Effects of enhanced insect feeding on the faecal microbiota and transcriptome of a family of captive common marmosets (*Callithrix jacchus*)

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Abstract

Common marmosets have been widely used in biomedical research for years. Nutritional control is an important factor in managing their health, and insect intake would be beneficial for that purpose because common marmosets frequently feed on insects in natural habitats. Here, we examined the effect of enhanced insect feeding on the gut by analysing the faecal microbiota and transcripts of captive marmosets. A family consisting of six marmosets was divided into two groups. During the seven-day intervention period, one group (the insect feeding group, or Group IF) was fed one cricket and one giant mealworm per marmoset per day, while the other (the control group, or Group C) was not fed these insects. RNA was extracted from faecal samples to evaluate the ecology and transcripts of the microbiota, which were then compared among time points before (Pre), immediately after (Post), and two weeks after intervention (After) by total RNA sequencing. The gut microbiota of marmosets showed *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* as dominant phyla. Linear discriminant analysis showed differential characteristics of microbiota with and without insect feeding treatment. Further analysis of differentially expressed genes revealed increases and decreases in *Bacteroidetes* and *Firmicutes*, respectively, corresponding to the availability of insects under both Post and After conditions. Significant changes specific to insect feeding were also detected within the transcriptome, some of which were synchronized with the fluctuations

in the microbiota, suggesting a functional correlation or interaction between the two. The rapid changes in the microbiota and transcripts may be deeply connected to the original microbiota community shaped by marmoset feeding ecology in the wild. The results were informative for identifying the physiological impact of insect feeding to produce a better food regimen and for detecting transcripts that are currently unidentifiable.

Introduction

After successful confirmation of the germline transmission of transgenes [1], common marmosets (*Callithrix jacchus*) have been increasingly used in various medical and biological areas [2]. Breeding methods for captive marmosets have been well established [3, 4, 5], while some health problems, such as diarrhoea and wasting, have been observed in many laboratories [6]. Marmoset wasting syndrome (MWS, or wasting marmoset syndrome, WMS) is a well-known health problem endemic to captive marmoset colonies and has been documented for several decades [7, 8]. The syndrome consists of various symptoms, but diarrhoea, anorexia, and anaemia are frequently observed [9, 10]. Several causes have been suggested to explain the variable symptoms, and malnutrition is thought to be one of the important factors for the aetiology of MWS [6, 7].

In the wild, common marmosets are known to maintain highly exudativorous (i.e., highly dependent on tree exudates, such as gum) diets [11], but they also feed on a variety of food items, such as insects, fruits, and small animals [12, 13]. Among them, insects are important nutritional resources because they account for 30-70% of their diet [3]. They eat various insects, such as grasshoppers, crickets, cicadas, and cockroaches [12]. Guidelines [3, 4] recommend providing captive marmosets with complete commercial food, insects and produce (vegetables and fruits). Although insects seem to have important nutritional roles in their health, the unique

63 impact of insects on the physiological functions of marmosets has not been clarified. In the present
64 study, we aimed to determine the effect of insect feeding on captive marmosets by analysing the
65 microbiota and transcripts extracted from faecal samples.

66 The microbiota of common marmosets has been documented in several captive groups.
67 One study [14] that compared the microbiota of faecal samples from individuals with and without
68 WMS revealed differences in the abundance of only anaerobic, not aerobic, bacteria. The numbers
69 of lactobacilli were lower in the WMS group than in the non-WMS group, whereas those of
70 *Bacteroides-Fusobacteria* and *Clostridia* were higher in the WMS group than in the non-WMS
71 group. The group with a higher rate of chronic diarrhoea had a lower proportion of
72 *Bifidobacterium* than the other group, but there was no significant difference between the groups
73 in terms of the Shannon diversity (H') index [15]. Because daily feeding regimens vary at each
74 facility, the microbiota should vary accordingly. However, there are still insufficient data to
75 characterize the gut microbiota distribution of captive common marmosets. In the present study,
76 we aimed to obtain basic data on the microbiota of marmosets by a total RNA sequencing (total
77 RNA-seq) analysis method [16].

78 The total RNA-seq approach can be used to simultaneously describe microbial ecology
79 and the transcriptome. It has been widely used in studies of marine ecology [17, 18], soil microbes
80 [19, 20] and animal gut microbiota [21, 22] to obtain information from all domains of microbial

inhabitants, including eucaryotes, archaea, and bacteria, without a strong PCR bias. Additionally, this approach can describe the gene expression patterns among samples, similar to standard transcriptome analyses, by using short-read alignment tools [23, 24, 25]. Therefore, we employed total RNA-seq to describe the activities of the whole microbial community in marmoset guts under our experimental conditions.

In the present study, we evaluated the effects of enhanced insect feeding for seven days on the gut microbiota and transcriptome by comparing groups with and without insect feeding. The main advantage of analysing both microbiota and transcripts simultaneously is to understand functional characteristics that would be attributable to ecological changes in the marmoset gut microbiota. The weekly weight and daily faecal scores were recorded to monitor the general health status throughout the experimental period. RNA was extracted from faecal samples from different timepoints (before (Pre), immediately after (Post), and after (After) the experimental intervention), and DNA was then sequenced and annotated with a database for taxonomic identification. In human subjects, the microbiota was reported to dramatically modify microbiota diversity after a change in diet for five days [26]. Thus, insect feeding is thought to have a substantial impact on the physiological states of marmosets, who preferentially eat insects in wild habitats [12].

Materials and Methods

Subjects

Six healthy adult common marmosets (*Callithrix jacchus*) from a family consisting of one mother (9 y) and five offspring (one male and three females, aged 3-4 y) were used in this study. The mean weight was 460 g, with a range from 374 g to 499 g. The mother was obtained from a company (Clea, Tokyo, Japan), and the offspring were laboratory born and raised by their own parents. They were living in a cage (w 70 x d 70 x h 180 cm) vertically separated by a metal mesh plate to prevent fighting; thus, they were physically separated but visually, acoustically, and olfactorily accessible to each other. The cage was placed in a breeding room on a 12-hour light-dark cycle and maintained at 28° and 50% of the temperature and humidity, respectively. According to this housing condition, the marmosets were divided into two groups that differed in terms of the amount of insect intake per week, as described below. After the study finished, the animals were not sacrificed, as the study did not include examination of postmortem specimens.

Diets

The marmosets were fed commercially available pelleted foods (CMS-1M, Clea, Tokyo, Japan; SPS, Oriental Yeast, Tokyo, Japan) daily ad libitum in the morning and vegetables and fruits in the afternoon, in addition to a variety of food items such as yogurt,

boiled eggs, acacia gum, cottage cheese, and small dried sardines. Different probiotic preparations (*Bifidobacterium bifidum* (Biofermine), Biofermin Seiyaku, Hyogo, Japan; *Bifidobacterium animalis* subsp. *lactis* (LKM512), Meito, Tokyo, Japan; *Bifidobacterium longum* and *Bifidobacterium infantis* (LAC-B), Kowa, Aichi, Japan) were added to the meals or given orally (1/2 to 1 tablet per head) when the animals had softened faeces or diarrhoea. Until the beginning of the current study, frozen house crickets (*Acheta domestica*), which were defrosted at the time of feeding, were given to all animals in the colony once per week (usually on Wednesday).

Materials

For the insect feeding treatment, we used two different species, a house cricket (Tsukiyono farm, Gunma, Japan) and a giant mealworm (*Zophobas atratus*, Sagaraya, Kumamoto, Japan), which were commercially available and were kept frozen when they were delivered to the laboratory. They were brought back to room temperature to thaw just before feeding. These species have been reported to have similar amounts of protein, while the giant mealworm is much fattier than the cricket with higher calories [27].

Procedures

Experimental design

The family was divided into two experimental groups, each with three subjects. One group (Group IF, consisting of three offspring females) was fed one cricket and one giant mealworm per day for seven continuous days. The other group (Group C, consisting of the mother, one offspring female, and one offspring male) was fed one cricket per week in the middle of the week, which was the regular food regimen in our colony. These insects were fed manually by the caretakers to each subject during the daytime.

There were three points of faecal sampling in the study: Pre, Post, and After. Pre samples were collected just before the one-week insect feeding period in the experimental group. Post samples were collected the day after the end of insect feeding, and After samples were collected two weeks after the insect feeding treatment.

Sample preservation

To analyse the microbiota and the transcripts of the faeces from the marmosets, samples were collected from the clean stainless floor of the breeding cages within 30 minutes of defecation early in the morning (8:30-10:30 AM) when they usually frequently defecated. Three pieces of faeces were collected from each group, and the faeces were immediately put into 10 ml RNAlater (Thermo Fischer Scientific, Waltham, MA, US) and manually mixed well with a sterile spatula to homogenize them in the liquid. Using the same procedure, three tubes consisting of three faecal pieces in 10 ml RNAlater were prepared for each group at each

sampling point. The tubes were allowed to stand for 24 hours at room temperature. Then, they were stored at -80°C in a refrigerator until cDNA construction.

Faecal and insect RNA extraction, sequencing, and taxonomic annotation

Faecal RNA was purified by using the RNeasy PowerMicrobiota kit (Qiagen, Hilden, Germany). The kit was operated with an automatic system, QIAcube (Qiagen, Hilden, Germany), according to the protocol (RNA_RNeasyPowerMicrobiota_StoolOrBiosolid_IRTwithDNAse_V1.qpf) provided by the manufacturer. The concentration of RNA was measured with a Qubit 2.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). For library construction, 10 ng of obtained RNA was processed using the SMARTer Stranded RNA-seq kit for Illumina (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. After the concentration of DNA was evaluated by qPCR using the KAPA Library Quantification kit (KAPA Biosystems, Wilmington, MA, USA), the libraries were loaded onto an Illumina MiSeq sequencer and then sequenced using MiSeq Reagent kits v2 500 cycles (Illumina, San Diego, CA, USA) to obtain 250 bp paired-end reads. The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers DRA008965 and DRA008966 for the marmoset and insect microbiota, respectively.

We used a mapping-based total RNA-seq pipelines [16] to analyse both rRNA and mRNA profiles to identify the taxonomy of the microbiota and to search for their functions. The obtained raw paired-end reads were trimmed by using Trimmomatic-0.35 [28] with seed mismatch settings: palindrome clip: simple clip threshold = 5:30:7, minimum read length of 100 bp, head crop of 6 bp and a specification to remove SMARTer kit-specific adaptor sequences. Then, trimmed paired-end reads were directly mapped to the SSU rRNA database SILVA release 128 rep-set data with 99% identity by Bowtie2 [23] with local mode default condition as a "best-hit" analysis. The data were transformed to BAM format for expression analysis. Mapped reads were counted by using eXpress [29] to obtain counting data against the SSU rRNA sequence database. Count data were combined with taxonomy data provided from SILVA release 128 (taxonomy all, 99% identity, taxonomy_7_levels.txt) by R [30].

To analyse the metatranscriptome, paired-end reads were assembled by using the Trinity v2.4.0 program package [31] with paired-end mode default settings. Open reading frames (ORFs) and the encoded protein sequences were predicted using Transdecoder. LongORF script in the TransDecoder v.3.0.0 program package (https://transdecoder.github.io/). The ORF data (longest_orfs.cds) were used as the reference database for read mapping. Mapping was performed as described above for SSU rRNA analysis. Functional annotation of the identified ORFs was conducted with the Trinotate-3.0.1 program package [32]

188 (https://trinotate.github.io/). The obtained functional annotations were combined with read
189 count data by R.

190 The obtained read count data were normalized according to Love et al. [33] using the
191 TCC package in R. Additionally, SSU rRNA reads or ORFs with less than 10 mapped reads in
192 total from all samples in the original count data were excluded by R.

193 ## Data analysis

194 The general health condition of the subjects was evaluated by weight and faecal score.
195 The weight was measured once during each period of the experiment (i.e., Pre, Post, and After)
196 as a part of the weekly physical examination performed by our laboratory. The faecal scores
197 were measured daily by visual inspection of the faecal shape based on three levels (partially
198 adopted from [34]): 3 corresponded to normal faeces (solid, with little liquid), 2 corresponded to
199 loose faeces (globules with liquid but still formed), and 1 corresponded to diarrhoea (mostly
200 globules, a large amount of liquid, and partially muddy).

201 The relative abundance of the communities with normalized read counts was analysed
202 and visualized at the phylum and genus levels according to the groups (C and IF) and timing of
203 the sampling (Pre, Post, and After). To evaluate the diversity/similarity of the microbes in the
204 faecal samples, the Shannon and Chao1 indices were calculated and statistically analysed by
205 using the RStudio software environment (ver. 1.3.1093 [35]). To see the similarity relationships

206 among each condition of the groups in microbiota and transcripts, dendrograms were created by
207 the clustering function in RStudio with the unweighted pair group method with arithmetic
208 average (UPGMA) method of agglomeration using the Bray-Curtis index. For visualization of
209 the distance of similarity among the conditions in spaces, nonmetric multidimensional scaling
210 (nMDS) was conducted using the Bray-Curtis index, and the differences were statistically
211 analysed by permutational multivariate analysis of variance (PERMANOVA).

212 To visualize the phylogenetic relationships of the microbes depending on the
213 experimental conditions, we used the LEfSe program package [36] to conduct linear
214 discriminant analysis (LDA) and to make a cladogram by following the instructions published
215 online (https://huttenhower.sph.harvard.edu/galaxy/). For LDA, the LEfSe program was run
216 with the threshold of 2.0 and an alpha value of 0.05 for both ANOVA (Kruskal-Wallis) and the
217 Wilcoxon test.

218 To evaluate the changes in gene expression of microbiota and transcripts caused by the
219 insect feeding treatment, differentially expressed genes (DEGs) were analysed using a pipeline
220 ("EEE-baySeq", 37), with a false discovery rate of 5%. To obtain DEGs, the datasets with
221 groups (C and IF) and conditions (Pre, Post, and After) were divided into six groups (G1, G2,
222 G3, G4, G5, and G6). G1, G2, and G3 corresponded to the Pre, Post, and After conditions of
223 Group C, respectively. G4, G5, and G6 corresponded to those of Group IF. Because G1, G2,

G3, and G4 were the conditions in which no insect feeding was executed, while G5 and G6 included insect feeding with different timings, any difference observed in G5 and G6 was of most interest in this study. Each group was compared to "others", which included all conditions except for that group (e.g., G1 vs. others). The analysis also included the comparison among all the categories to find any differences (named "G7": G1 vs. G2 vs. G3 vs. G4 vs. G5 vs. G6). Thus, eight DEG patterns were obtained involving six categories (no DEG, DEG G1, DEG G2, DEG G3, DEG G4, DEG G5, DEG G6, DEG G7). Then, for the microbes and transcripts associated with with any of the DEG patterns, the direction (i.e., larger or smaller than those of other categories) was determined: G1 > others, G2 > others, G3>others, G4>others, G5>others, G6>others, G1 < others, G2 < others, G3 < others, G4 < others, G5 < others, and G6 < others. For G7, only DEGs where G5 or G6 ranked among the top comparisons (i.e., G5>G3>G4>G6>G1>G2, for example) were considered for further analysis (G5>anywhere, G5<anywhere, G6>anywhere, and G6<anywhere). To overview the changes among the conditions, microbes with DEGs were presented at the phylum and genus levels. To detect the possible relationships, the microbiota and transcripts with DEGs were combinatorily clustered in each DEG category by using the ComplexHeatmap package in RStudio.

Results

General health condition

The faecal scores of the group subjected to the insect feeding treatment (Group IF) decreased (i.e., increase in the frequency of loose stools) in the After condition, as shown by the bars in Fig 1, but this was not statistically significant (F (2, 54) = 1.29, p = 0.283). Although the weights of the two groups were significantly different by 2-way ANOVA (F (1, 12) = 11.78, p = 0.005), they were stable during the whole experimental period, as shown by the lack of significance both among the conditions (F (2, 12) = 0.073, p = 0.930) and between the interaction of the group and condition (F (2, 12) = 0.011, p = 0.999), as shown in the lines in Fig 1.

