technical support and guidance for metagenomics sequencing and critically discussed the data. NP and MP contributed to ethical permission, patient selection and clinical data acquisition and critically discussed the data. All the authors provided criticisms and read and approved the manuscript.

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Competing interests:

The authors declare no competing interests.

Consent for publication:

All authors have given their consent for the publication of this manuscript.

Figure and Table Legend

Table 1. Study characteristics of the control, RIF, and the UE groups. Data are expressed as the mean ± SD or n/N (%).RIF, recurrent implantation failure; UE, unexplained infertility; BMI, body–mass index; Veg, Vegetarian; NA, Not applicable. Differences between the 3 groups were assessed by 1-factor ANOVA and Tukey's multiple comparison test was used for post-hoc comparisons, if $P<0.05$. In the RIF group, two participants belonged to the RPL category. a. statistically significant difference between CON and RIF.

Figure 1. Box plots of α-diversity indices of the gut bacterial microbiomes of the control (CON, N = 11), RIF (RIF, N = 10), and the UE (UE, N=10) groups: (a) Shannon and (b) Chao 1 indices. The *P* values were determined by the Kruskal-Wallis test.

Figure 2. Differences in community composition (β-diversity) between the control (CON, Red, N = 11), RIF (RIF, Green, N = 10), and the UE group (UE, Cyan N=10) groups. Comparisons are based on the PCoA plots of Bray-Curtis (left) and Jaccard distances (right). Each principal coordinate axis represents the proportion of variance. Non-parametric multivariate analysis of variance (PERMANOVA)

was used to calculate statistical differences between the groups. In the Bray-Curtis-based PCoA, the first and second axes of the PCoA accounted for 21.5% of the total variance (PERMANOVA, $P < 0.05$, $R2 = 0.12$). In the Jaccard based PCoA, the first and second axes explained 23.4% of the total variance (PERMANOVA, $P<0.05$, $R2 = 0.10$).

Figure 3. The bar chart shows the comparisons of relative abundances of top gut bacterial taxa between control (CON, N = 11), RIF (RIF, N = 10), and the UE (UE, N=10) groups at (a) the phylum (b) family and (c) genus levels. No differences in relative abundance were found between the phylum and family levels ($P>0.05$, Kruskal-Wallis test). Six bacterial genera showed statistically significantly ($P<0.05$, Kruskal-wallis test) differential abundance across the three groups are shown.

Figure 4. Distinct taxa of the gut bacteria determined by linear discriminant analysis effect size (LEfSe) analysis in the control (CON, N = 11), RIF (RIF, N = 10), and the UE (UE, N=10) groups. (a) The cladogram shows the taxa that were significantly elevated between the groups (b) Taxa with an LDA score significant threshold >3 are shown ($P < .05$; LDA score 3).

Figure 5. The bar charts show taxonomic comparisons of the vaginal bacteria between the control (CON, N = 8), RIF (RIF, N = 8), and the UE (UE, N=8) groups at (a) the phylum, (b) family (c) genus level (d) species levels. No differences in relative abundance were found at any levels ($P > 0.05$, Kruskal-Wallis test).

Figure 6. Differentially abundant vagina bacteria between the control (CON, N = 8), RIF (RIF, N = 8), and the UE (UE, N=8) groups as determined by linear discriminant analysis effect size (LEfSe) analysis. Taxa with a LDA score significant threshold >3 are shown ($P < .05$; LDA score 3).

Figure 7. The bar charts show taxonomic comparisons of different *Lactobacillus* spp. of the vagina between the control (CON, N = 8), RIF (RIF, N = 8), and the UE (UE, N=8) groups. No significant differences in relative abundance were found between at any levels ($P > 0.05$, Kruskal-Wallis test).

Supplementary figures

Figure 1S. Rarefaction analysis of Gut and Vaginal Microbial diversity of the controls, RIF and UE groups. (A) Rarefaction curve of α-diversity of the gut bacteria in control (CON, N = 11), RIF (RIF, N = 10), and the UE (UE, N=10) groups (B) Rarefaction curve of α-diversity of the vaginal bacteria in control (CON, N = 8), RIF (RIF, N = 8), and the UE (UE, N=8) groups. The x-axis shows the number of sequences per sample, and the y-axis shows the rarefaction measure. When the curve plateaus, it shows that the sequencing data volume is sufficient to reveal most of the microbial information in the sample. The values of the y-axis reflect the community diversity of microbiota. The sequence number in the chart shows the sequence number of the sample. OTU, operational taxonomic unit.

Figure 2S. Rarefaction analysis of microbiome diversity sequences per sample: (a) gut (n=31) and (b) vaginal samples (n=24) (Operational taxonomic units (OTUs) for each sample at 97% of similarity). Each graph represents mean (column) and SD (bars).

Figure 3S. Influence of diet on α-diversity between vegetarian (n=21) and non–vegetarian participants (n=10). No significant difference in α-diversity between these groups was found ($P > 0.05$, the Mann-Whitney U test).

Figure 4S. The bar chart shows taxonomic comparisons of the gut bacteria between the controls (CON, N=11) and the infertile cohort (the RIF plus UE groups, N=20) at the genus level with statistical significance values ($P > 0.05$, Mann-Whitney U test).

Table 1

Characteristics	Control (n=11)		RIF (n=10)		UE (n=10)	
Age (Years)	27.9±3.8		34.5±4.8[a]		30.8±3.42	
BMI (kg/m^2)	21.62±4.05		25.9±3.31[a]		23.92±3	
Nulligravida	NA		2 (20%)		NA	
Nullipara			8 (80%)		10 (100%)	
Dietary Information	Veg	Non-Veg	Veg	Non-Veg	Veg	Non-Veg
	5 (45%)	6 (54%)	4 (40%)	6 (60%)	9 (90%)	1 (10%)

Figure 1(a) Kruskal-Wallis , P=0.049; H =6.00

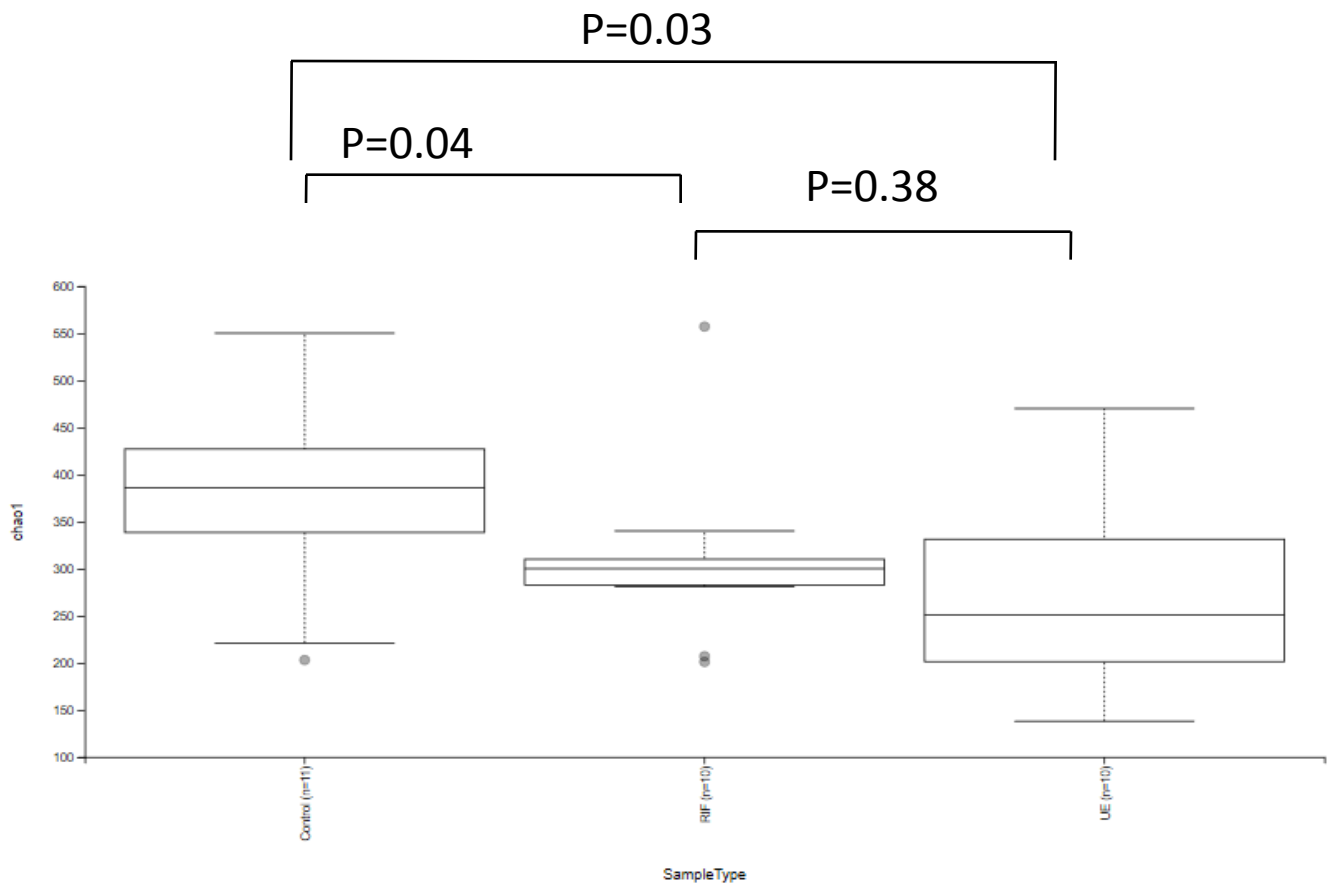


Figure 1(b)