< Fig 1 around here >

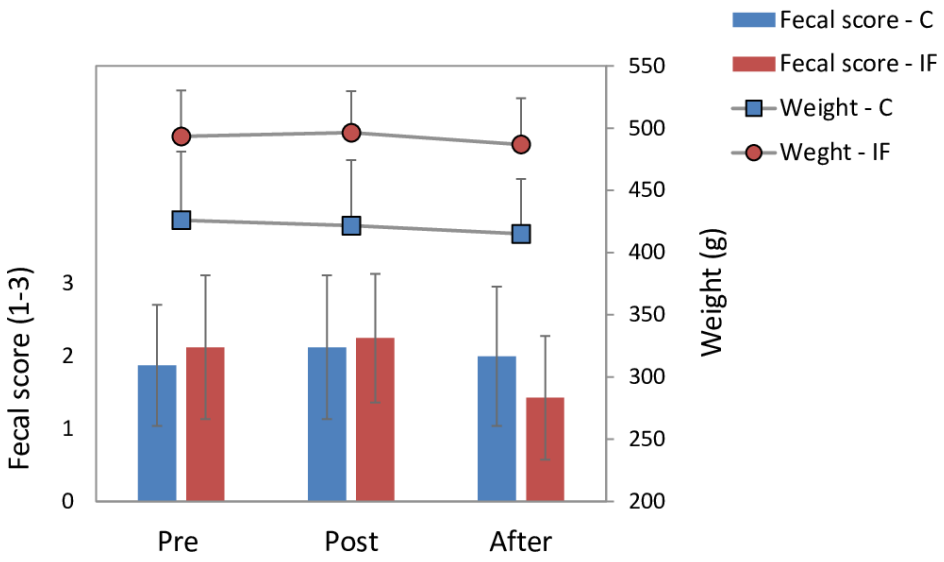

Fig 1. Faecal scores (bars) and weight (lines) during the experimental periods for Groups C (blue) and IF (red). Error bars show the standard deviation of the mean from three subjects. The faecal scores were recorded daily, and the weight was recorded individually once during each period.

252

253 ## Whole microbiota community of the marmoset gut

254 The total number of read counts normalized against the SSU rRNA sequences
255 generated from the 18 faecal samples of the six common marmosets at the Pre, Post, and After
256 conditions were 355,905.34, with an average of 19,772.52 counts per sample (see Supporting
257 Information of S1 Table for the normalized count data of the annotated microbes). The
258 difference in normalized read counts per sample between Groups C and IF was not significant (t
259 (16) = -0.16, p = 0.88, mean ± standard deviation (SD) for Group C: 65.66 ± 2.63; Group IF:
260 66.03 ± 6.49).

261 Fig 2a shows the relative abundance of the microbiota at the phylum level for each
262 group across the conditions. The phyla accounting for more than 0.5% of the total read count
263 were listed, and those accounting for less than 0.5% and unable to be assigned to any phylum
264 were categorized as "others". *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*
265 were abundant in every sample of both groups. By observing the same data at the genus level,
266 with more than 0.5% abundance, as shown in Fig 2b, *Veillonellaceae (*phylum *Firmicutes)* and
267 *Bifidobacteriaceae* (phylum *Actinobacteria*) were dominant under all conditions.

268

269 <Fig 2 around here>

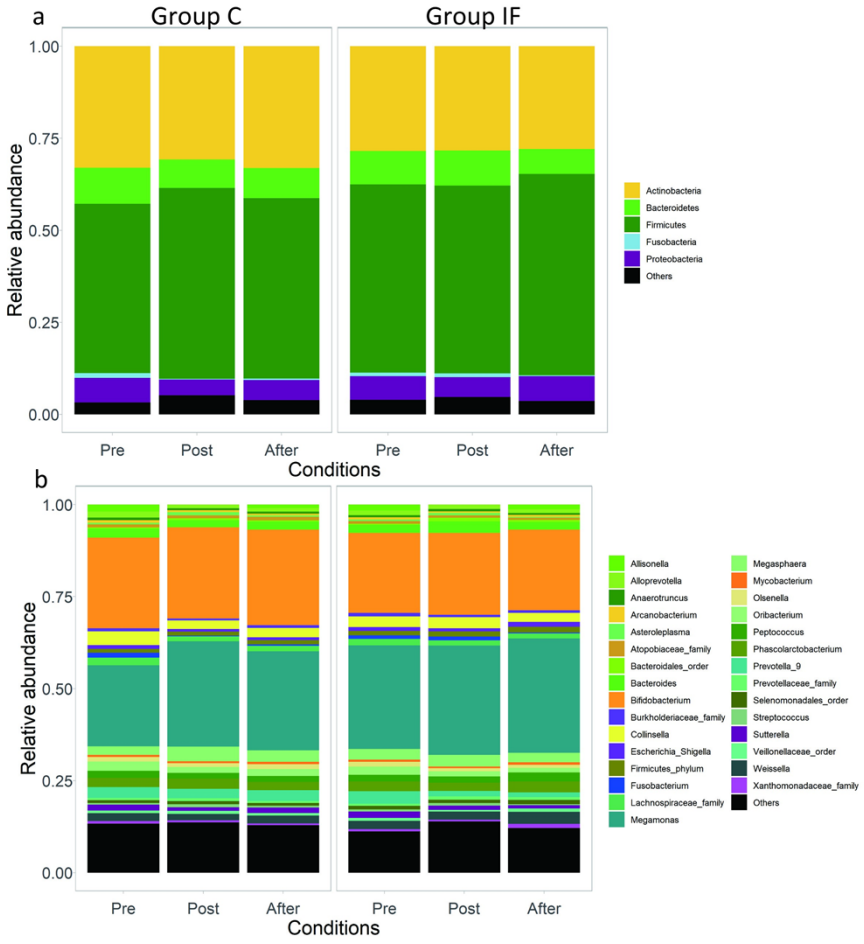

Fig 2. Relative abundance of microbes at the phylum (a) and genus (b) levels for Groups C (left) and IF (right) under the experimental conditions (Pre, Post, and After).

Microbial diversity under each condition

Shannon and Chao1 indices were used to determine the diversity of the microbial communities within the samples of each group, as shown in Fig 3. ANOVA of the Shannon indices after applying the general linear model (GLM) revealed a significant difference among the conditions (Pre, Post, After, $F = 7.065$, $p = 0.008$) but not between the groups ($F = 0.292$, $p = 0.597$). Similar

276 results were obtained using the Chao1 index with the GLM (conditions: F=7.092, p = 0.007;

277 groups: F=1.051, p = 0.323).

278 <Fig 3 around here>

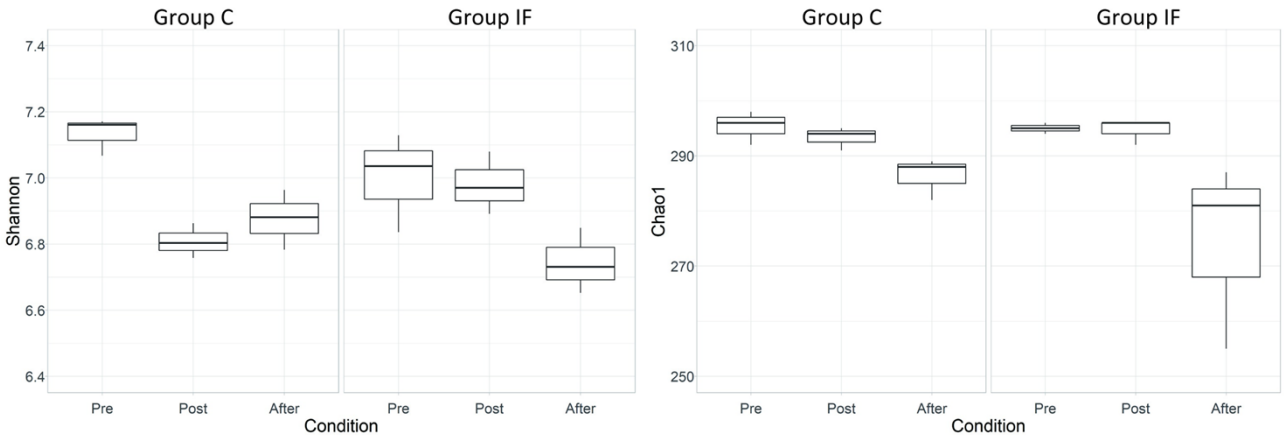

Fig 3. Shannon (left) and Chao1 (right) indices used to visualize the diversity within the samples of each group for the three conditions (left for Group C and right for Group IF in each panel, respectively).

279

280 Similarity of the microbial communities among the conditions

281 To determine the similarities of the microbial communities among the groups, the Bray Curtis

282 index was calculated and used to generate the cluster dendrogram among the conditions of each

283 group (Fig 4a), together with the differentially coloured squares and lines for individual

284 conditions of two groups. For microbiota clustering, the Post conditions of Group IF (yellow

285 solid line squares) were closely agglomerated.

286 <Fig 4 around here>

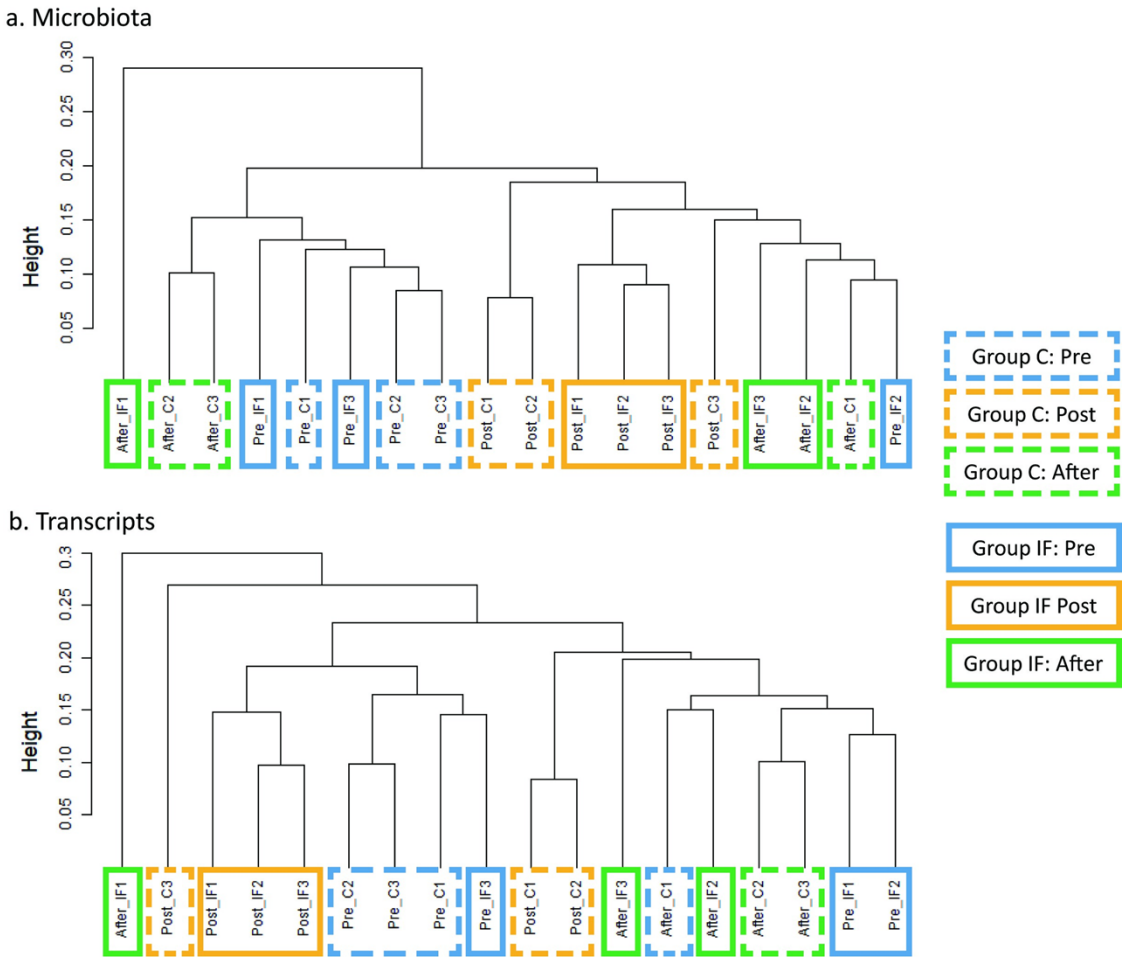

Fig 4. Cluster dendrograms for microbiota (a) and transcripts (b). Pre, Post, and After conditions are differentially coloured (sky-blue, yellow, and green), with dotted and solid lines for Groups C and IF, respectively.

287

288 To spatially visualize the microbiota similarities, nMDS was applied, and the data

289 from each condition were shown in 2D space, as depicted in Fig 5a. To determine the effect of

290 insect feeding, PERMANOVA was performed after dividing the individuals into three groups:

291 (1) no insect feeding (three conditions for Group C and the Pre condition for Group IF), (2) Post

292 of Group IF, and (3) After of Group IF, and significant differences were found among the three

293 groups (F = 3.070, p = 0.005).

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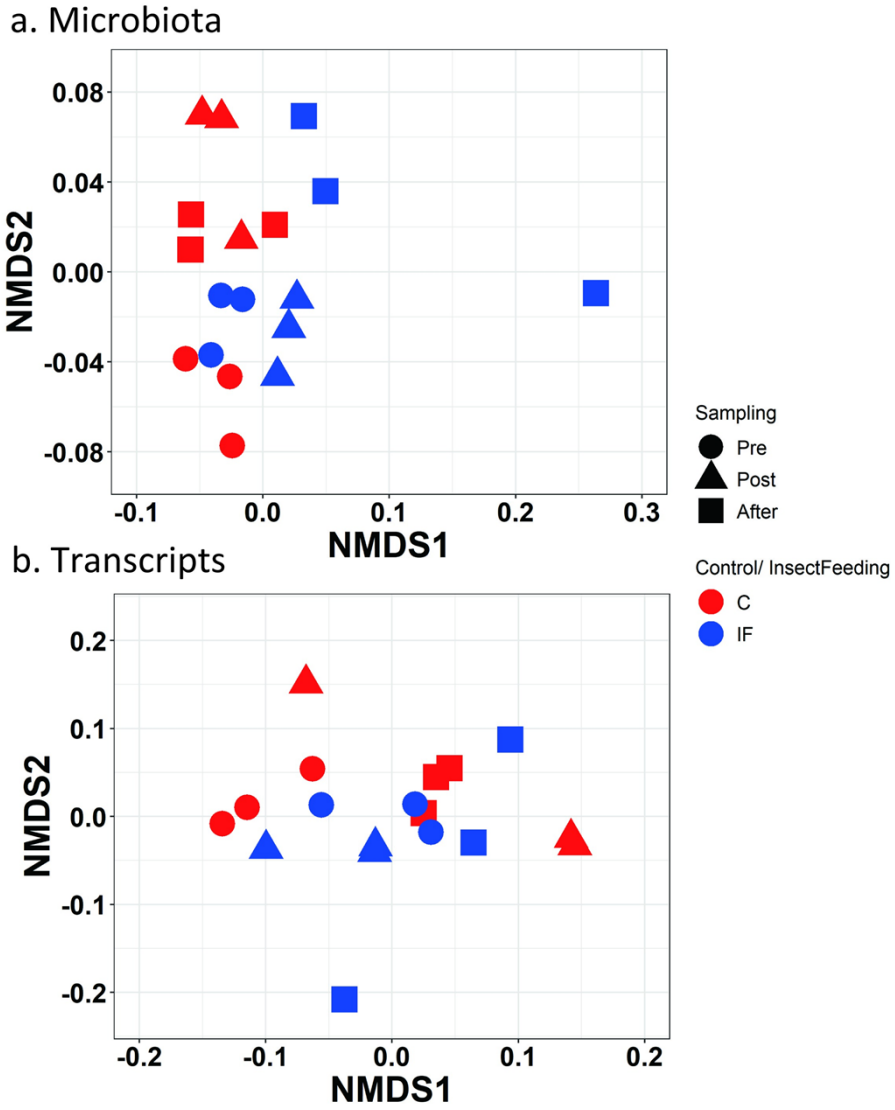

Fig 5. nMDS of the microbiota (a) and transcripts (b) data under the Pre (circle), Post (triangle), and After (square) conditions for Group C (red) and IF (blue).

295

Phylogenetic characterization of differentially abundant microbiota with and without insect feeding treatment

To characterize the specific microbes that emerged from the insect feeding treatment, we used the LEfSe analytical tool (see methods) on the normalized counts at multiple levels of taxonomy. The histogram in Fig 6a shows the LDA scores above the threshold (2.0) for the microbiota on various taxonomic levels ranked according to the effect size (alpha = 0.05) for the treatments with (IF: Post and After for Group IF) and without (NoIF: all conditions for Group C and Pre for Group IF) insect feeding. With IF, 5 taxonomic clades (three from the phylum *Proteobacteria* and two from *Bacteroidetes*) were differentially abundant. With NoIF, 8 taxonomic clades were detected by LDA (two from the phylum *Actinobacteria*, two from *Bacteroidetes*, and four from *Firmicutes*). Fig 6b shows the microbial abundance and taxonomic clades with differential characteristics for each treatment. The cladogram shows that abundant bacterial communities characteristic of IF were phylogenetically separatable from those characteristic of NoIF, as depicted by the red and green sectors without overlap.