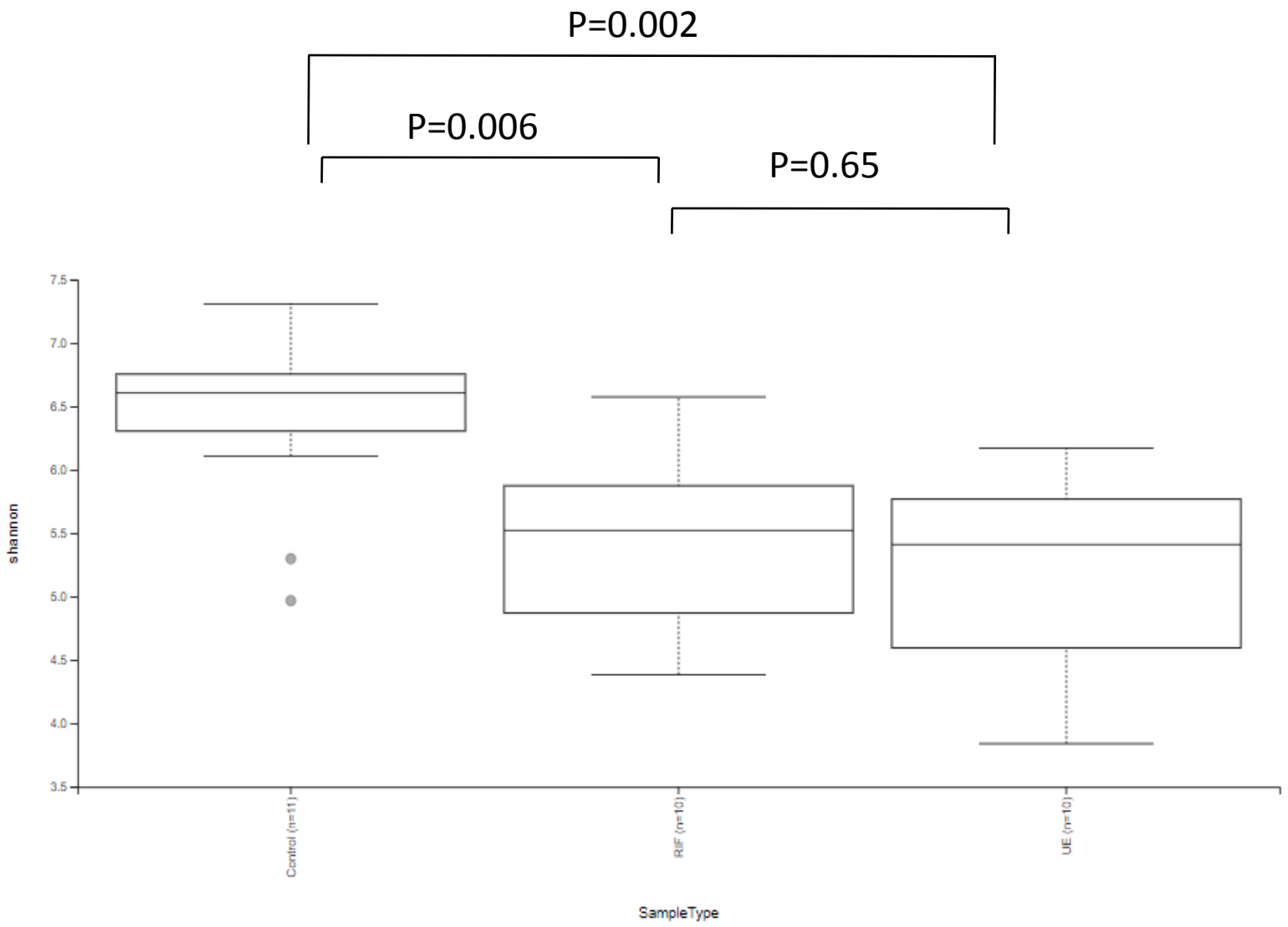
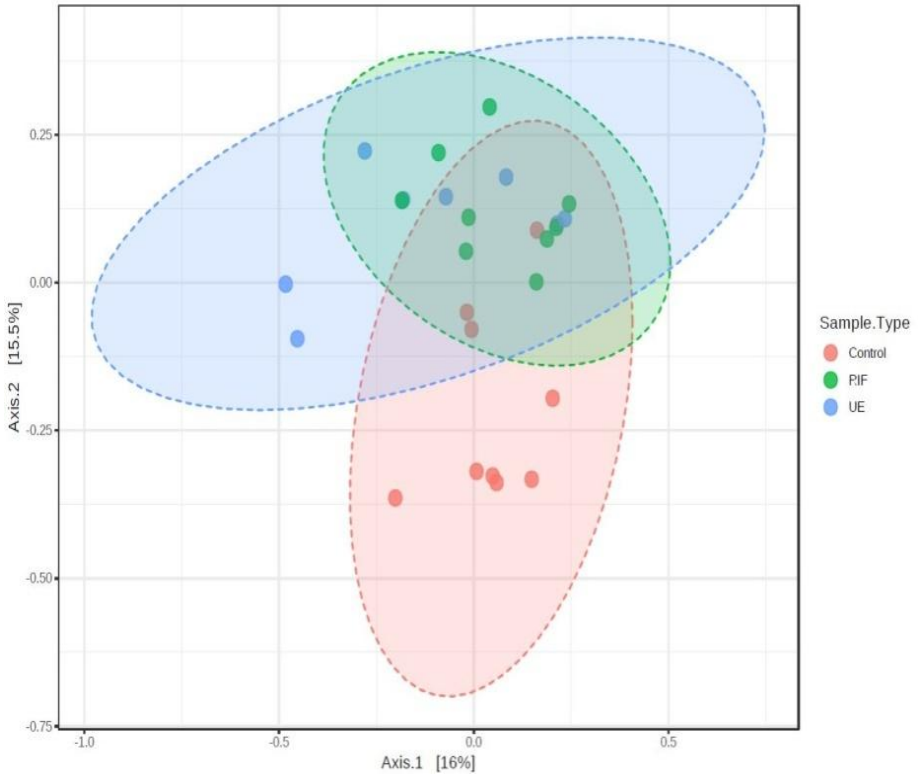

Kruskal-Wallis , P=0.003; H =11.6
P=0.002
P=0.006
P=0.65
shannon
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
Control (n=11)
RIF (n=10)
UE (n=10)
SampleType

Figure 2(a),(b)


0.25
0.00
-0.25
-0.50
-0.75
Axis.2 [15.5%]
-1.0
-0.5
0.0
0.5
Axis.1 [16%]
Sample.Type
Control
PIF
UE

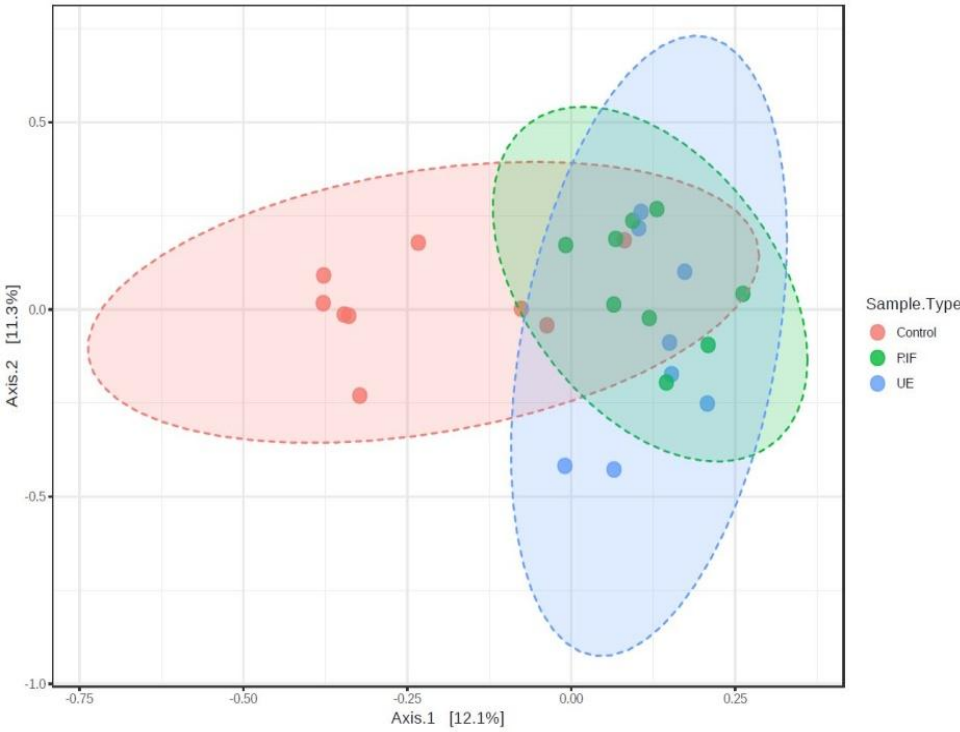

0.5
0.0
-0.5
-1.0
Axis.2 [11.3%]
-0.75
-0.50
-0.25
0.00
0.25
Axis.1 [12.1%]
Sample.Type
Control
PIF
UE

Figure 3(a)

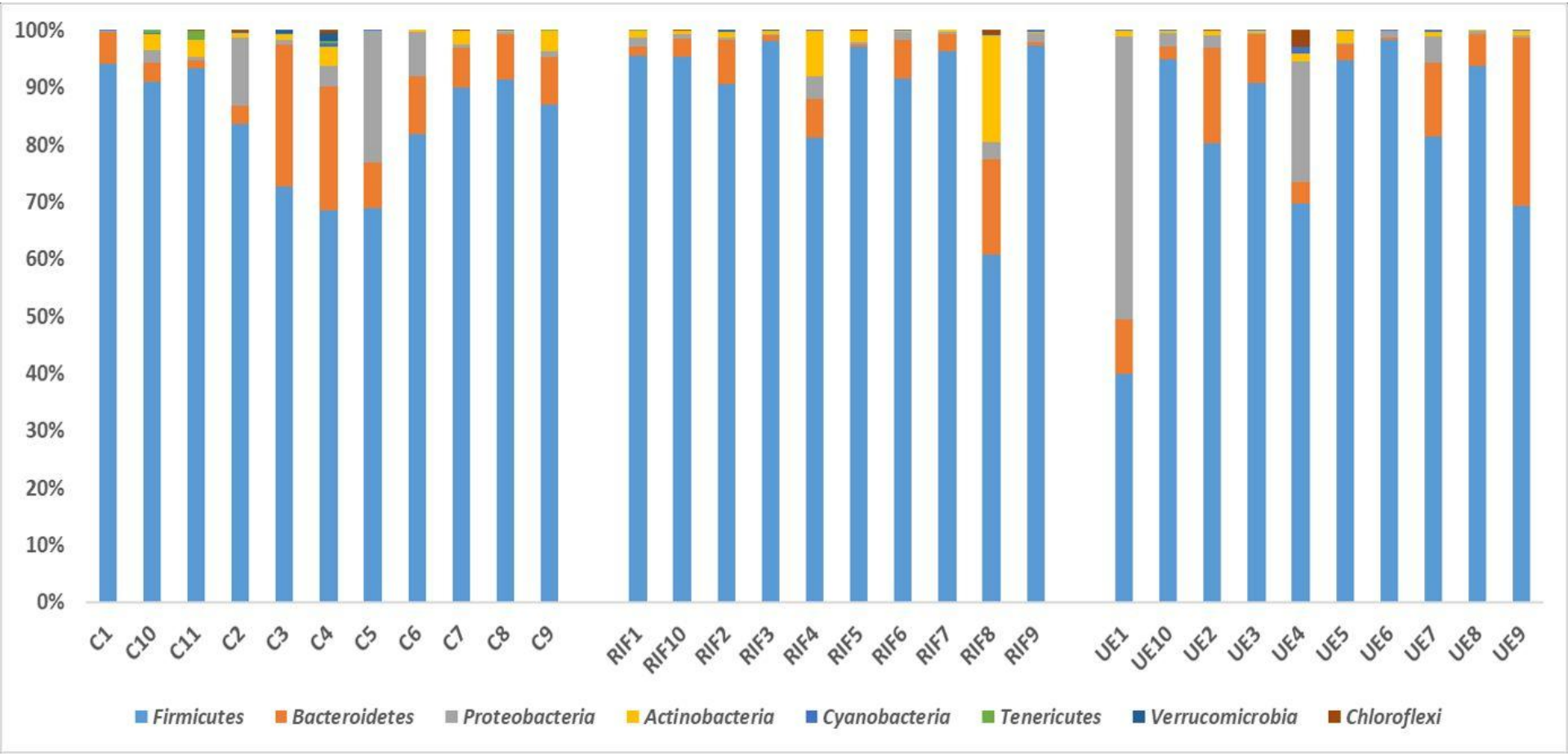

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C1
C10
C11
C2
C3
C4
C5
C6
C7
C8
C9
RIF1
RIF10
RIF2
RIF3
RIF4
RIF5
RIF6
RIF7
RIF8
RIF9
UE1
UE10
UE2
UE3
UE4
UE5
UE6
UE7
UE8
UE9
Firmicutes
Bacteroidetes
Proteobacteria
Actinobacteria
Cyanobacteria
Tenericutes
Verrucomicrobia
Chloroflexi