<Fig 6 around here>

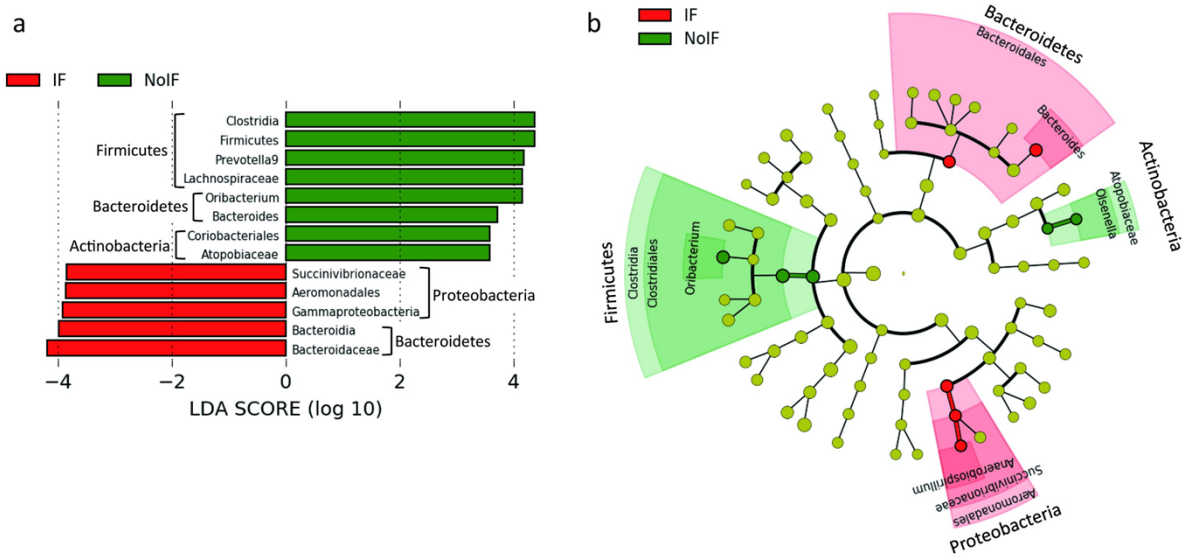

Fig 6. LEfSe characterization of the dominant microbial taxa according to the treatments with (IF: Post and After for Group IF) or without (NoIF: all conditions for Group C and Pre for Group IF) insect feeding. (a) LDA scores, above the threshold 2, of the microbial clades for each treatment, Insect feeding (red) and No insect feeding (green). (b) Cladogram based on the ranked list in (a) to visualize the relationships between the treatments and the phylogenetic relationships among the microbes. Phylogenetic clades are ordered from the centre of the circle, with narrower to broader taxonomic levels. Diameters of outer circles correspond to the relative abundance in the microbial community. Red and green points in the circles show the most abundant classes under the IF and NoIF treatments, respectively. Points in light green show the clades that are not significant.

312

313 ## Differentially expressed genes in microbiota among the conditions

314 The microbial communities fluctuated even without insect feeding treatment. Thus, to clarify

315 the microbes specifically changed in Group IF depending on the conditions, the DEGs were

316 analysed using the community data. Among 300 microbes annotated by the analysis, DEGs

317 were confirmed for 99 of them. The relative distribution of DEG categories is presented in Fig

318 7a. Of 99 microbes with DEGs, a total of 21.21% were upregulated in terms of the experimental

319 conditions (G5>others, G5>anywhere, G6>others, G6>anywhere), whereas a total of 20.20%

320 were downregulated (G5<others, G6<others). No DEGs were found for the G3<>others,

321 G5<anywhere, and G6<anywhere categories.

322 <Fig 7 around here>

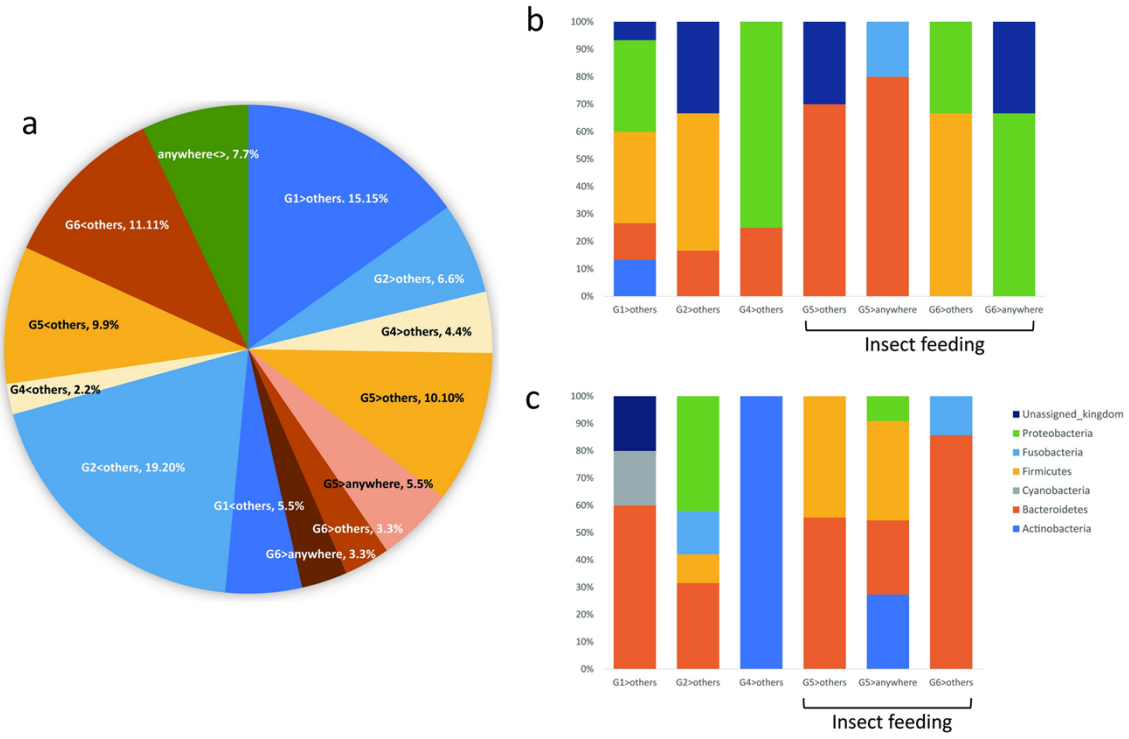

Fig 7. Distribution of DEG categories found in microbiota. (a) Relative distribution of the DEG categories. (b) Relative distribution of microbes in each DEG category at the phylum level, showing upregulated changes. Categories under the insect-feeding treatment were G5>others, G5>anywhere, G6>others, and G6>anywhere. (c) Relative distribution of microbes in each DEG category at the phylum level, showing downregulated changes. Categories under the insect-feeding treatment were G5>others, G5>anywhere, and G6>others.

323

Ninety-nine microbes with DEGs were further classified into phyla. Fig 7b and 7c show the microbes showing upregulated and downregulated changes with each DEG category, respectively. Among the upregulated phyla shown in Fig 7b, the distribution of *Bacteroidetes* was different under the Post condition of Group IF (i.e., G5>others and G5>anywhere). On the other hand, among the downregulated phyla in Fig 7c, Firmicutes showed different distributions in the same categories.

Fig 8 shows the normalized read counts of the microbes at the phylum level according to the DEG categories. The left two rows show upregulation, and the right row shows downregulation. In the case of the Post condition of Group IF (G5), *Bacteroidetes* appeared in both upregulated (Fig 8d, 8f) and downregulated (Fig 8k) categories, whereas *Firmicutes* only showed downregulated DEGs (Fig 8k). In the case of the After condition of Group IF (G6), *Actinobacteria* and *Bacteroidetes* only showed downregulated DEGs (Fig 8l), whereas *Firmicutes* and *Proteobacteria* showed both the upregulated (Fig 8e) and downregulated (Fig 8l) categories.

<Fig 8 around here>

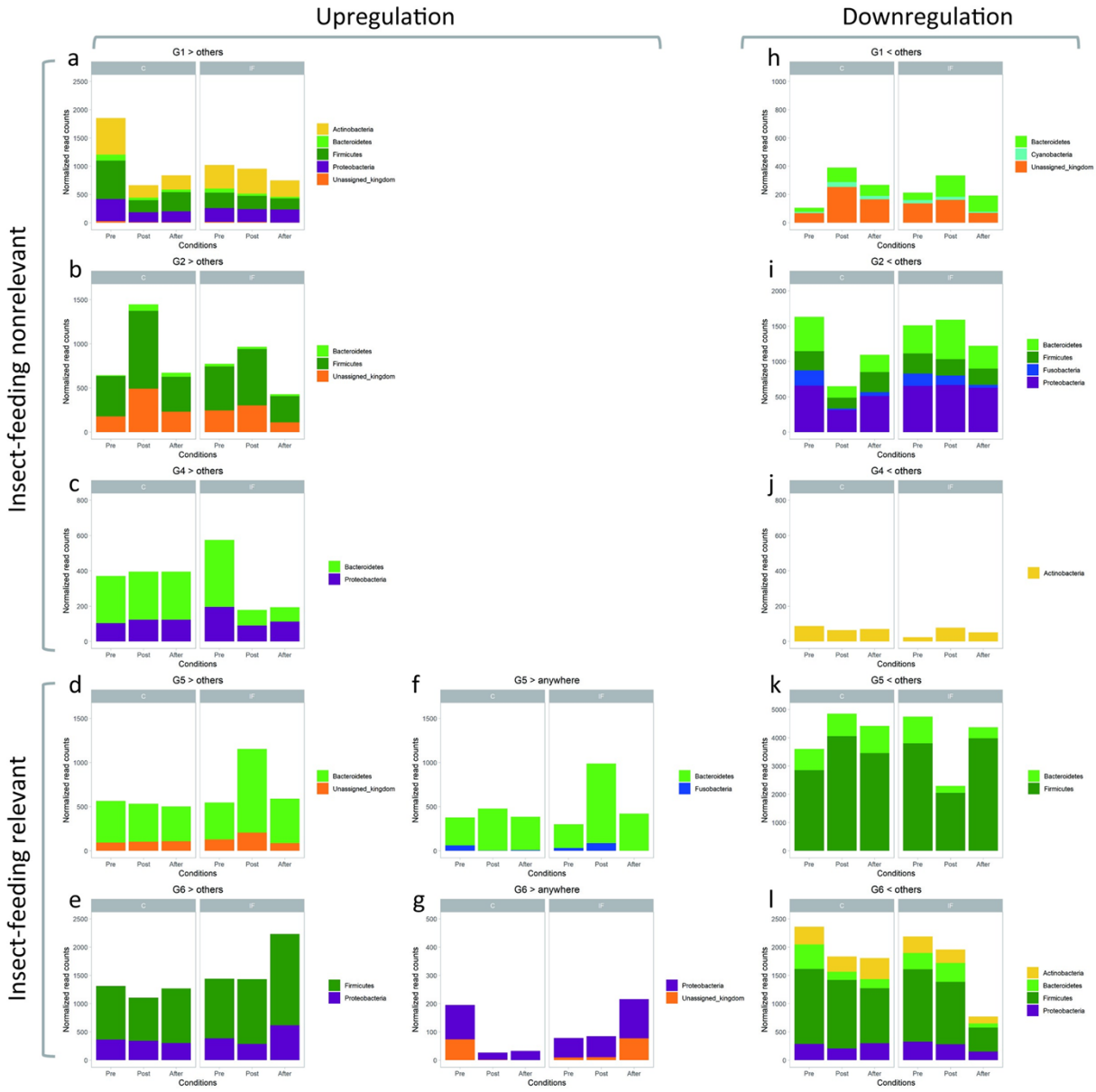

Fig 8. Normalized read counts of the microbes identified by the differentially expressed gene (DEG) analysis of faecal SSU rRNA at the phylum level. In each panel, the left and the right three bars show Pre, Post, and After conditions for Group C and Group IF, respectively. Black arrows in the panels indicate the groups which have DEGs compared with the other groups. (a-g) Upregulated DEGs, with insect-feeding nonrelevant (a, b, c) and insect-feeding relevant (d, e, f, g) conditions. (h-l) Downregulated DEGs with insect-feeding nonrelevant (h, i, j) and insect-feeding relevant (k, l) conditions. No DEG was found under the After condition in Group C (G3). For f, and g, data for each microbiota were compared with those of the other groups separately. In other graphs, data for each microbiota were compared with the total of the other groups.

341

342

343 To examine the changes according to the conditions (Pre, Post, and After) separately
344 in the groups, Fig 9 shows the heatmap of the microbes at the genus level (for microbes unable
345 to be annotated at the genus level, upper taxonomies (i.e., order, family) were assigned) with
346 DEGs. The blue squares indicate the results relevant to the insect feeding treatment (Post (G5)
347 and After (G6) conditions). In the upregulated category of the Post condition (G5>others,
348 G5>anywhere), the genera *Bacteroides*, *Parabacteroides*, *Prevotella9* (phylum *Bacteroidetes*)
349 and *Fusobacterium* (*Fusobacteria*) were listed, whereas in the After condition (G6>others,
350 G6>anywhere), the genera *Weissella* (*Firmicutes*) and *Escherichia-Shigella* (*Proteobacteria*)
351 were listed as having DEGs. In the downregulated category of the Post condition (G5<others),
352 the genera *Bacteroides* (*Bacteroidetes*), *Allisonella, Megamonas,* and *Weissella* (*Firmicutes)*
353 were found to have DEGs. In the After condition (G6<others), the genera were *Collinsella,*
354 *Olsenella* (*Actinobacteria*), *Alloprevotella, Parabacteroides* (*Bacteroidetes*), *Streptococcus,*
355 *Erysipelotrichaceae UCG-004, Oribacterium* (*Firmicutes*), and *Sutterella* (*Proteobacteria*).

356

357 <Fig 9 around here>

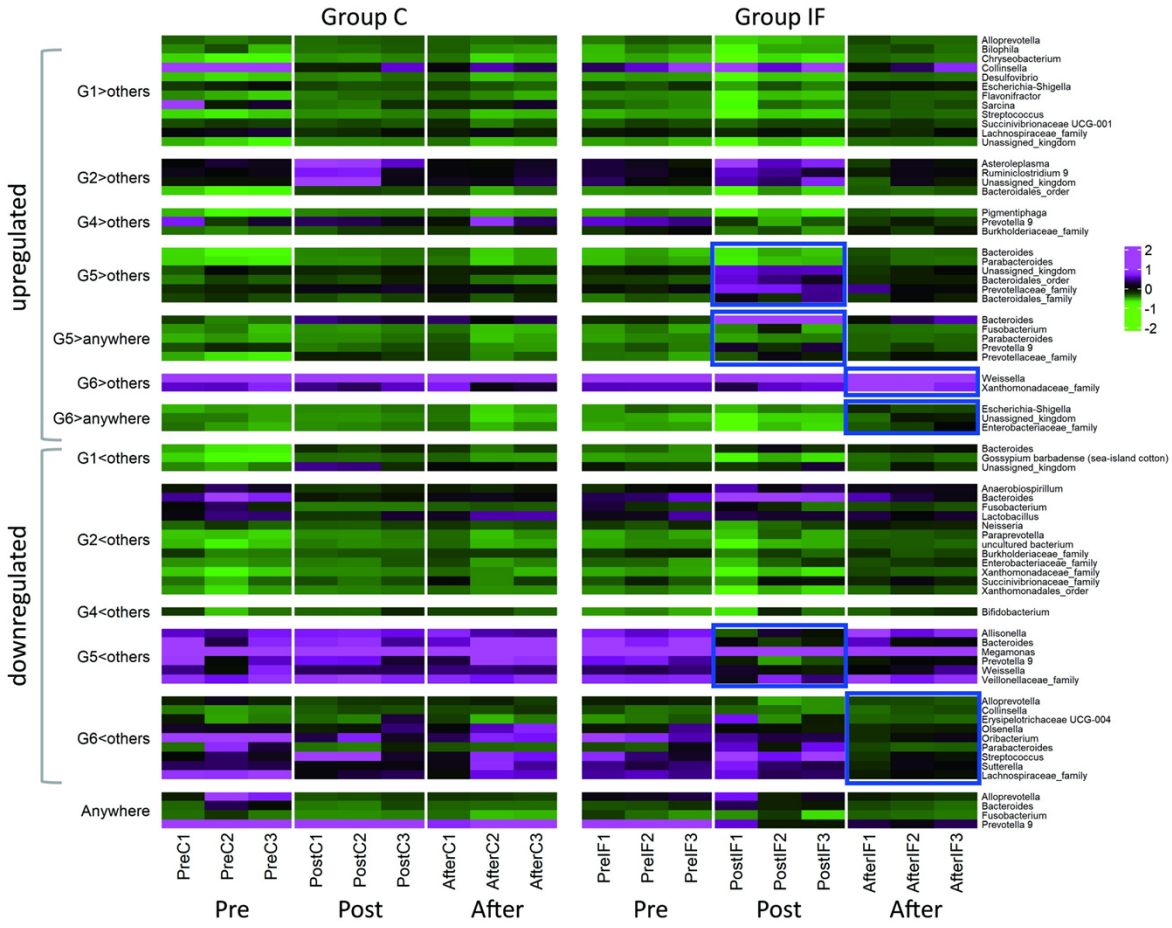

Fig 9. Heatmap of the SSU rRNA data (genus level) with DEGs. Groups C and IF are presented on left and right of the panel, respectively. For each group, the upper and lower parts present the data from upregulated and downregulated genera, respectively. G1: Pre condition for Group C; G2: Post condition for Group C; G4: Pre condition for Group IF, G5: Post condition for Group IF; G6: After condition for Group IF; AW: DEGs from any comparisons among the conditions. G3 is not presented because DEGs were not found under this condition (After condition of Group C). The heatmap with blue squares on the right indicates the changes in microbiota in accordance with the experimental treatments (i.e., G5 and G6 of Group IF).

358

359 LEfSe (Fig 6) revealed some genera with differential changes relevant to the insect

360 feeding treatment, and thus we could identify the specific microbes of those genera by

361 combining the results with the DEG results (i.e., selecting the DEG (G5 and G6) microbes in the
362 genera found to be treatment-relevant by LEfSe). Fig 10 shows the normalized counts of such
363 microbes from the genera *Bacteroides* (phylum *Bacteroidetes*), *Olsenella*, (phylum
364 Actinobacteria) and *Oribacterium* (phylum *Firmicutes*). In *Bacteroides* (Fig 10a and 10b),
365 there were microbes showing both upregulation and downregulation, but they were all relevant
366 to the Post condition of Group IF (i.e., G5). On the other hand, the microbes in *Oribacterium*
367 and *Olsenella* showed downregulation relevant to the After condition of Group IF (i.e., G6, Fig
368 10c and 10d, respectively).

369 <Fig 10 around here>

370

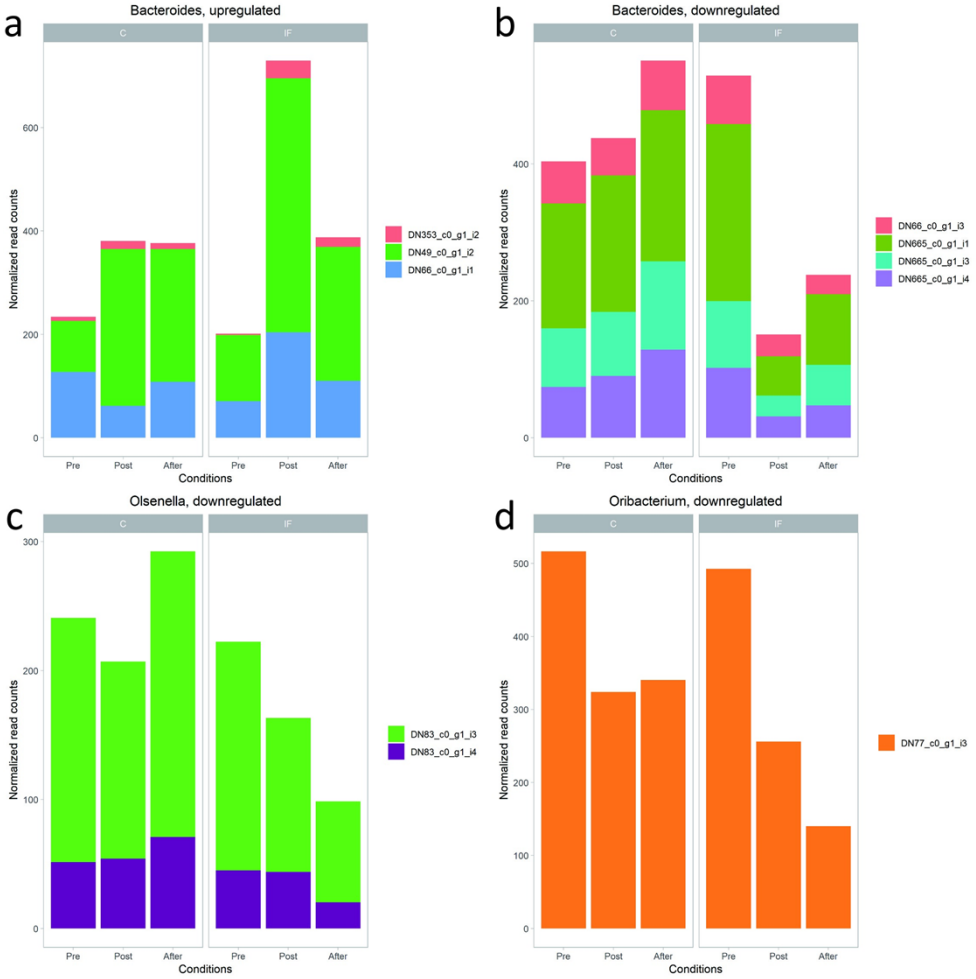

Fig 10. Results overlapped from the LEfSe and DEG analysis, showing the microbes specifically expressed just after the insect feeding treatment (a, b) and two weeks after the treatment (c, d), indicated by the black arrows. (a) Upregulation found under the Post condition in three microbes of the genus *Bacteroides*. (b) Downregulation found under the Post condition in four microbes of the genus *Bacteroides*. (c) Downregulation found under the After condition in two microbes of the genus *Olsenella*. (d) Downregulation found under the After condition in a microbe of the genus *Oribacterium*.

371

372 # Transcriptomes of groups IF and C

373 Altogether, 407 different transcript IDs were classified by BLASTP (see Supporting

374 information of S2 Table for normalized count data of transcripts). Among those, 72 were

375 analysed with DEGs, among which 83% were classified as “hypothetical proteins” from various
376 bacteria (Fig 11a). By classifying those proteins with an e-value above 1.0e+8, it was found that
377 transcripts originating from *Bacteroidetes* and *Firmicutes* were abundant, as shown in Fig 11b
378 and 11c. In the case of insect feeding-relevant conditions, both *Bacteroidetes* and unclassified
379 bacteria were abundant in both upregulated (G5>others) and downregulated (G5<others)
380 categories. *Firmicutes* characteristically increased in the downregulated category under the
381 After condition (G6<others). *Proteobacteria* appeared only in the upregulated category under
382 the After condition (G6>others).

383 <Fig 11 around here>

384

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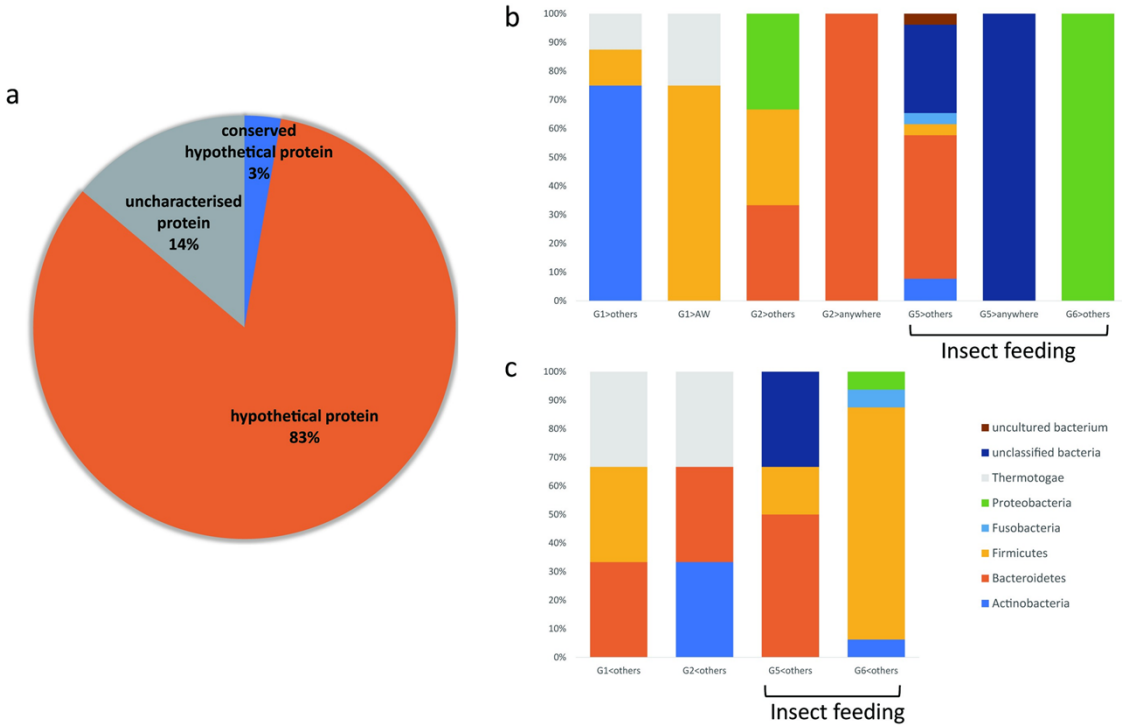


Fig 11. Relative distribution of DEG categories found in transcripts. (a) Relative distribution of transcript functions of DEGs. (b) Relative distribution of transcripts in each DEG category at the phylum level, showing upregulated changes. Categories under the insect-feeding treatments were G5>others, G5>anywhere, and G6>others. (c) Relative distribution of transcripts in each DEG category at the phylum level, showing downregulated changes. Categories under the insect-feeding treatment were G5>others and G6>others.

386

387 # Relationship of the changes between the microbiota and

388 # transcriptome

389 The functional significance of transcripts was evaluated by analysing the similarity of

390 changes between the microbiota and transcriptome and whether such changes closely interacted

391 with each other according to the experimental conditions. Thus, we performed clustering

392 analysis with Pearson's product moment correlation coefficient among the SSU rRNA and

393 transcriptome data with DEGs within 6 categories. Fig 12 shows the heatmap of the changes in
394 the microbiota and transcriptome listed together in each DEG category, according to the
395 experimental period (indicated on the bottom of the heatmap) in Groups C (left) and IF (right).
396 The dendrogram located on the right of the heatmap shows the results of Group IF. Note that the
397 microbiota and transcriptomes that behaved similarly were near each other in the heat map with
398 a short clustering distance. The blue squares indicate the results relevant to the insect feeding
399 treatment. In the case of the upregulated categories, microbes and transcripts tended to cluster
400 separately. In the case of the downregulated categories, especially G6<others, they were more
401 intermixed.
402
403 <Fig 12 around here>

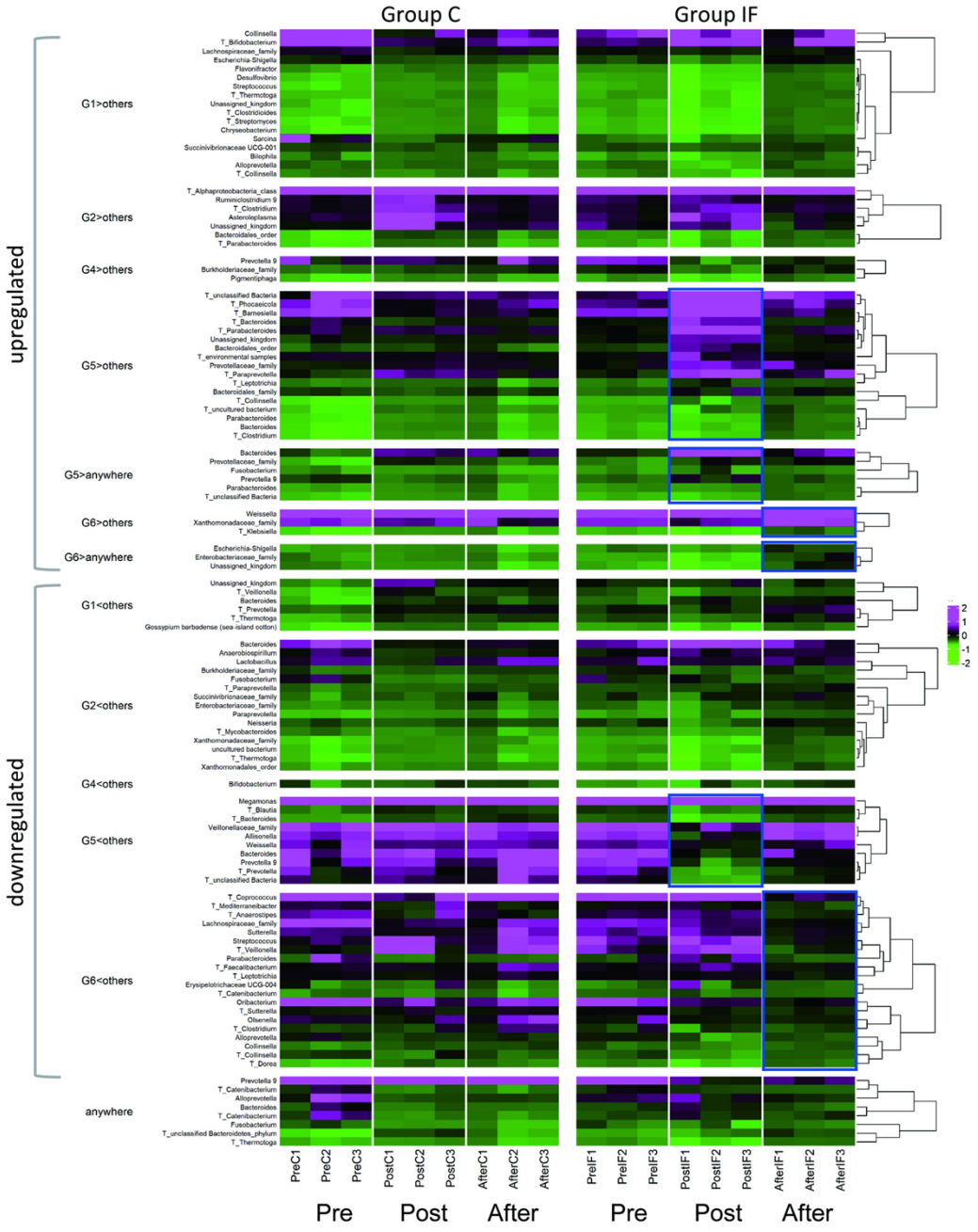


Fig 12. Heatmap with clustering among the SSU rRNA (genus level) and annotated transcripts with DEGs. Transcript data are presented with T with the genera name of the originating microbes. Increases and decreases are shown in magenta and green, respectively. Hierarchical clustering enabled items from the microbiota and transcriptome data to be listed together, where items with similar characteristics were listed close to each other. The data relevant to the experimental treatment (insect feeding) are emphasized by the blue squares. Upregulated and downregulated DEGs are located upper and lower panels of the figure, respectively.

Microbiota and transcripts of the insects fed to the marmosets

To determine whether the significant changes in the abundance of the microbial community in the samples of Group IF after insect feeding were attributable to the insects themselves fed to the marmosets, we analysed the microbiota of crickets and mealworms from the same lot as those fed to the subjects, using the same protocol described in the methods (see Supporting information of S3 and S4 Tables for normalized count data of the microbes and transcripts, respectively). As shown in Fig 13, abundant microbial phyla accounting for more than 0.5% of the total reads were *Opisthokonta* (eucaryotes), *Archaeplastida* (eucaryotes), and *Firmicutes* for the crickets and *Opisthokonta*, *Archaeplastida*, *Firmicutes*, *Proteobacteria*, and *Cyanobacteria* for the meal worms. The relative abundance of the kingdom *Bacteria* was 1.09 and 7.26% of the total reads from the crickets and worms, respectively, while it was 96.38% of the total reads from the faecal samples of marmosets. All the remaining microbes were in the kingdom *Eukaryota* (98.91% and 92.04% for crickets and mealworms, respectively), which was 0.28% in the case of the marmoset sample.