Figure 3(b)

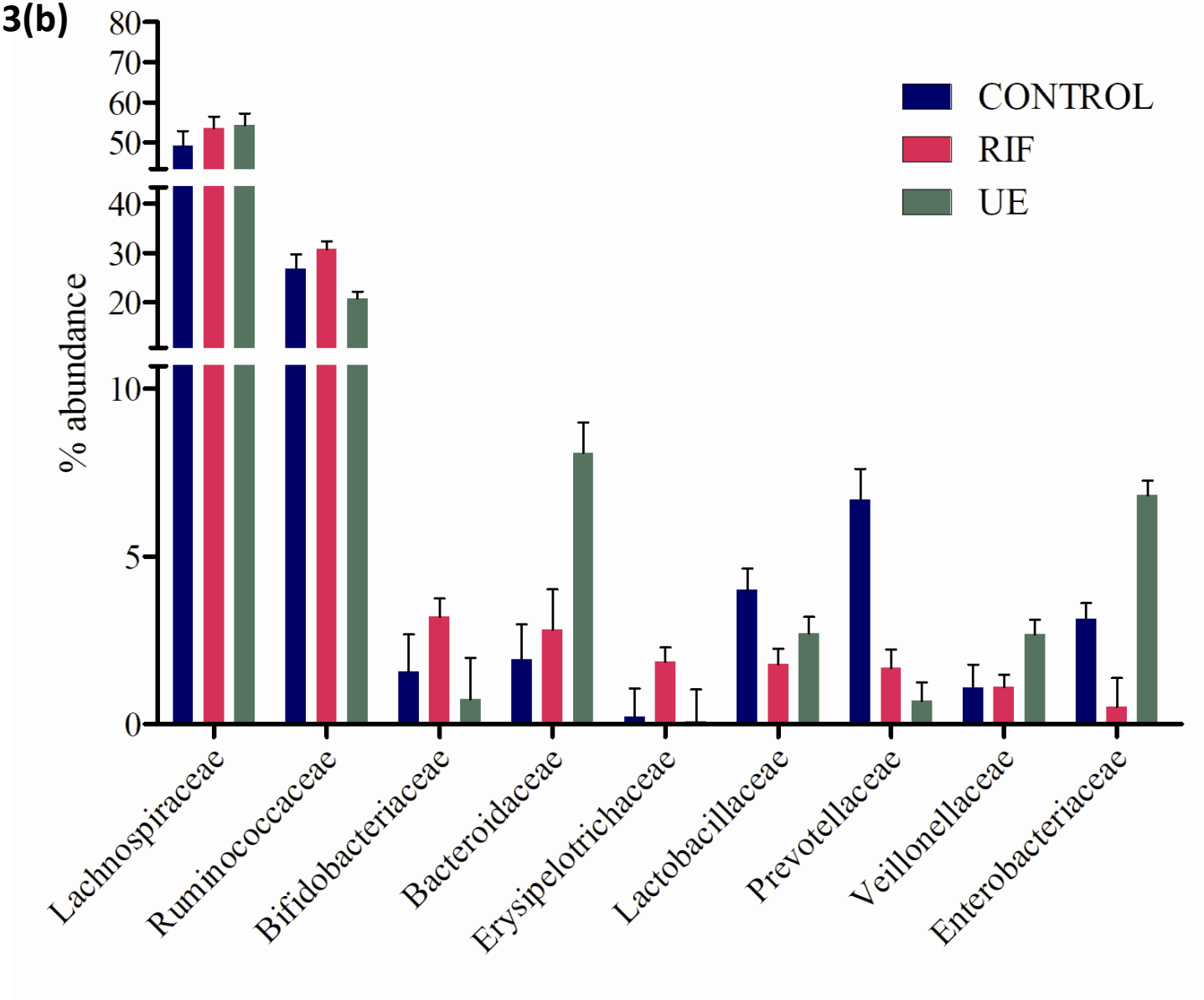
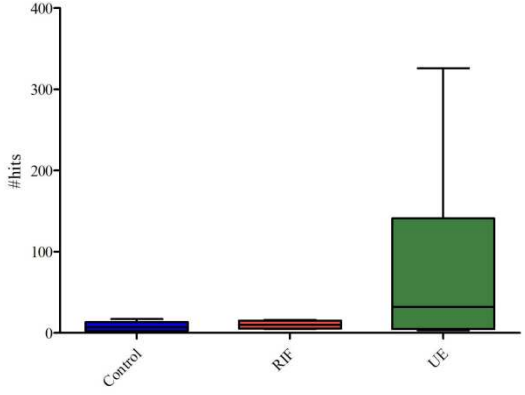
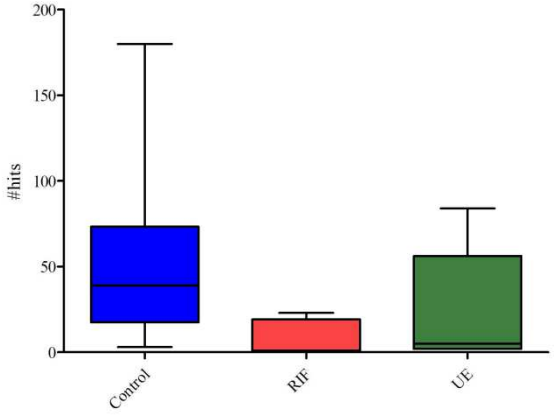

80
70
60
50
40
30
20
10
5
0
% abundance
CONTROL
RIF
UE
Lachnospiraceae
Ruminococcaceae
Bifidobacteriaceae
Bacteroidaceae
Erysipelotrichaceae
Lactobacillaceae
Prevotellaceae
Veillonellaceae
Enterobacteriaceae

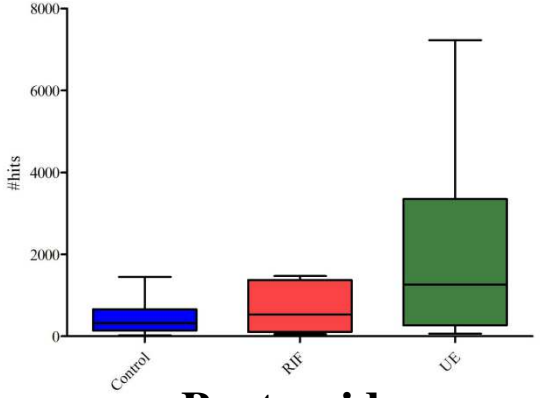
Figure 3(c)



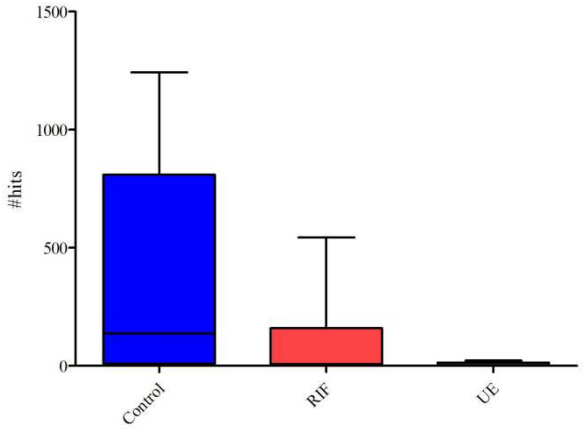
Hungatella



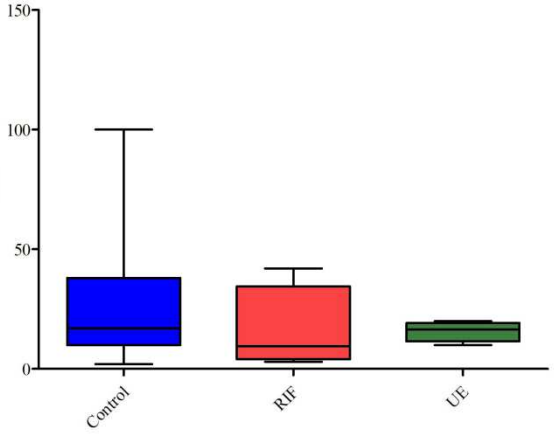
Ruminococcaceae UCG-010



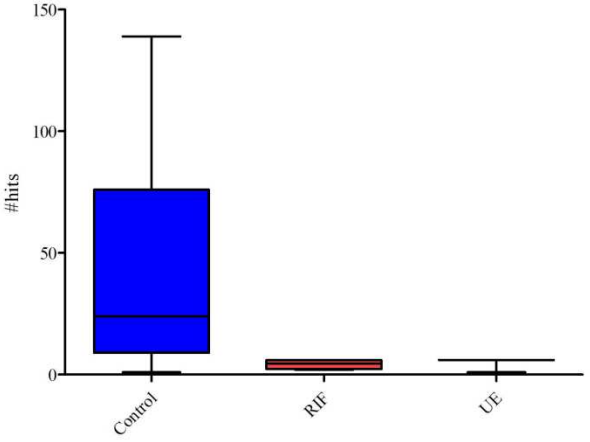
Bacteroides



Prevotella_9



Ruminococcaceae UCG-004



Sutterella

Figure 4(a)