<Fig 13 around here>

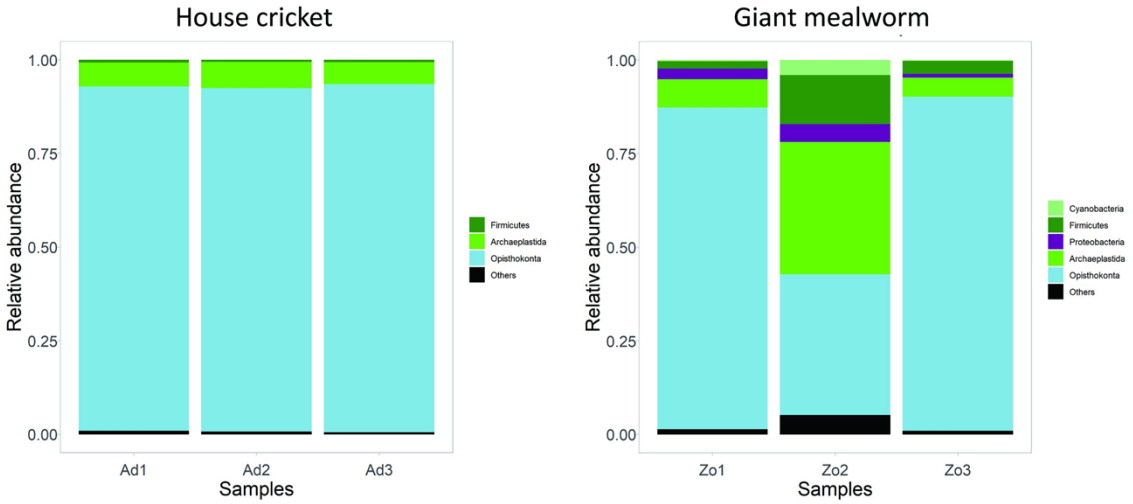


Fig 13. Relative abundance of microbes at the phylum level for house cricket (left) and giant meal worm (right).

421

422 # Discussion

423 The present study described the characteristics of the gut microbiota of captive

424 common marmosets by using the total RNA sequencing method, with *Firmicutes*,

425 *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* as dominant phyla. Then, we showed that

426 enhanced insect intake for only one week modified the microbiota population in the gut, which

427 interacted with the transcripts simultaneously extracted from the faecal samples. Changes

428 observed in the microbiota were not attributable to the insects themselves. More specifically,

429 microbes in the phyla *Bacteroidetes* and *Firmicutes* showed corresponding changes in their

430 abundance under the insect feeding treatment at the different sampling points. *Bacteroidetes*

431 showed both an increase and decrease upon just finishing the treatment (Post condition),

followed by a decrease after two weeks (After condition), while *Firmicutes* showed a decrease at the Post condition, followed by both an increase and decrease at the After condition. These results corresponded well to the changes in the abundance of transcripts having the same homologous phyla of origin. Overall, the current study indicated that a partial change in the diet for seven days had an impact on the host marmosets' microbiota and that insect feeding naturally observed in wild populations of common marmosets has special roles.

Treatment-relevant changes observed in the microbes belonging to the phyla *Bacteroidetes* and *Firmicutes*

Treatment with insect feeding differentially affected the marmosets' gut microbiota at different times. As indicated by LEfSe (Fig 6), sets of microbes in the phylum *Bacteroidetes* appeared in both categories, whereas those of the phyla *Proteobacteria* (insect feeding) and *Actinobacteria* and *Firmicutes* (no insect feeding) appeared in either of the categories. Additionally, DEG analysis with 6 patterns showed the exact changes according to the groups and conditions (Figs 8 and 9). Because the phylum Bacteroidetes showed both an increase and a decrease under Post conditions and a decrease under After conditions and the phylum *Firmicutes* showed a decrease under Post conditions and an increase and decrease under After conditions, one possible interpretation of these results would be that *Bacteroidetes* and *Firmicutes* had opposite responses to insect feeding. That is, microbes in those phyla may have

shown rapid changes corresponding to the availability of insects. Adding one more sampling point during the insect feeding treatment would clarify the above possibility, which was not examined in the current study.

The results from the studies on other species treated with animal-concentrated diets would be comparable to those obtained in the current experiment. In a human study, after taking an animal-based diet for five days, an increase was found in species belonging to the genus *Bacteroides*, whereas a decrease was found in those of *Firmicutes,* but there were some species belonging to *Firmicutes* that showed increased in abundance after the treatment [26]. This study also detected an increase in the abundance of bacteria in the phylum *Proteobacteria*, including the species *Bilophila wadsworthia*, which is known to be stimulated by increased bile acid responsible for fat intake [38]. In our study, both an increase and a decrease in the abundance of *Proteobacteria* were observed under After, not Post conditions (Figs 8e, 8g, and 8l), suggesting a gradual change in the metabolism related to bile acid in the gut of the host. A previous study on dogs fed a raw meat diet for 14 days [39] observed 7 genera showing an increase after the treatment, and only the genus *Bacteroides* was in common with our current study findings. Chickens fed *Tenebrio molitor* larvae for 54 days showed an increase in *Firmicutes* and a decrease in *Bacteroidetes* at the phylum level [40], which corresponded to some of our results (Figs 8d, 8f, and 8e).

468

469 # General impact of insect feeding on the gut

470 The abundances at the phylum and genus levels, together with two indices of α
471 diversity (Shannon and Chao1 indices), did not differ from each other between Groups C and
472 IF. At the macroscopic level, insect feeding for seven days did not affect the general community
473 of the intestinal microbiota, which was in common with the results of human adults treated with
474 25 g cricket powder per day for 14 days [41]. However, after focusing on the pattern of the
475 changes corresponding to the experimental treatment by calculating the β diversity, there were
476 significant differences between the conditions with and without insect feeding. The same
477 patterns, no difference in α diversity but a significant difference in β diversity, were observed in
478 a study on human subjects treated for 5 days with an animal-based diet [26]. A study examining
479 the effects of an animal-based diet on the microbiota in dogs reported that faeces became firm
480 and that the Shannon H' index increased after raw beef was added to commercial food for 14
481 days [39]. The reason for the lack of a significant change in α diversity indices (Shannon and
482 Chao1) after insect feeding in the current study would partially be attributable to unexpected
483 fluctuations observed in the samples of Group C. Thus, evaluation of the stability of the
484 microbial community would be necessary to compare the effects of short-term intervention
485 (seven days) by taking additional samples in the Pre period, for example.

For microbiota, the samples just after the insect feeding treatment were clearly distinguishable from other samples, as shown in Fig 4a. By examining the distances among the samples, conditions with insect feeding (Post and After conditions of Group IF) were closely grouped and more distance from the other samples. Additionally, as the results of the PERMANOVA indicated, there were also differences between the Post and After conditions of Group IF, suggesting that changes caused by the insect feeding treatment had impacts on microbiota, which were long lasting for the bacterial community even after the treatment ended. On the other hand, PERMANOVA did not detect a significant difference in the transcript data between the conditions with and without insect feeding, although the samples under the Post conditions were closely clustered in Fig 4b, and the plots were separately located in Fig 5b. The reason for this difference between the microbiota and transcriptome was not identified by the current results, so some possibilities, such as length of the treatment and quantity of the fed insects, need to be examined to determine the changes in the transcripts.

Limitations and future perspectives of the study

Our analysis using total RNA-seq, which could concurrently detect the dynamics of the microbiota and transcripts, was effective in searching for functional genes that are currently unidentifiable after establishing a metagenomic database of the transcriptome with a full-length cDNA library. However, the functional significance of the transcriptomes could only be inferred

by the microbiota that showed similar changes across the experimental conditions. In the next step, we need to capture a wider view that would integrate the changes in microbiota, transcripts, and host responses to understand the effect of feeding insects, considering that the subjects have a long history of foraging.

In the present study, we used frozen crickets and giant mealworms instead of live mealworms. The results might have been different from those obtained in the study using live insects. The use of live insects as a feeding regimen is also recommended for marmosets in terms of enrichment purposes [4]. Nevertheless, the present study showed that feeding common marmoset insects changes their physiological status by balancing the microbiota to modulate metabolites. Consideration must be given to how much and what types of insects we should feed captive marmosets because there is a risk of overeating in the breeding cages but not in bushes in the wild. For example, some insect larvae are rich in fat and lack calcium, and it is therefore recommended to feed the insects a high calcium diet before feeding the insects to marmosets [3]. Insects with a high phosphorous-calcium rate should not be provided in abundance to prevent the malabsorption of calcium [4]. Thus, further studies are clearly needed to determine the long-term effect of insect feeding in captive animals and what types of insects are most beneficial for their health while simultaneously monitoring the changes in the faecal microbiota and transcriptome.

Conclusions

The present study showed that adding insects to the regular food regimen for seven days could have a distinct effect on the microbiota and transcripts of captive common marmosets. The total RNA-seq method was used to analyse the microbiota and transcripts simultaneously, and the correlational analysis suggested that they did interact with each other. Thus, enhanced insect feeding could activate the physiological dynamics that have been evolutionarily developed in this species in wild habitats. The obtained results help us to understand the interaction between the host and the microbiota via food sources and suggest that the feeding ecology in wild habitats is an important key to developing food regimens appropriate for the microbiota of common marmosets.

List of abbreviations

ANOVA: analysis of variance

Group C: control group.

Group IF: insect feeding group

LDA: linear discriminant analysis

LEfSe: linear discriminant analysis effect size

MWS: marmoset wasting syndrome

ORF: open reading frame

PERMANOVA: permutational multivariate analysis of variance

SSU rRNA: small subunit ribosomal ribonucleic acid

total RNA-seq: total RNA sequencing

WMS: wasting marmoset syndrome

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Experiment Committees at the RIKEN Brain Science Institute (H27-2-203) and was conducted in accordance with the Guidelines for Conducting Animal Experiments of the RIKEN Brain Science Institute. Consent to participate was not applicable to the study.

Consent for publication

N/A

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

Authors declare no competing financial interests.

Funding

Grants of Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) at RIKEN from Japan Agency for Medical Research and Development (JP18dm0207001) and the Science Research Promotion Fund for Private University at Azabu University from the Ministry of Education, Culture, Sports, Science, and Technology of Japan

supported the purchase of the materials for RNA extraction and preservation of faecal samples and the costs of sequencing.

Authors' contributions

Y.Y., S.M., and S.K. designed and executed the experiments, analysed the data, wrote the main manuscript text, and prepared the Figs. S.M. developed and applied the total RNA-seq pipeline for analysing microbiota and transcriptome data. S.K. supervised the health status of the subjects; H. M, T. K, and A.I. coordinated manuscript writing. All authors reviewed the manuscript.

Acknowledgements

N/A

References

1. Sasaki E, Suemizu H, Shimada A, Hanazawa K, Oiwa R, Kamioka M, et al. Generation of transgenic non-human primates with germline transmission. Nature 2009 May 28;459(7246):523-527.

2. 't Hart BA, Abbott DH, Nakamura K, Fuchs E. The marmoset monkey: a multi-purpose preclinical and translational model of human biology and disease. Drug Discovery Today 2012 November 2012;17(21):1160-1165.

583 3. National RC. Nutrient requirements of nonhuman primates: second revised edition.

584 Washington, DC: The National Academies Press; 2003.

585 4. Bairrão Ruivo E ed.: EAZA Best practice guidelines for Callitrichidae – 3.1 Edition. 3.1st

586 ed.: Beauval Zoo; 2017.

587 5. Rensing S, Oerke A. CHAPTER 10 - Husbandry and management of New World Species:

588 marmosets and tamarins. In: Sonia W, editor. The Laboratory Primate London: Academic Press;

589 2005. p. 145-162.

590 6. Cabana F, Maguire R, Hsu CD, Plowman A. Identification of possible nutritional and stress

591 risk factors in the development of marmoset wasting syndrome. Zoo Biol 2018 Mar;37(2):98-

592 106.

593 7. Shimwell M, Warrington BF, Fowler JS. Dietary habits relating to 'wasting marmoset

594 syndrome' (WMS). Lab Anim 1979 Apr;13(2):139-142.

595 8. Tucker MJ. A survey of the pathology of marmosets (*Callithrix jacchus*) under experiment.

596 Lab Anim 1984 Oct;18(4):351-358.

597 9. Chalmers DT, Murgatroyd LB, Wadsworth PF. A survey of the pathology of marmosets

598 (*Callithrix jacchus*) derived from a marmoset breeding unit. Lab Anim 1983 Oct;17(4):270-279.

599 10. Juan-Salles C, Prats N, Resendes A, Domingo M, Hilton D, Ruiz JM, et al. Anemia,

600 myopathy, and pansteatitis in vitamin E-deficient captive marmosets (*Callithrix spp*.). Vet

601 Pathol 2003 Sep;40(5):540-547.

602 11. Ferrari SF. Ecological differenciation in the Callitrichidae. In: Rylands AB, editor.

603 Marmosets and tamarins: systematics, behaviour, and ecology. Oxford: UK.: Oxford University

604 Press; 1993. p. 314-328.

605 12. Stevenson MF, Rylands AB. The marmosets, genus *Callithrix*. In: Mittermeier RA, Cimbra-

606 Filho A, Fonseca GAB, editors. Ecology and behavior of neotropical primates, Vol.2

607 Washington, D.C.: World Wildlife Fund; 1988. p. 131-222.

608 13. Rylands AB, de Faria DS. Habitats, feeding ecology, and home range size in the genus

609 Callithrix. In: Rylands AB, editor. Marmosets and tamarins: systematics, behaviour, and

610 ecology. Oxford: UK.: Oxford University Press; 1993. p. 262-272.

611 14. Lewis DH, Stein FJ, Sis RF, McMurray DN. Fecal microflora of marmosets with wasting

612 marmoset syndrome. Lab Anim Sci 1987 Feb;37(1):103-105.

613 15. Shigeno Y, Toyama M, Nakamura M, Niimi K, Takahashi E, Benno Y. Comparison of gut

614 microbiota composition between laboratory-bred marmosets (*Callithrix jacchus*) with chronic

615 diarrhea and healthy animals using terminal restriction fragment length polymorphism analysis.

616 Microbiol Immunol 2018 Nov;62(11):702-710.

617 16. Moriya, S. Simple mapping-based quantification of a mock microbial community using total
618 RNAseq data. PLOS ONE 2021; 16(7):e0254556.
619 17. McCarren J, Becker JW, Repeta DJ, Shi Y, Young CR, Malmstrom RR, et al. Microbial
620 community transcriptomes reveal microbes and metabolic pathways associated with dissolved
621 organic matter turnover in the sea. Proc Natl Acad Sci USA 2010;107(38):16420.
622 18. Aylward FO, Eppley JM, Smith JM, Chavez FP, Scholin CA, DeLong EF. Microbial
623 community transcriptional networks are conserved in three domains at ocean basin scales. Proc
624 Natl Acad Sci USA 2015;112(17):5443.
625 19. Urich T, Lanzén A, Qi J, Huson DH, Schleper C, Schuster SC. Simultaneous assessment of
626 soil microbial community structure and function through analysis of the meta-transcriptome.
627 PLOS ONE 2008;3(6):e2527.
628 20. Tveit AT, Urich T, Svenning MM. Metatranscriptomic analysis of arctic peat soil
629 microbiota. Appl Environ Microbiol 2014;80(18):5761.
630 21. Li F, Henderson G, Sun X, Cox F, Janssen PH, Guan LL. Taxonomic assessment of rumen
631 microbiota using total RNA and targeted amplicon sequencing approaches. Frontiers in
632 Microbiology 2016;7:987.

633 22. Elekwachi CO, Wang Z, Wu X, Rabee A, Forster RJ. Total rRNA-seq analysis gives insight
634 into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized
635 RNA isolation method. Frontiers in Microbiology 2017;8:1814.
636 23. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature Methods
637 2012;9:357.
638 24. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of
639 short DNA sequences to the human genome. Genome Biol 2009;10(3):R25.
640 25. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform.
641 Bioinformatics 2009;25(14):1754-1760.
642 26. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet
643 rapidly and reproducibly alters the human gut microbiome. Nature 2014 Jan 23;505(7484):559-
644 563.
645 27. Finke MD. Complete nutrient composition of commercially raised invertebrates used as
646 food for insectivores. Zoo Biol 2002;21(3):269-285.
647 28. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
648 data. Bioinformatics 2014;30(15):2114-2120.
649 29. Roberts A, Pachter L. Streaming fragment assignment for real-time analysis of sequencing
650 experiments. Nature Methods 2012;10:71.