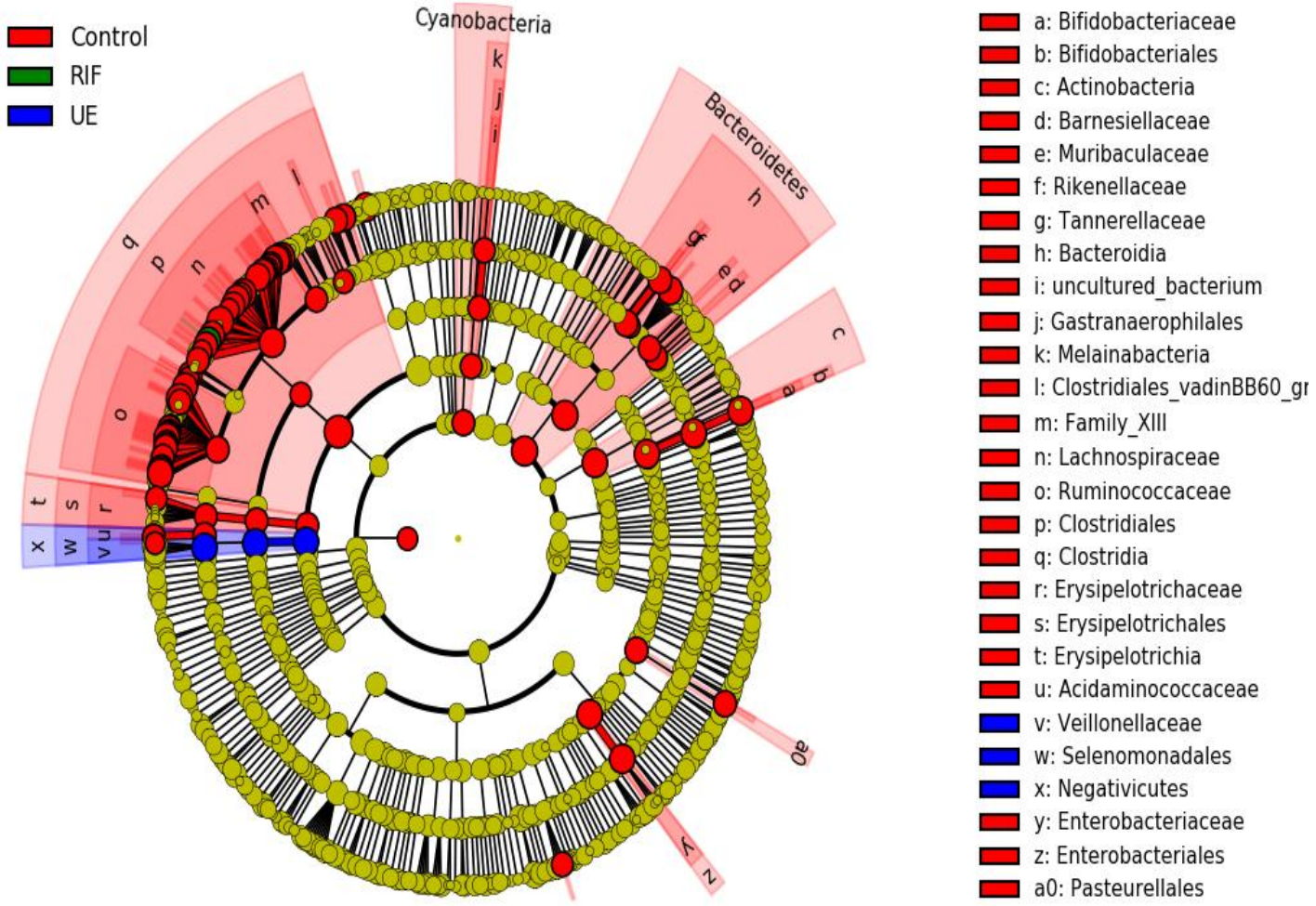

Control
RIF
UE
Cyanobacteria
Bacteroidetes
a: Bifidobacteriaceae
b: Bifidobacteriales
c: Actinobacteria
d: Barnesiellaceae
e: Muribaculaceae
f: Rikenellaceae
g: Tannerellaceae
h: Bacteroidia
i: uncultured_bacterium
j: Gastranaerophilales
k: Melainabacteria
l: Clostridiales_vadinBB60_gr
m: Family_XIII
n: Lachnospiraceae
o: Ruminococcaceae
p: Clostridiales
q: Clostridia
r: Erysipelotrichaceae
s: Erysipelotrichales
t: Erysipelotrichia
u: Acidaminococcaceae
v: Veillonellaceae
w: Selenomonadales
x: Negativicutes
y: Enterobacteriaceae
z: Enterobacteriales
a0: Pasteurellales

Figure 4(b)

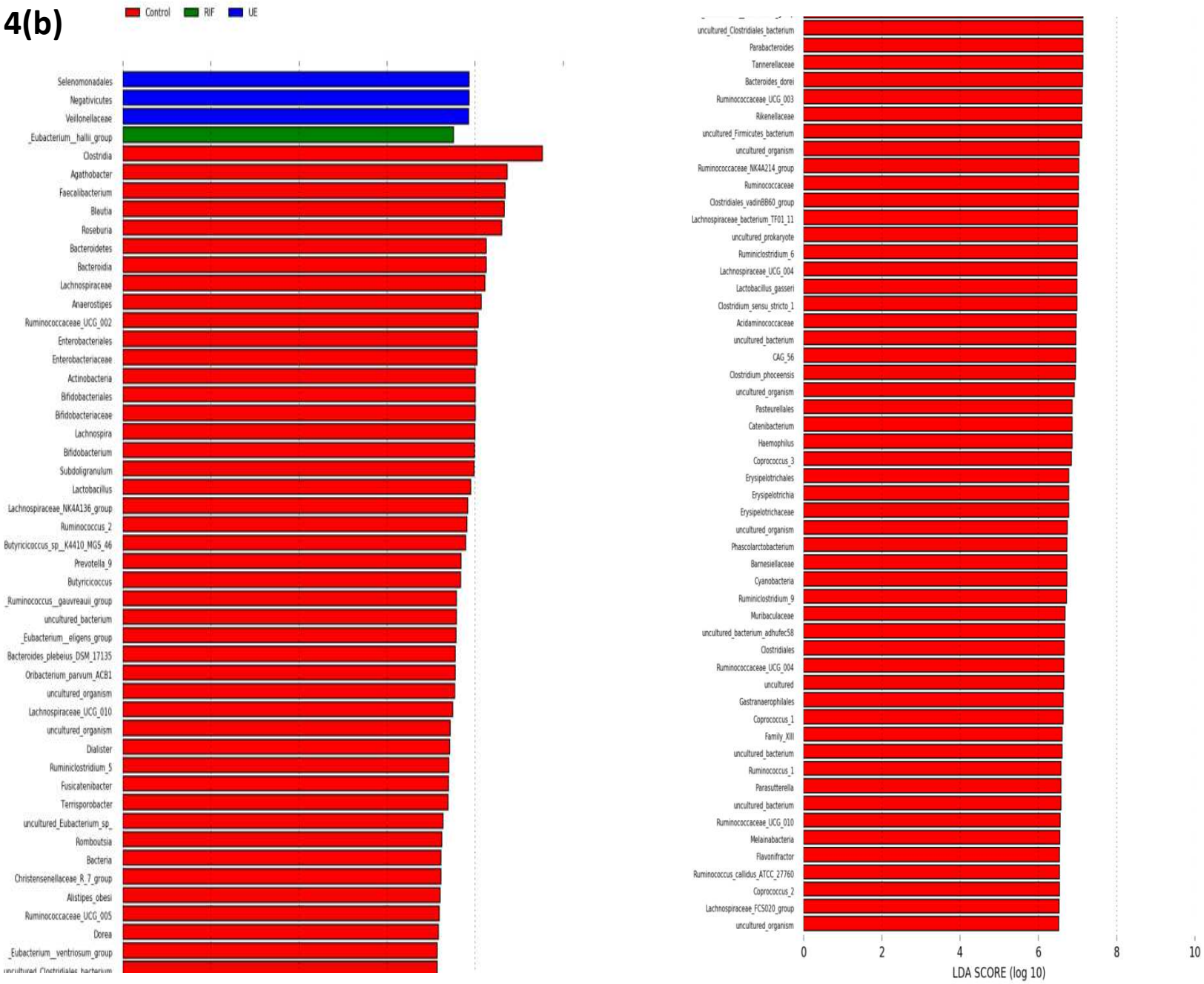

Control
RIF
UE
Selenomonadales
Negativicutes
Veillonellaceae
Eubacterium_hallii_group
Clostridia
Agathobacter
Faecalibacterium
Blautia
Roseburia
Bacteroidetes
Bacteroidia
Lachnospiraceae
Anaerostipes
Ruminococcaceae_UCG_002
Enterobacteriales
Enterobacteriaceae
Actinobacteria
Bifidobacteriales
Bifidobacteriaceae
Lachnospira
Bifidobacterium
Subdoligranulum
Lactobacillus
Lachnospiraceae_NK4A136_group
Ruminococcus_2
Butyricicoccus_sp._K4410_MGS_46
Prevotella_9
Butyricicoccus
Ruminococcus_gauvreauii_group
uncultured_bacterium
Eubacterium_eligens_group
Bacteroides_plebeius_DSM_17135
Oribacterium_parvum_ACB1
uncultured_organism
Lachnospiraceae_UCG_010
uncultured_organism
Dialister
Ruminiclostridium_5
Fusicatenibacter
Terrisporobacter
uncultured_Eubacterium_sp._
Romboutsia
Bacteria
Christensenellaceae_R-7_group
Alistipes_obesi
Ruminococcaceae_UCG_005
Dorea
Eubacterium_ventriosum_group
uncultured_Clostridiales_bacterium
Parabacteroides
Tannerellaceae
Bacteroides_dorei
Ruminococcaceae_UCG_003
Rikenellaceae
uncultured_Firmicutes_bacterium
uncultured_organism
Ruminococcaceae_NK4A214_group
Ruminococcaceae
Clostridiales_vadinBB60_group
Lachnospiraceae_bacterium_TF01-11
uncultured_prokaryote
Ruminiclostridium_6
Lachnospiraceae_UCG_004
Lactobacillus_gasseri
Clostridium_sensu_stricto_1
Acidaminococcaceae
uncultured_bacterium
CAG-56
Clostridium_phoceensis
uncultured_organism
Pasteurellales
Catenibacterium
Haemophilus
Coprococcus_3
Erysipelotrichales
Erysipelotrichia
Erysipelotrichaceae
uncultured_organism
Phascolarctobacterium
Barnesiellaceae
Cyanobacteria
Ruminiclostridium_9
Muribaculaceae
uncultured_bacterium_adhufec58
Clostridiales
Ruminococcaceae_UCG_004
uncultured
Gastranaerophilales
Coprococcus_1
Family_XIII
uncultured_bacterium
Ruminococcus_1
Parasutterella
uncultured_bacterium
Ruminococcaceae_UCG_010
Melainabacteria
Flavonifractor
Ruminococcus_callidus_ATCC_27760
Coprococcus_2
Lachnospiraceae_FCS020_group
uncultured_organism
0
2
4
6
8
10
LDA SCORE (log 10)

Figure 5(a)

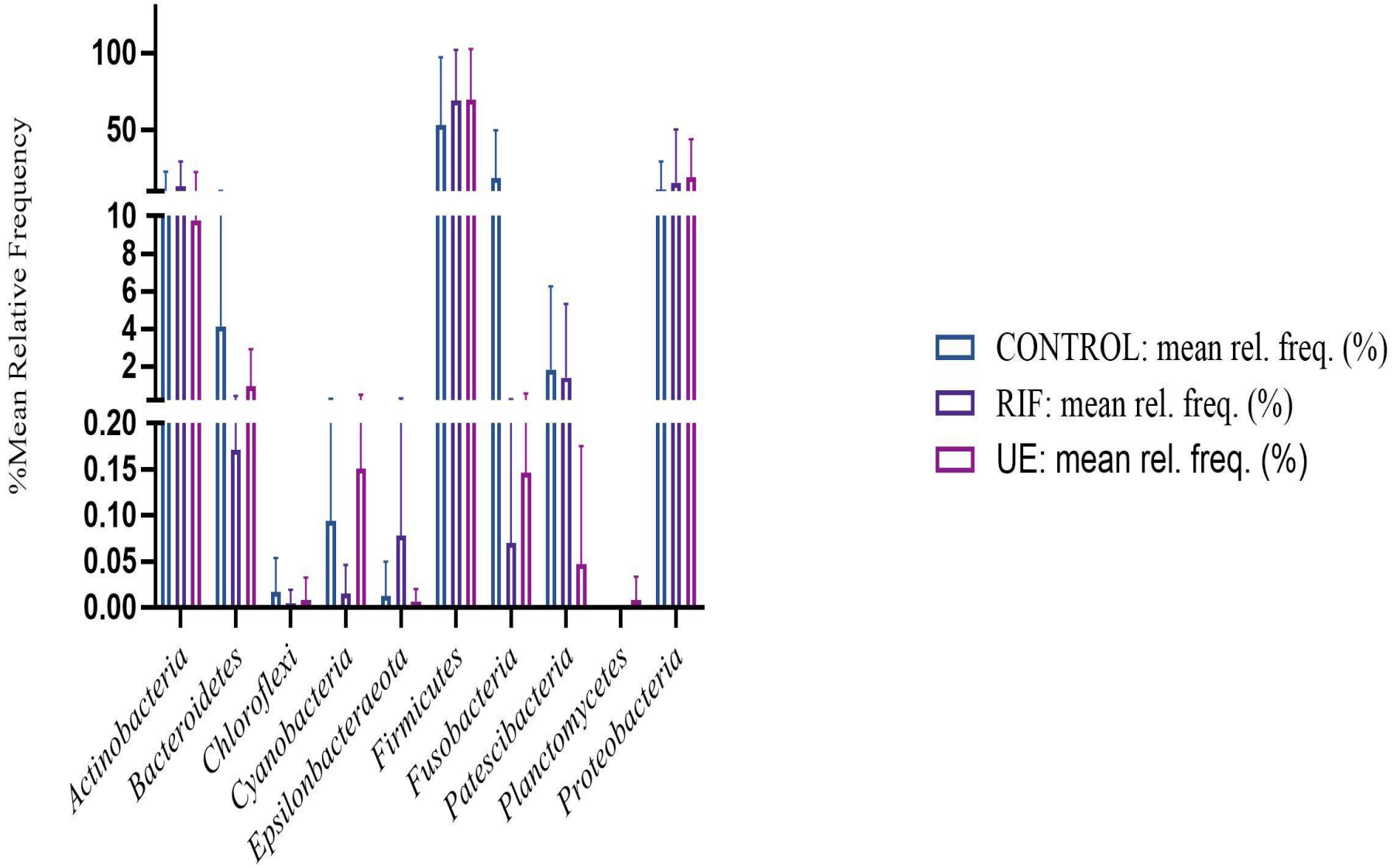

%Mean Relative Frequency
100
50
10
8
6
4
2
0.20
0.15
0.10
0.05
0.00
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Epsilonbacteraeota
Firmicutes
Fusobacteria
Patescibacteria
Planctomycetes
Proteobacteria
CONTROL: mean rel. freq. (%)
RIF: mean rel. freq. (%)
UE: mean rel. freq. (%)

Figure 5(b)

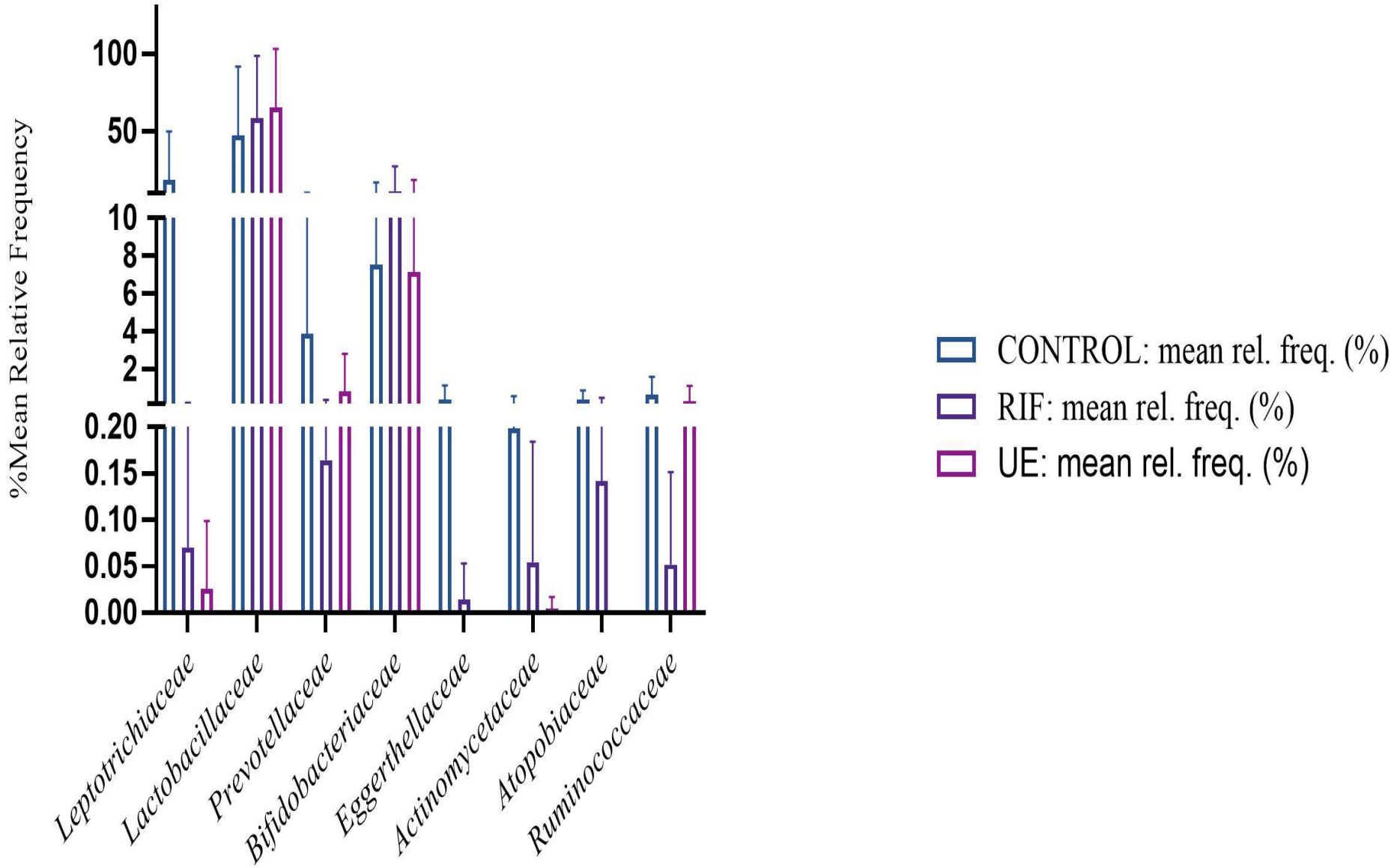

%Mean Relative Frequency
100
50
10
8
6
4
2
0.20
0.15
0.10
0.05
0.00
Leptotrichiaceae
Lactobacillaceae
Prevotellaceae
Bifidobacteriaceae
Eggerthellaceae
Actinomycetaceae
Atopobiaceae
Ruminococcaceae
CONTROL: mean rel. freq. (%)
RIF: mean rel. freq. (%)
UE: mean rel. freq. (%)

Figure 5(c)

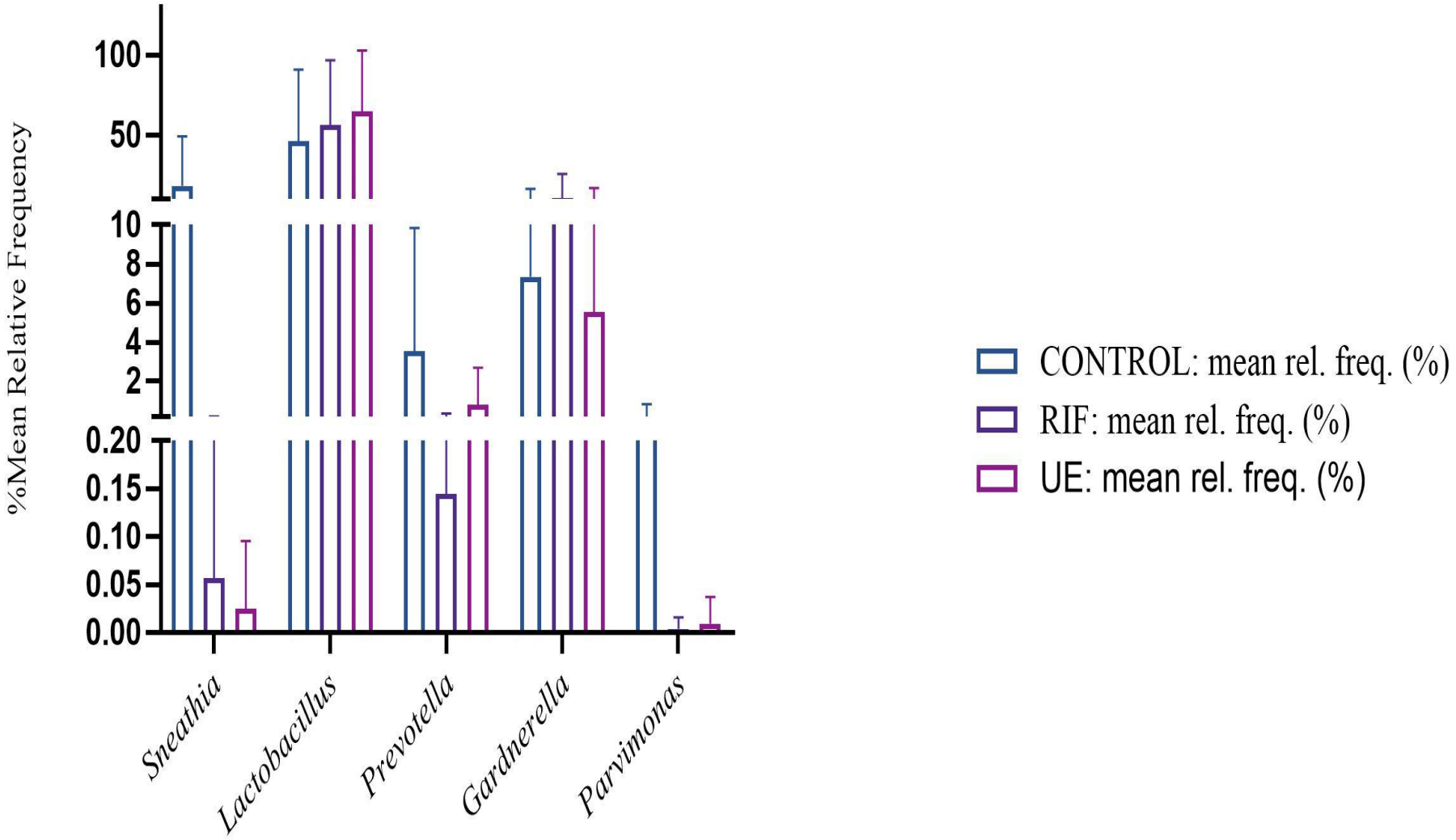

%Mean Relative Frequency
100
50
10
8
6
4
2
0.20
0.15
0.10
0.05
0.00
Sneathia
Lactobacillus
Prevotella
Gardnerella
Parvimonas
CONTROL: mean rel. freq. (%)
RIF: mean rel. freq. (%)
UE: mean rel. freq. (%)

Figure 5(d)