651 30. R Core Team: R: A language and environment for statistical computing. R Foundation for
652 Statistical Computing, Vienna, Austria. URL https://www.R-project.org/ 2019.
653 31. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length
654 transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol
655 2011;29:644.
656 32. Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Payzin-Dogru D, et al. A
657 tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors.
658 Cell Reports 2017;18(3):762-776.
659 33. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
660 RNA-seq data with DESeq2. Genome Biol 2014;15(12):550.
661 34. Yamazaki Y, Kawarai S, Morita H, Kikusui T, Iriki A. Faecal transplantation for the
662 treatment of *Clostridium difficile* infection in a marmoset. BMC Vet Res 2017 May
663 31;13(1):150-017-1070-z.
664 35. R Development Core Team. R: A language and environment for statistical computing. R
665 Foundation for Statistical Computing, Vienna, Austria. 2005.
666 36. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic
667 biomarker discovery and explanation. Genome Biol 2011;12(6):R60.

668 37. Osabe T, Shimizu K, Kadota K. Accurate classification of differential expression patterns in
669 a bayesian framework with robust normalization for multi-group RNA-seq count data.
670 Bioinform Biol Insights. 2019;13.
671 38. Reddy BS. Diet And Excretion Of Bile Acids. Cancer Res. 1981 Sep 1;41(9 Pt 2):3766–8.
672 39. Sandri M, Dal Monego S, Conte G, Sgorlon S, Stefanon B. Raw meat based diet influences
673 faecal microbiome and end products of fermentation in healthy dogs. BMC Vet Res 2017 Feb
674 28;13(1):65-017-0981-z.
675 40. Biasato I, Gasco L, De Marco M, Renna M, Rotolo L, Dabbou S, et al. Yellow mealworm
676 larvae (*Tenebrio molitor*) inclusion in diets for male broiler chickens: effects on growth
677 performance, gut morphology, and histological findings. Poult Sci 2018;97.
678 41. Stull VJ, Finer E, Bergmans RS, Febvre HP, Longhurst C, Manter DK, et al. Impact of
679 edible cricket consumption on gut microbiota in healthy adults, a double-blind, randomized
680 crossover trial. Sci Rep. 2018 Dec 1;8(1).

681
682

683 Supporting information

684 **S1 Table. Normalized counts of microbes annotated by QIIME 2 vsearch for Groups C**
685 **and IF.**

686 **S2 Table. Normalized counts of transcripts annotated by blastp for Groups C and IF.**

687 **S3 Table. Normalized counts of the microbes of the insects (house crickets and giant meal**

688 **worm).**

689 **S4 Table. Normalized counts of the transcripts of the insects.**

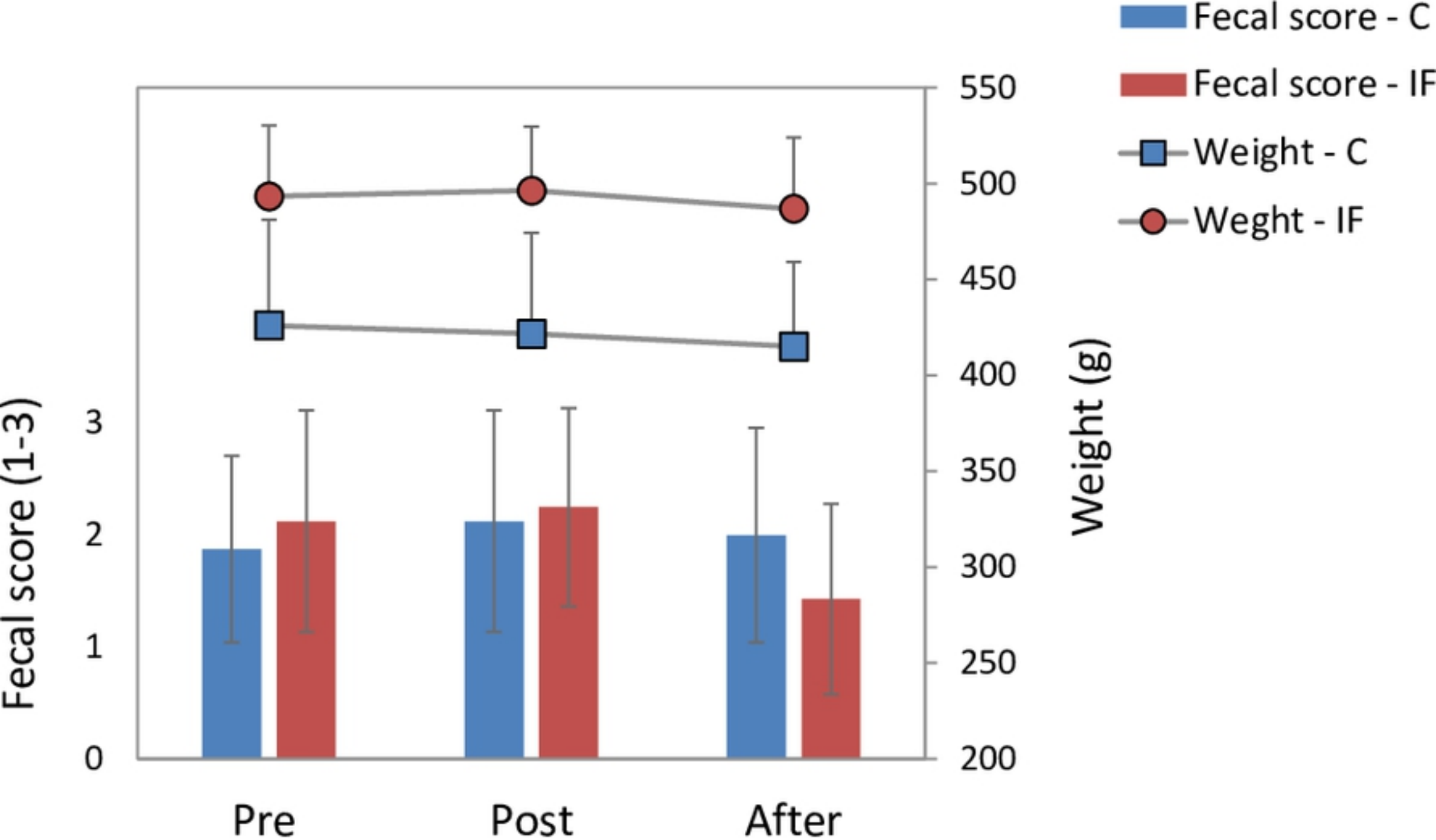

Fecal score - C
Fecal score - IF
Weight - C
Weght - IF
550
500
450
400
350
300
250
200
Weight (g)
3
2
1
0
Fecal score (1-3)
Pre
Post
After

Figure1

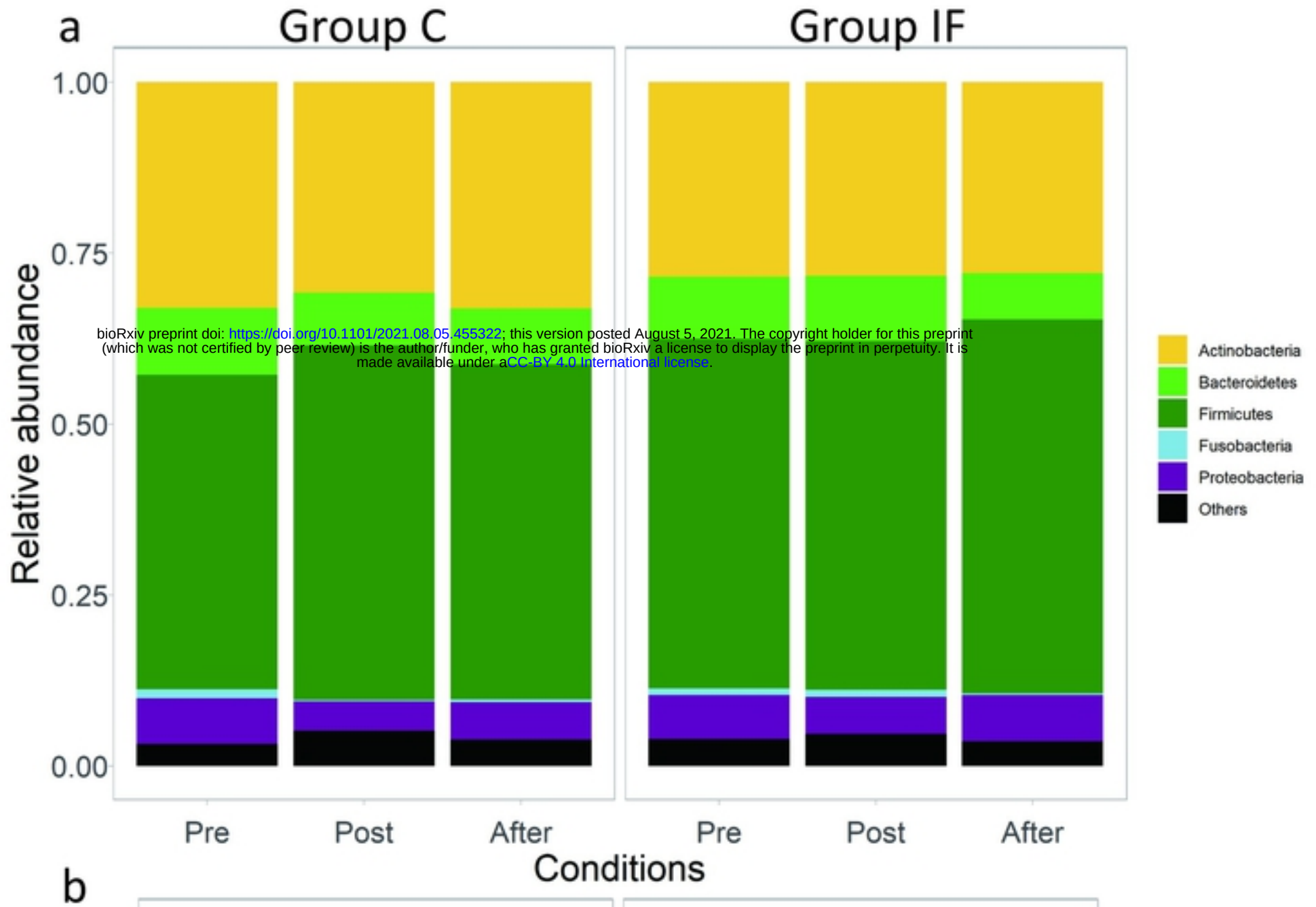


Figure2

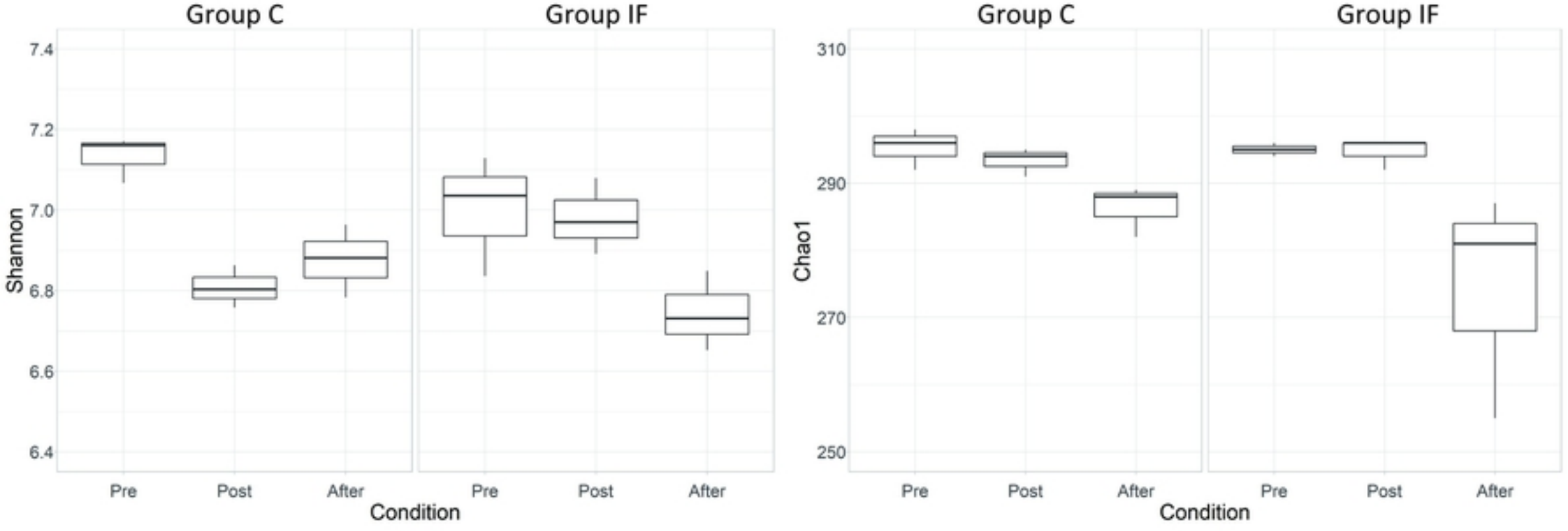
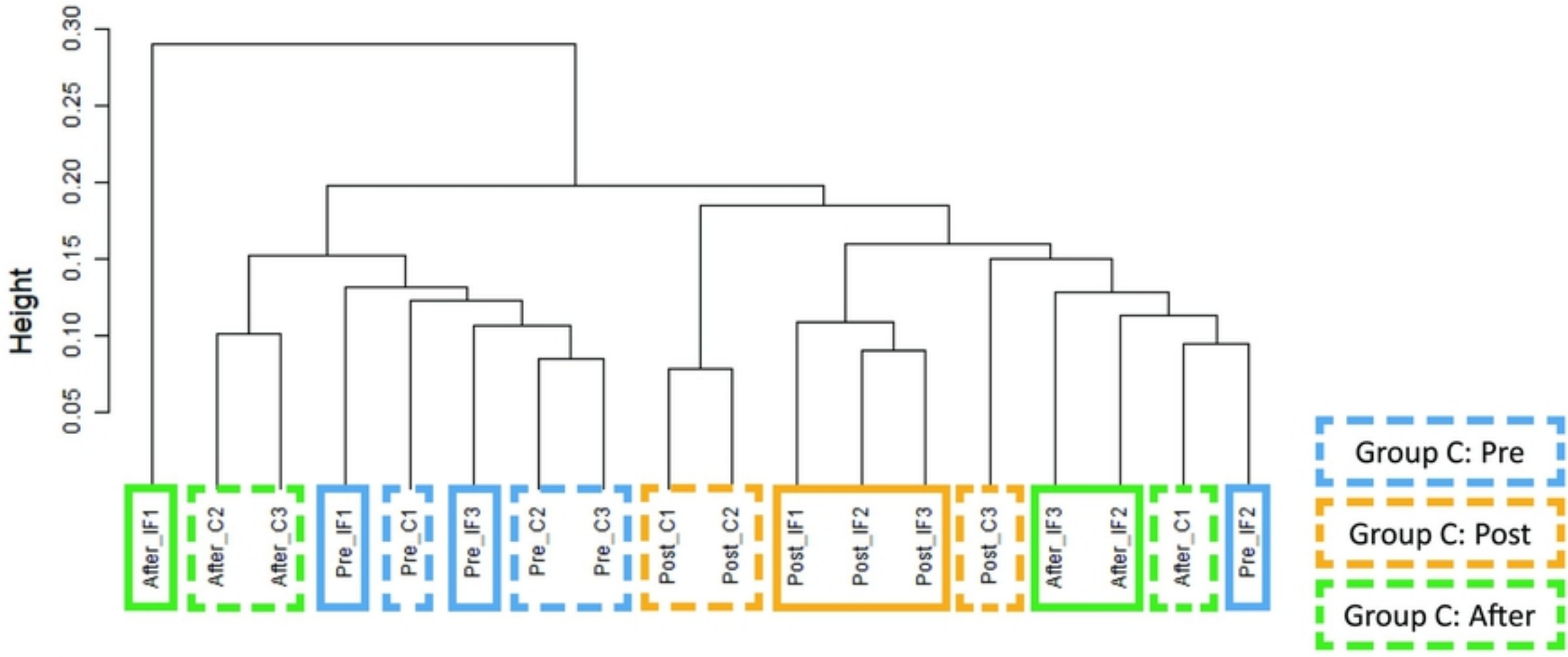

Group C
Group IF
Group C
Group IF
Shannon
7.4
7.2
7.0
6.8
6.6
6.4
Chao1
310
290
270
250
Pre
Post
After
Pre
Post
After
Condition
Pre
Post
After
Pre
Post
After
Condition

Figure3

a. Microbiota



b. Transcripts

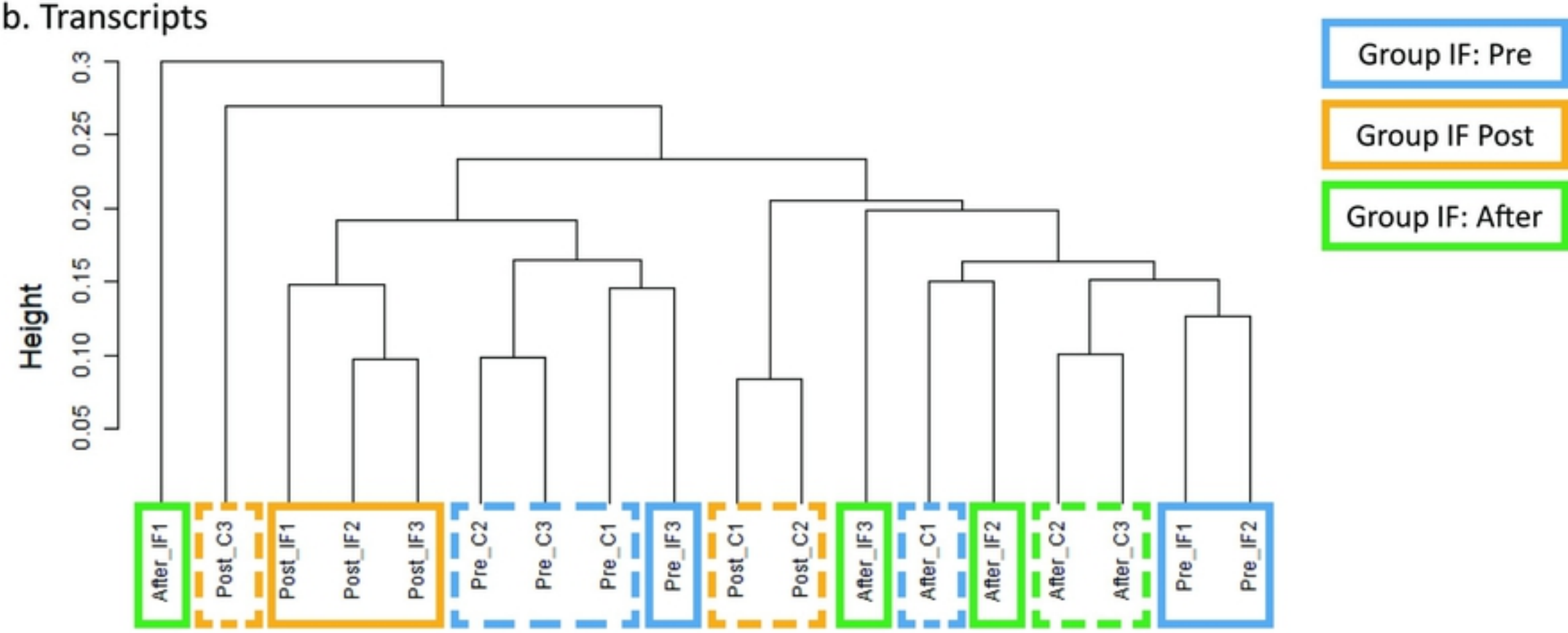
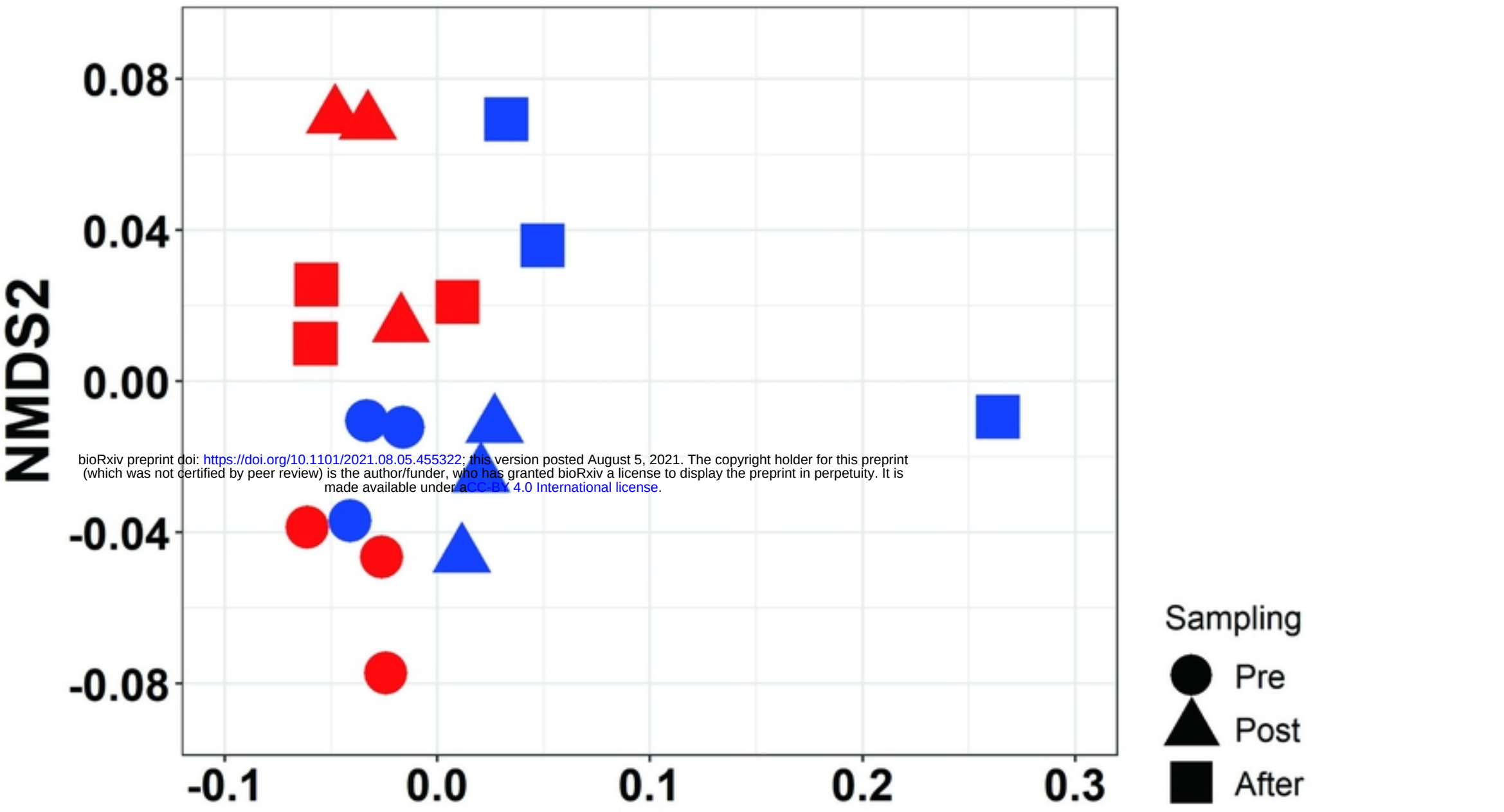


Figure4

a. Microbiota



b. Transcripts

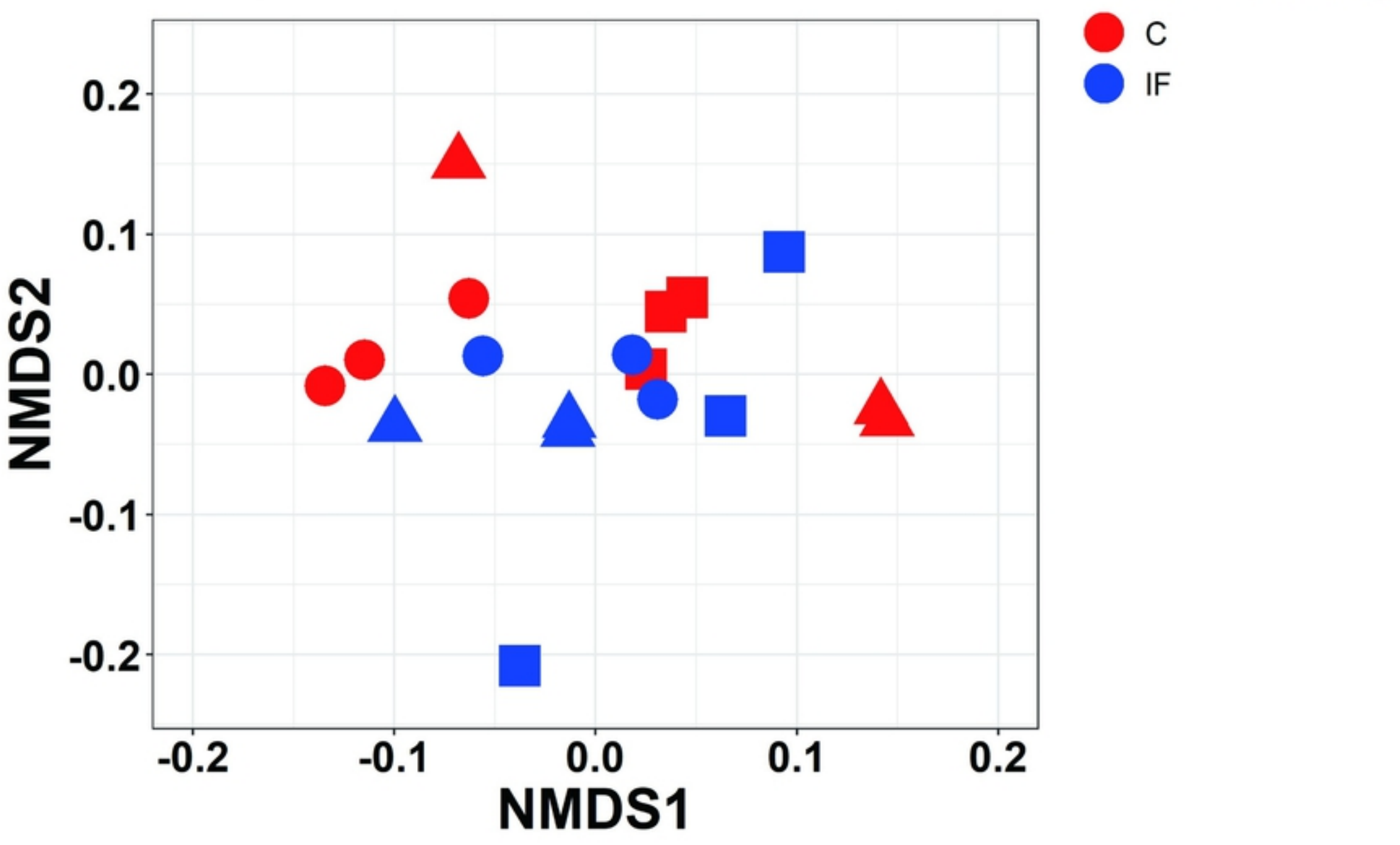


Figure5

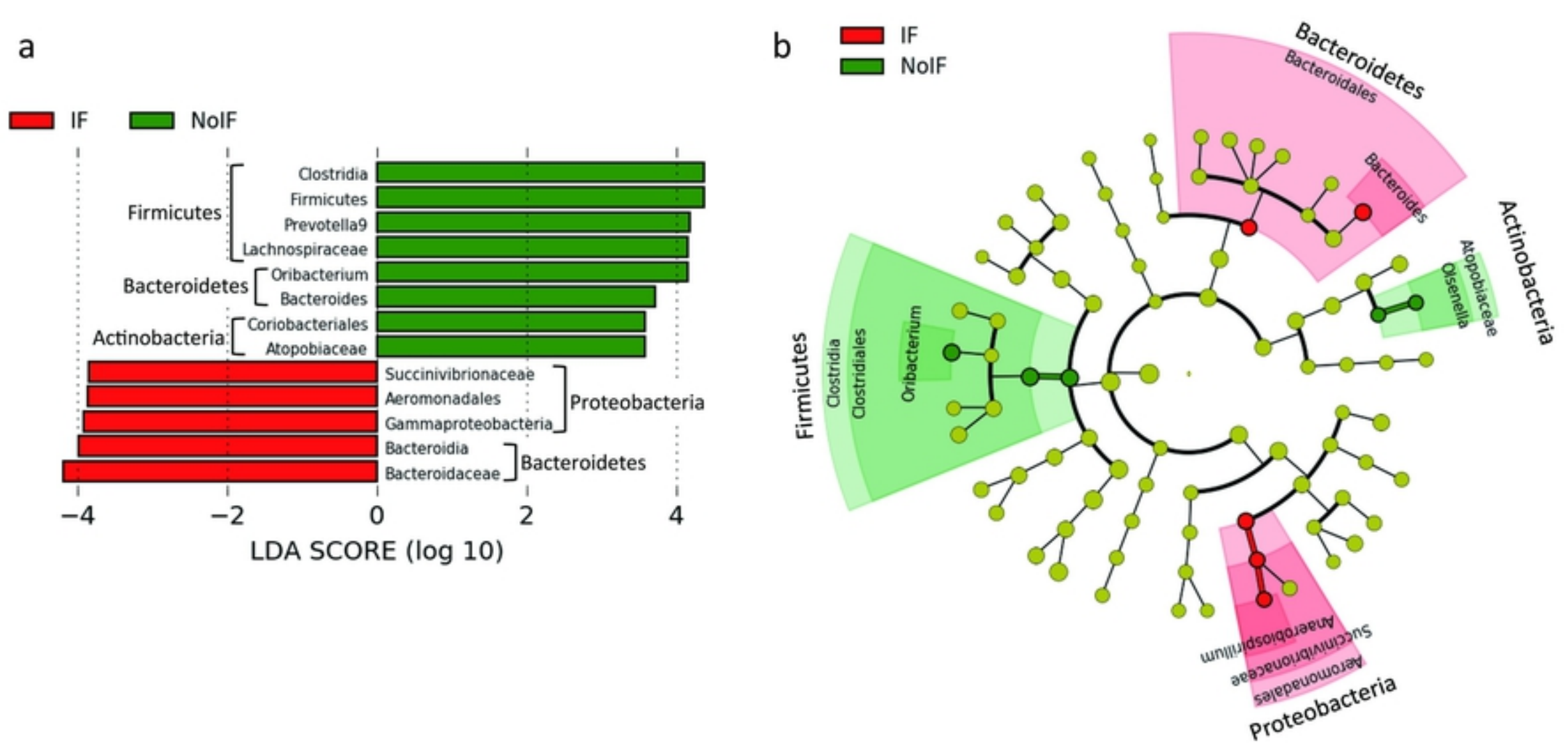
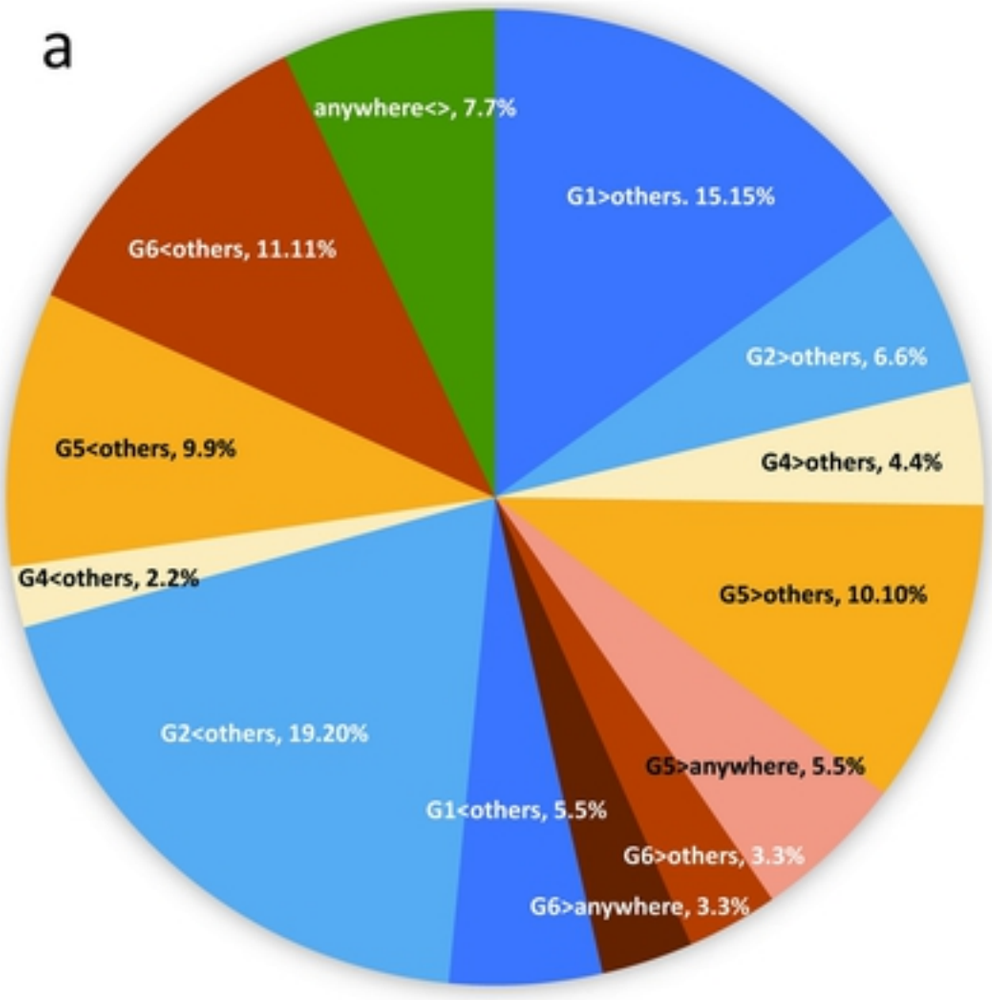
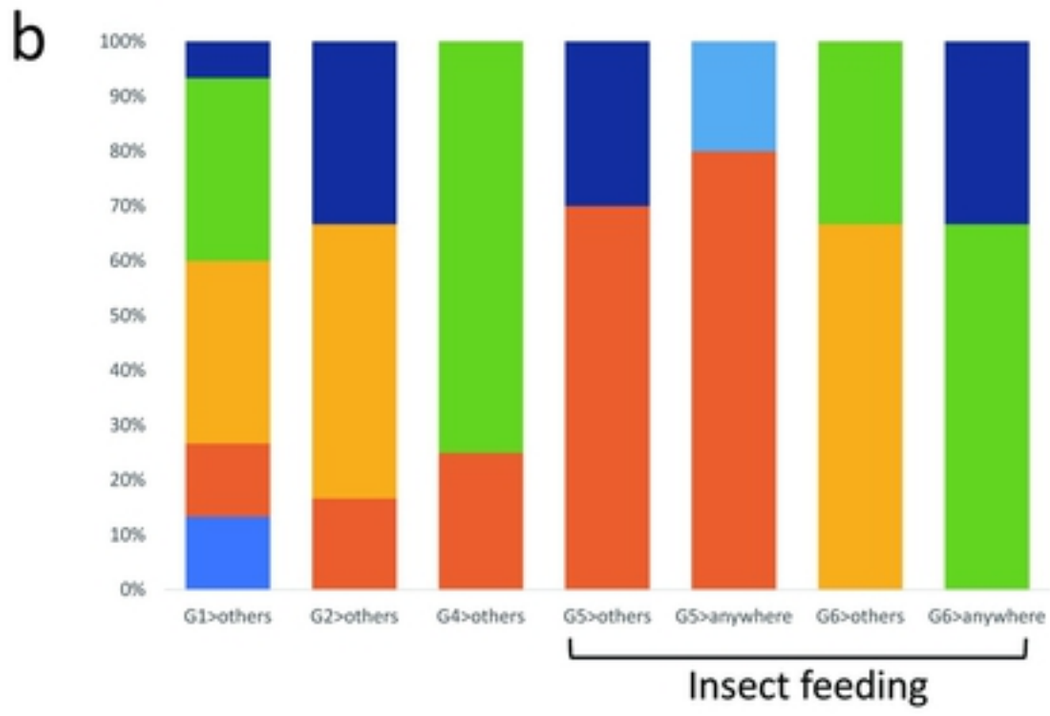