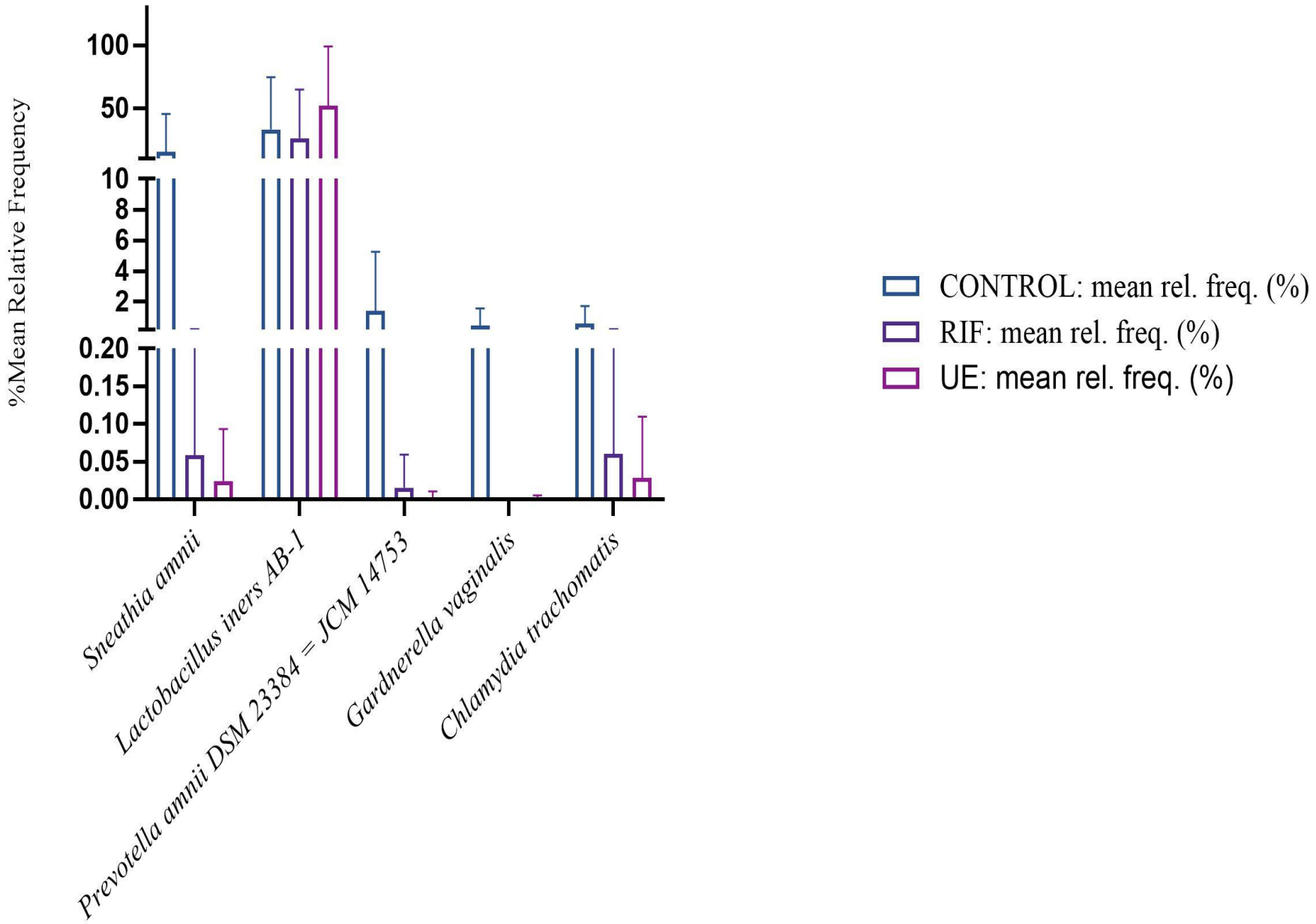

%Mean Relative Frequency
100
50
10
8
6
4
2
0.20
0.15
0.10
0.05
0.00
Sneathia amnii
Lactobacillus iners AB-1
Prevotella amnii DSM 23384 = JCM 14753
Gardnerella vaginalis
Chlamydia trachomatis
CONTROL: mean rel. freq. (%)
RIF: mean rel. freq. (%)
UE: mean rel. freq. (%)

Figure 6

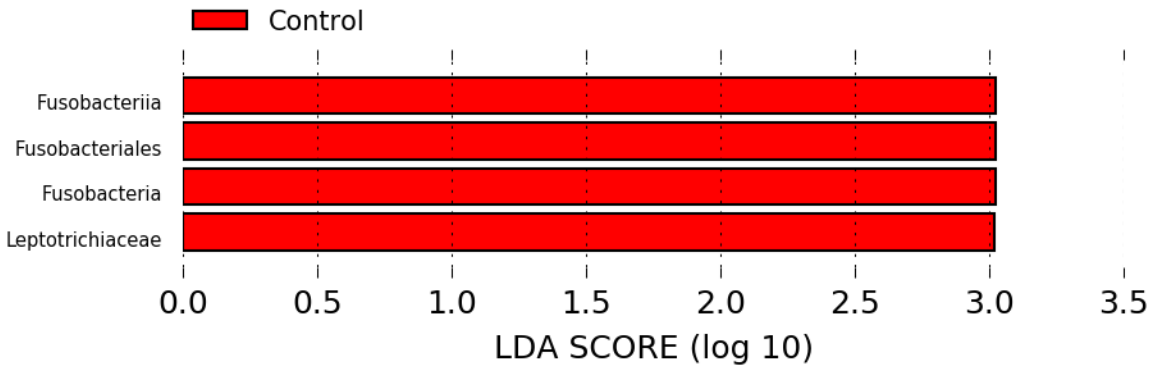
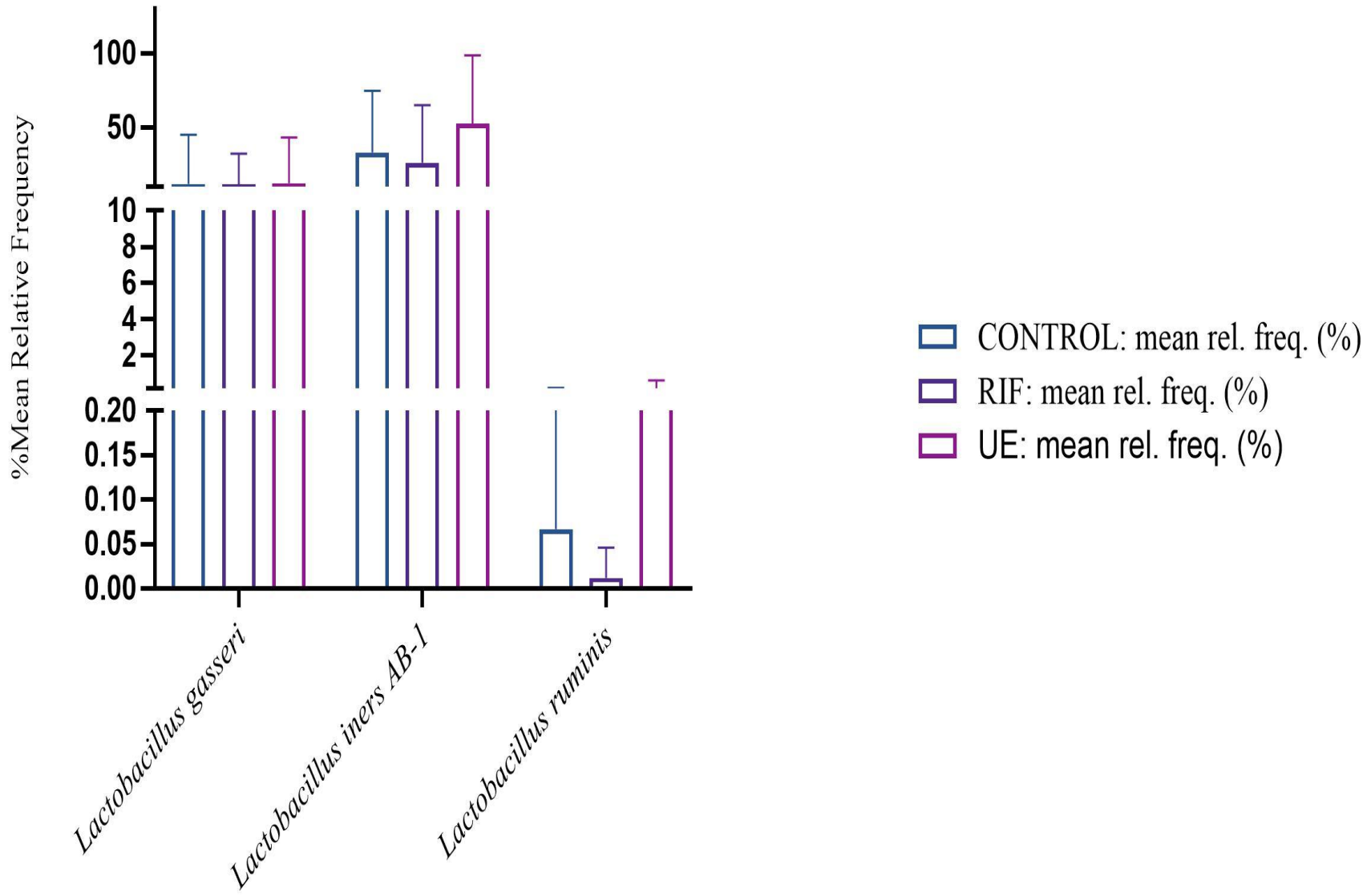

Control
Fusobacteriia
Fusobacteriales
Fusobacteria
Leptotrichiaceae
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
LDA SCORE (log 10)

Figure 7


%Mean Relative Frequency
100
50
10
8
6
4
2
0.20
0.15
0.10
0.05
0.00
Lactobacillus gasseri
Lactobacillus iners AB-1
Lactobacillus ruminis
CONTROL: mean rel. freq. (%)
RIF: mean rel. freq. (%)
UE: mean rel. freq. (%)

Supplementary figures

Figure 1S(a)

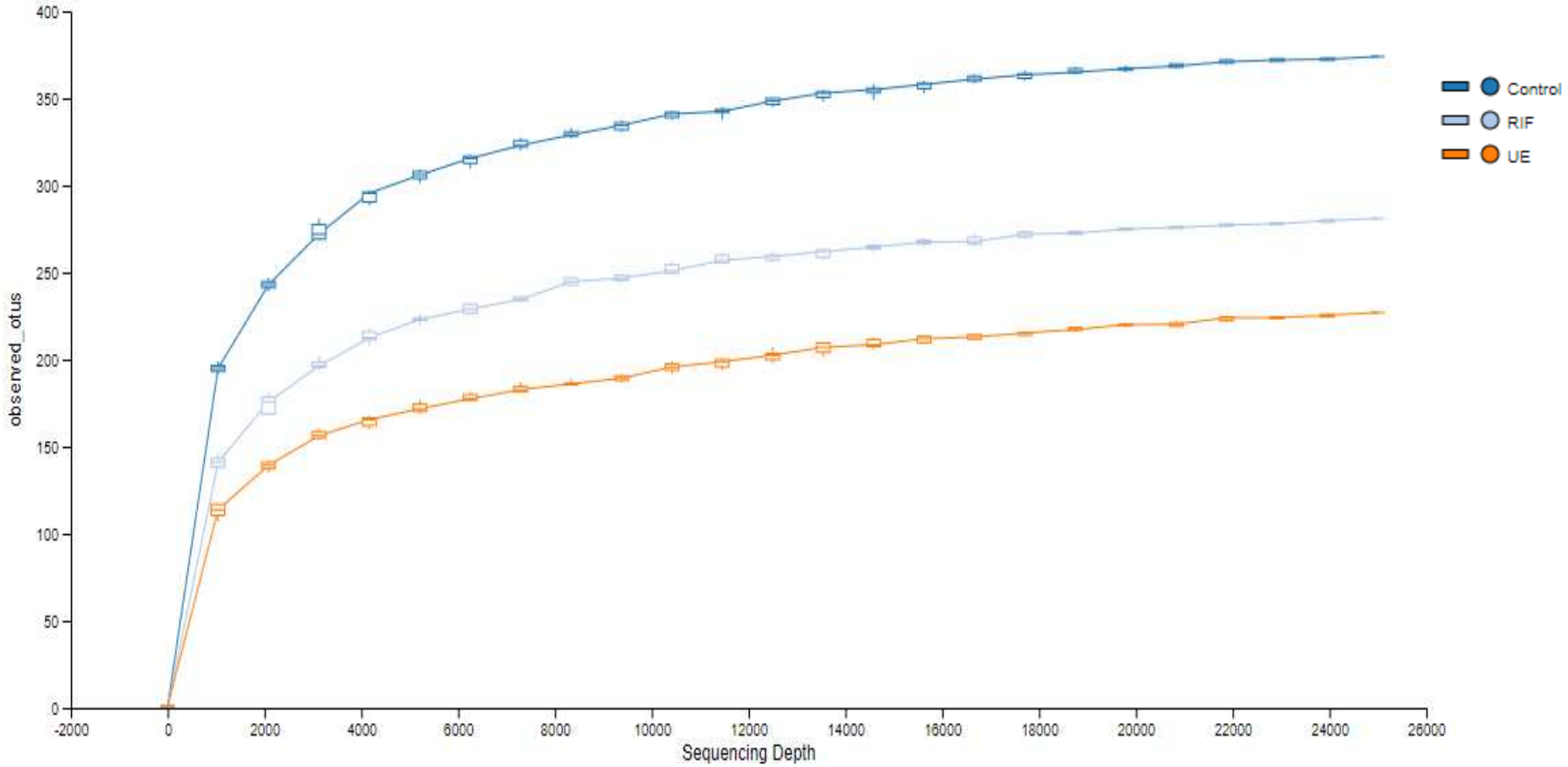

observed_otus
400
350
300
250
200
150
100
50
0
-2000
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
22000
24000
26000
Sequencing Depth
Control
RIF
UE

Figure 1S(b)

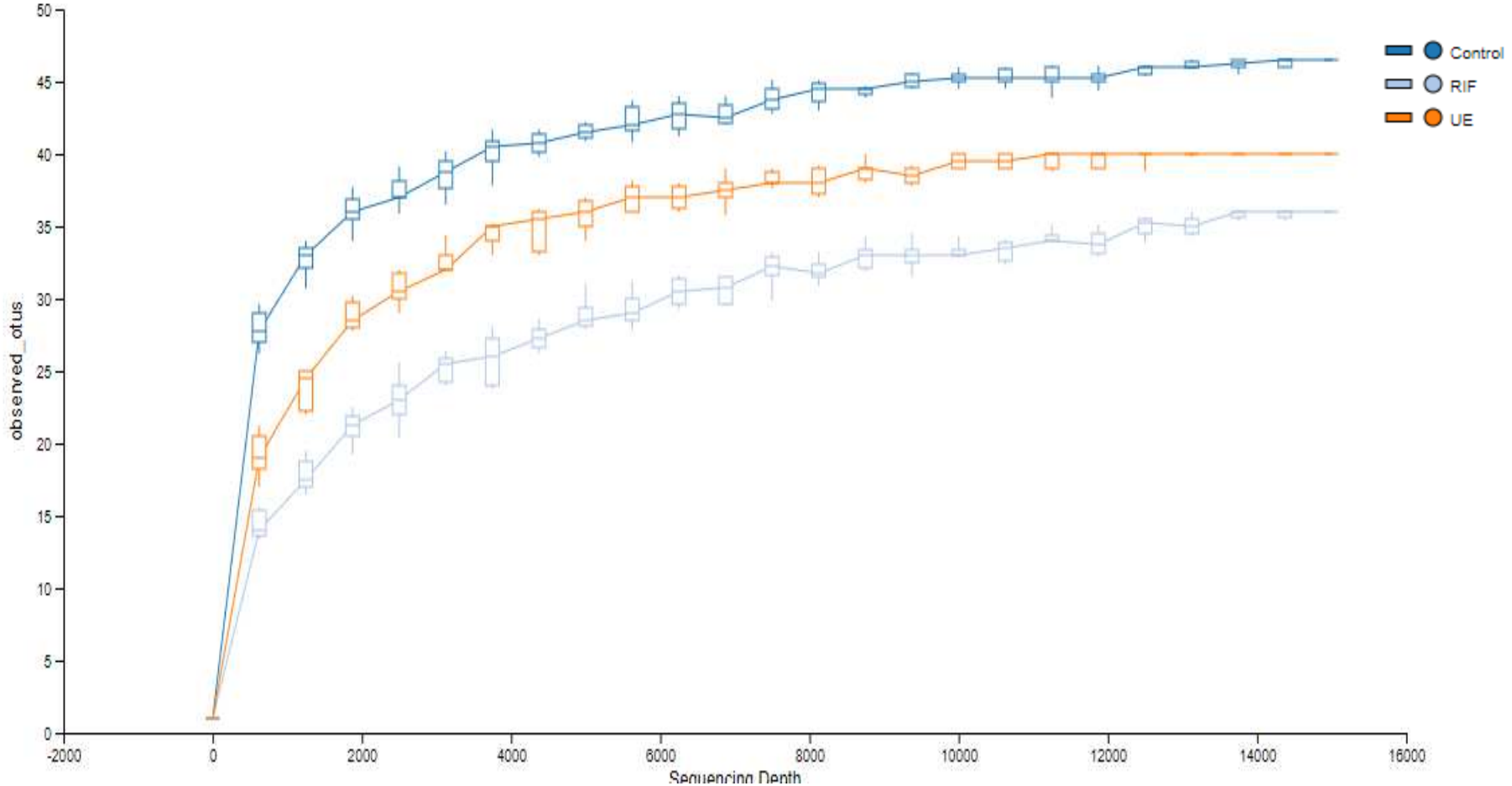

observed_otus
Sequencing Depth
Control
RIF
UE
0
5
10
15
20
25
30
35
40
45
50
-2000
0
2000
4000
6000
8000
10000
12000
14000
16000

Figure 2S(a)

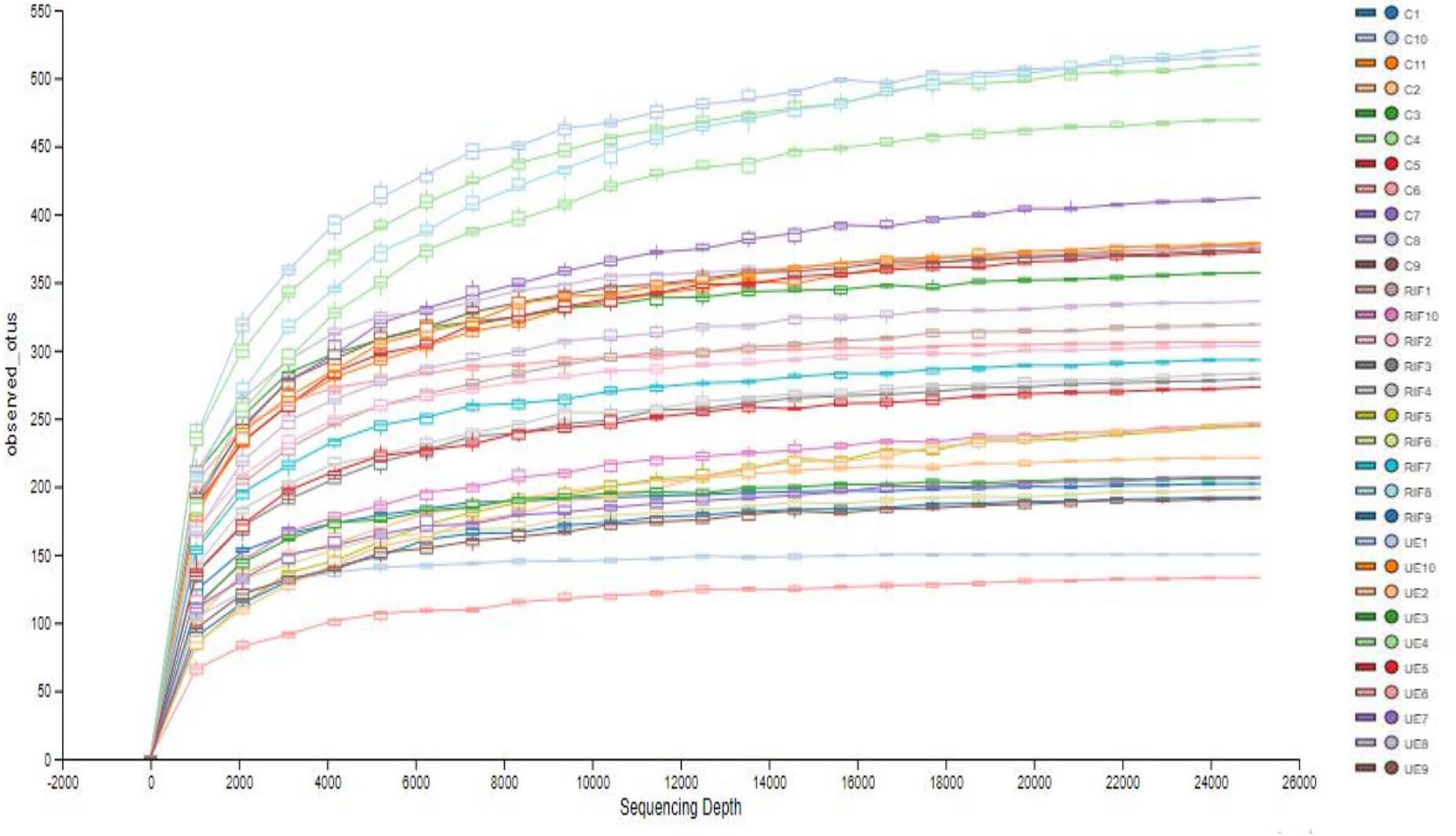

observed_otus
Sequencing Depth
0
50
100
150
200
250
300
350
400
450
500
550
-2000
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
22000
24000
26000
C1
C10
C11
C2
C3
C4
C5
C6
C7
C8
C9
RIF1
RIF10
RIF2
RIF3
RIF4
RIF5
RIF6
RIF7
RIF8
RIF9
UE1
UE10
UE2
UE3
UE4
UE5
UE6
UE7
UE8
UE9

Figure 2S(b)

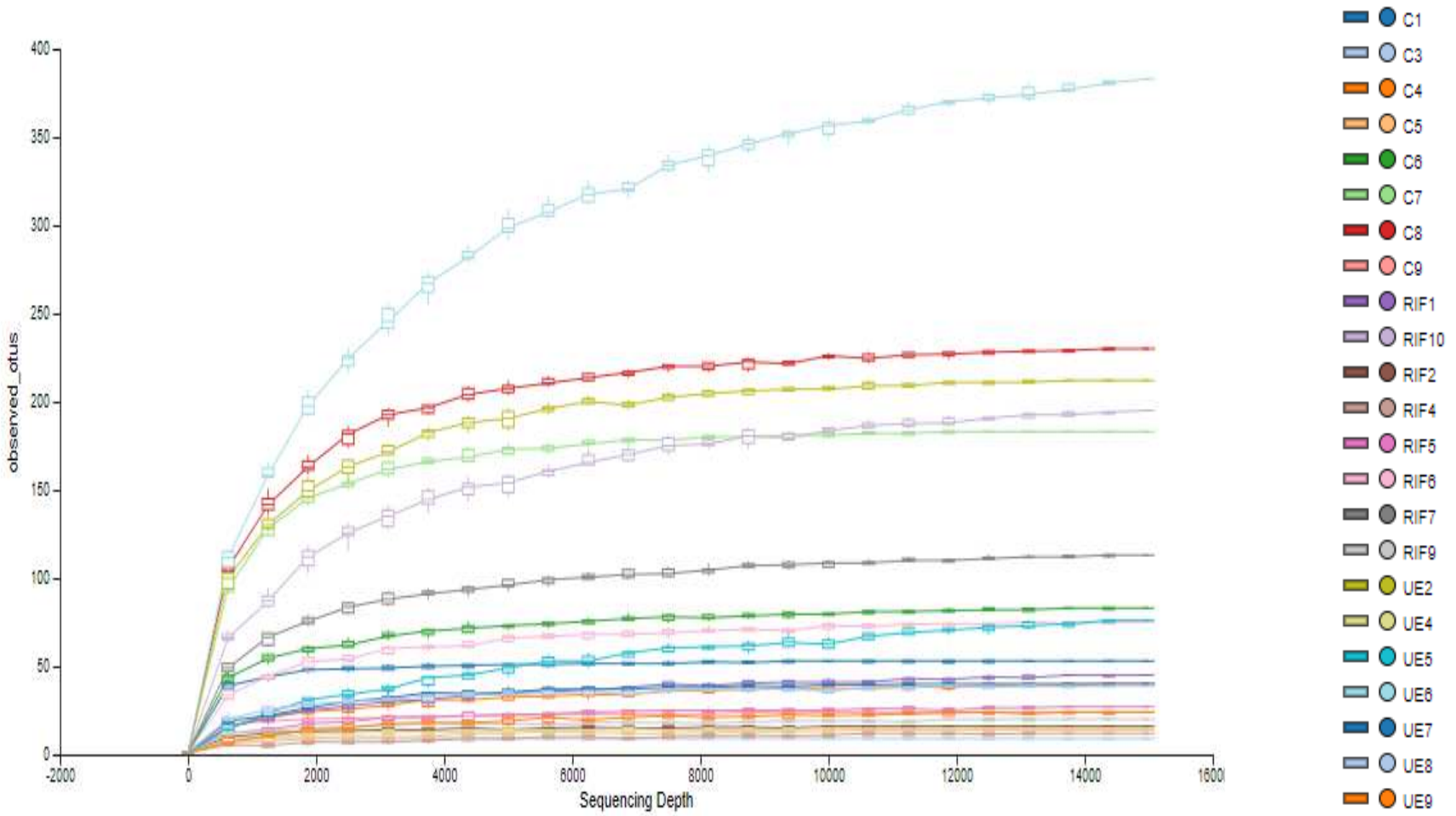

observed_otus
400
350
300
250
200
150
100
50
0
-2000
0
2000
4000
6000
8000
10000
12000
14000
1600
Sequencing Depth
C1
C3
C4
C5
C6
C7
C8
C9
RIF1
RIF10
RIF2
RIF4
RIF5
RIF6
RIF7
RIF9
UE2
UE4
UE5
UE6
UE7
UE8
UE9

Figure 3S

Kruskal-Wallis , P=0.150; H =2.06

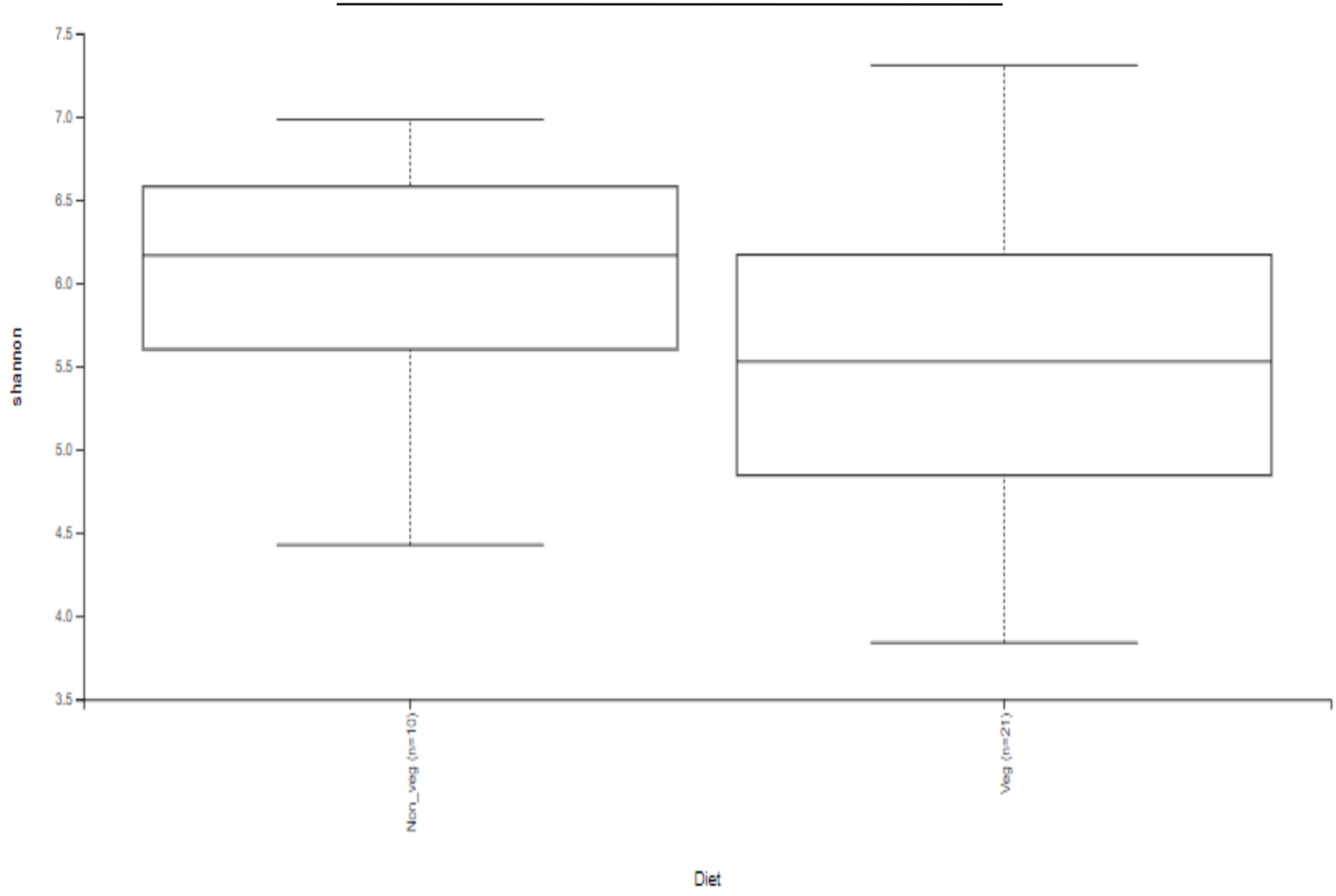
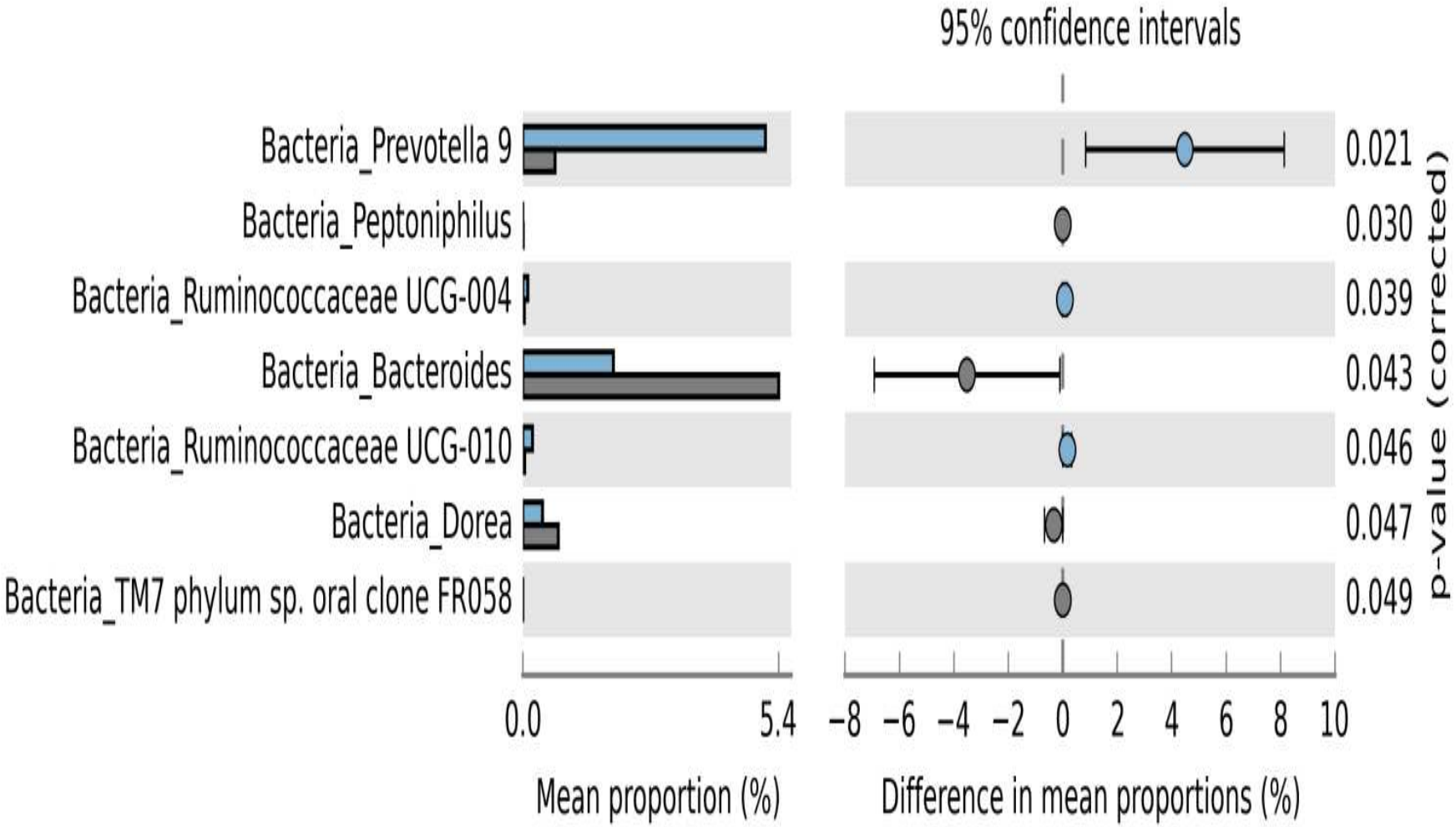


Figure 4S


95% confidence intervals
Bacteria_Prevotella 9
Bacteria_Peptoniphilus
Bacteria_Ruminococcaceae UCG-004
Bacteria_Bacteroides
Bacteria_Ruminococcaceae UCG-010
Bacteria_Dorea
Bacteria_TM7 phylum sp. oral clone FR058
0.021
0.030
0.039
0.043
0.046
0.047
0.049
p-value (corrected)
0.0
5.4
−8 −6 −4 −2 0 2 4 6 8 10
Mean proportion (%)
Difference in mean proportions (%)