a
IF
NoIF
Clostridia
Firmicutes
Prevotella9
Lachnospiraceae
Firmicutes
Oribacterium
Bacteroides
Bacteroidetes
Coriobacteriales
Atopobiaceae
Actinobacteria
Succinivibrionaceae
Aeromonadales
Gammaproteobacteria
Proteobacteria
Bacteroidia
Bacteroidaceae
Bacteroidetes
−4
−2
0
2
4
LDA SCORE (log 10)
b
IF
NoIF
Bacteroidetes
Bacteroidales
Bacteroides
Actinobacteria
Atopobiaceae
Olsenella
Firmicutes
Clostridia
Clostridiales
Oribacterium
Proteobacteria
Aeromonadales
Succinivibrionaceae
Anaerobiospirillum

Figure6


a
anywhere<>, 7.7%
G1>others. 15.15%
G6<others, 11.11%
G2>others, 6.6%
G5<others, 9.9%
G4>others, 4.4%
G4<others, 2.2%
G5>others, 10.10%
G2<others, 19.20%
G5>anywhere, 5.5%
G1<others, 5.5%
G6>others, 3.3%
G6>anywhere, 3.3%


b
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G1>others
G2>others
G4>others
G5>others
G5>anywhere
G6>others
G6>anywhere
Insect feeding

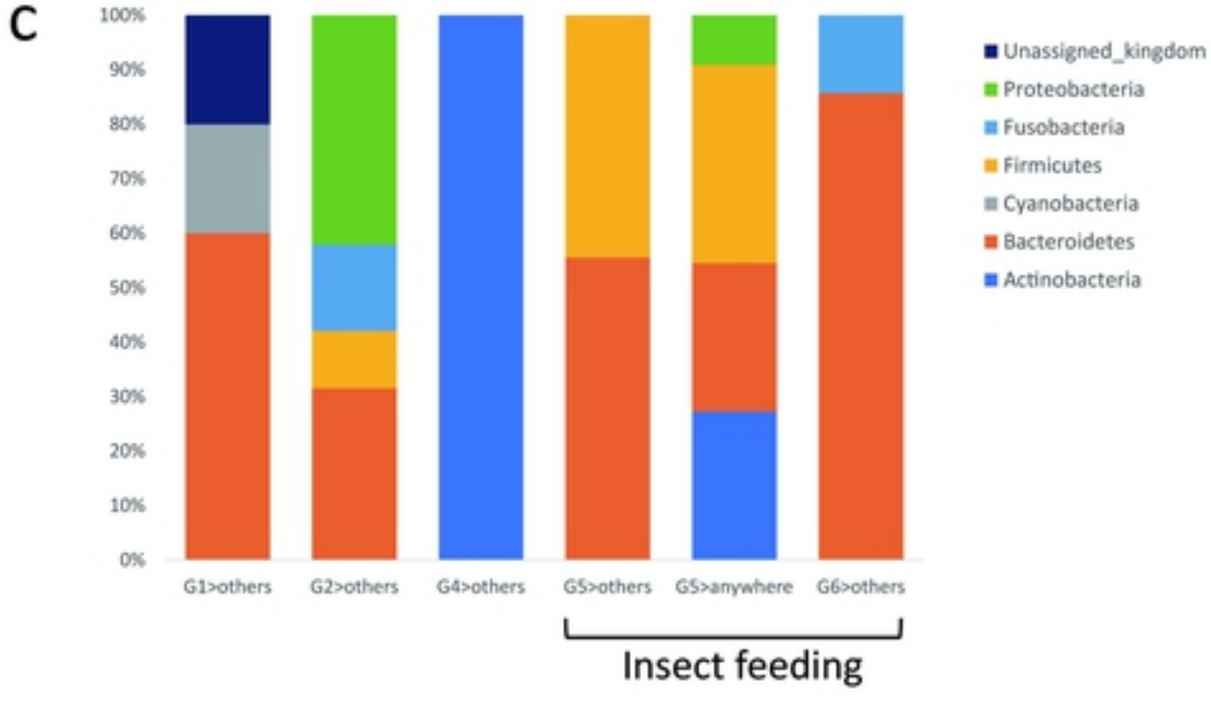

c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G1>others
G2>others
G4>others
G5>others
G5>anywhere
G6>others
Insect feeding
Unassigned_kingdom
Proteobacteria
Fusobacteria
Firmicutes
Cyanobacteria
Bacteroidetes
Actinobacteria

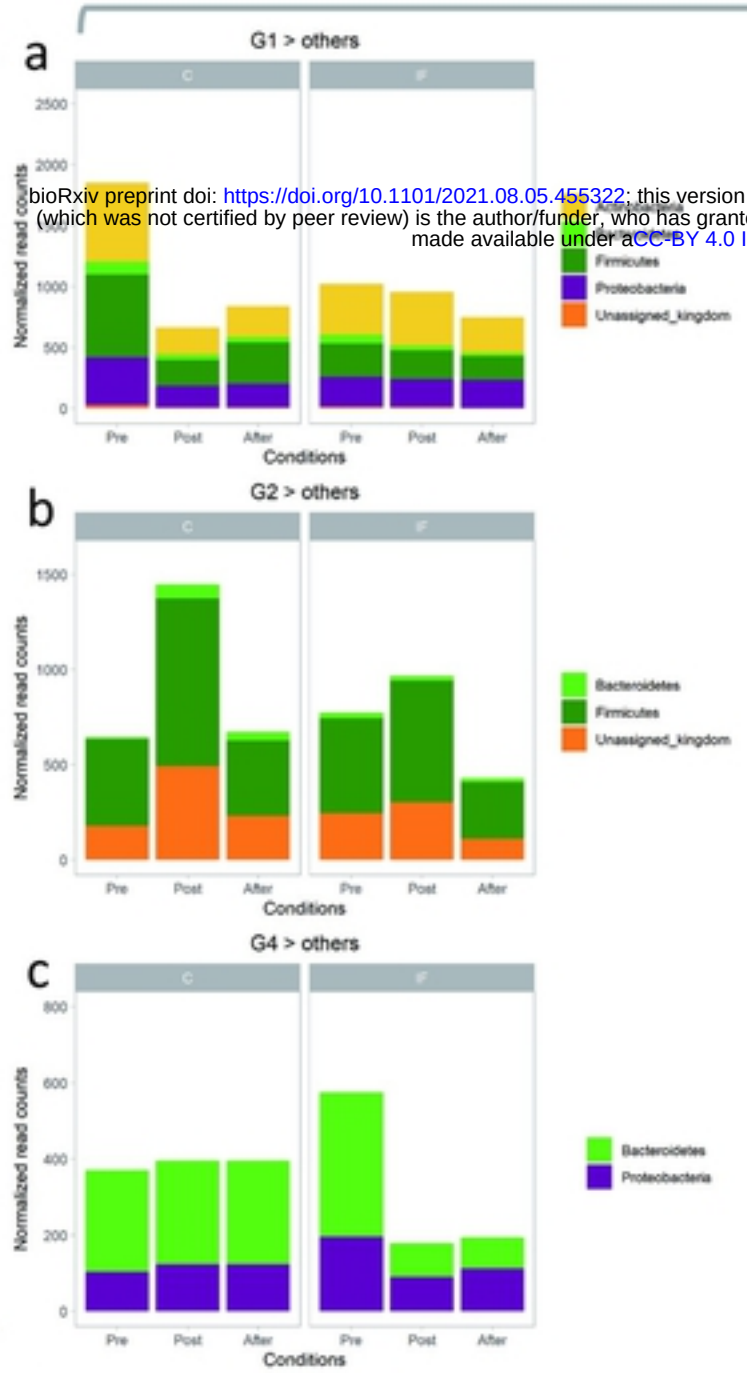
Figure7

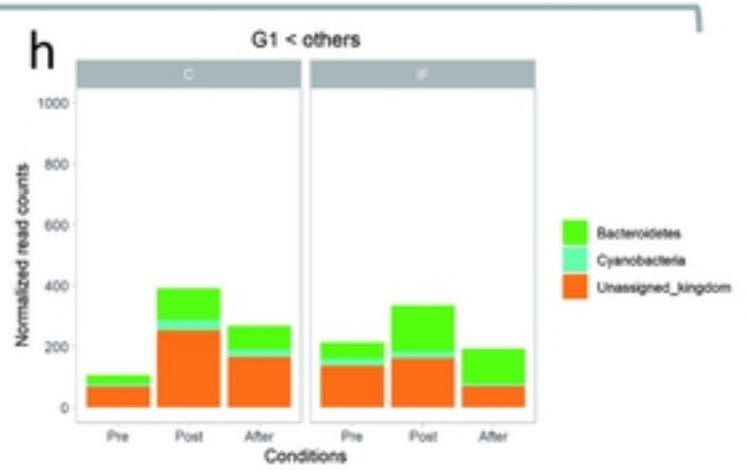
Upregulation

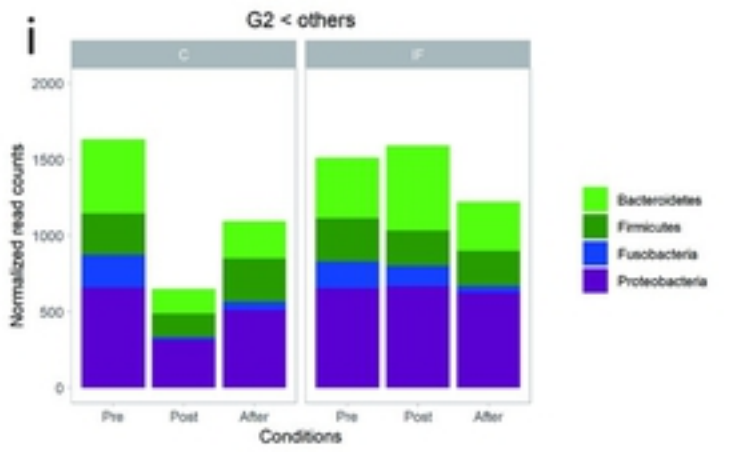
Downregulation

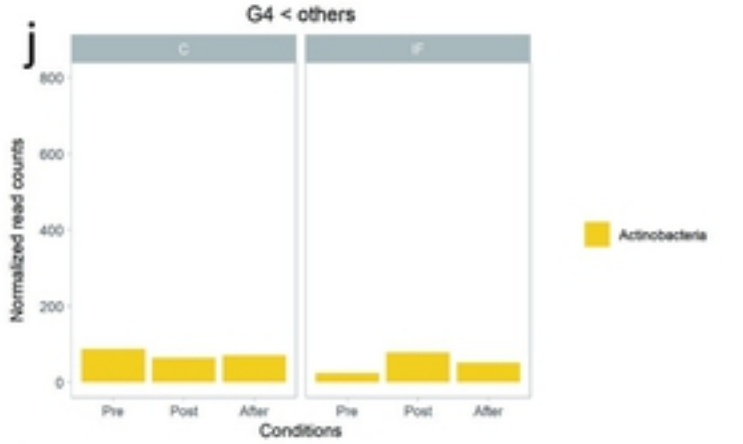
Insect-feeding nonrelevant

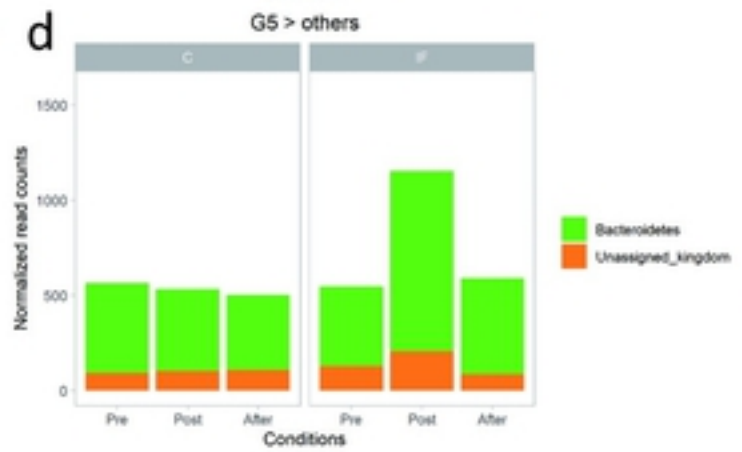
Insect-feeding relevant

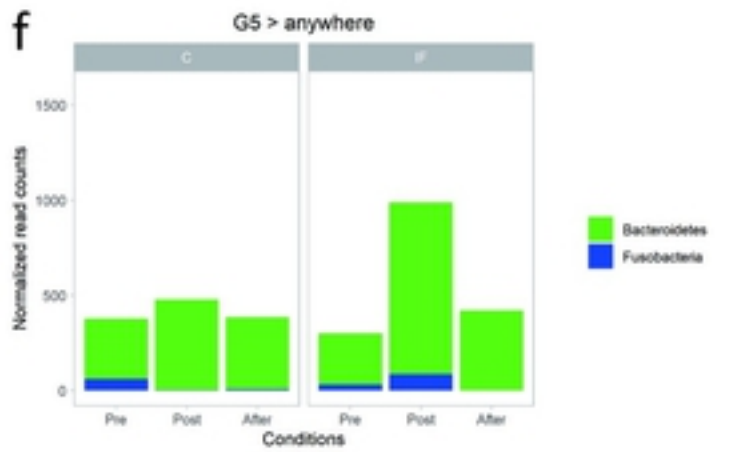


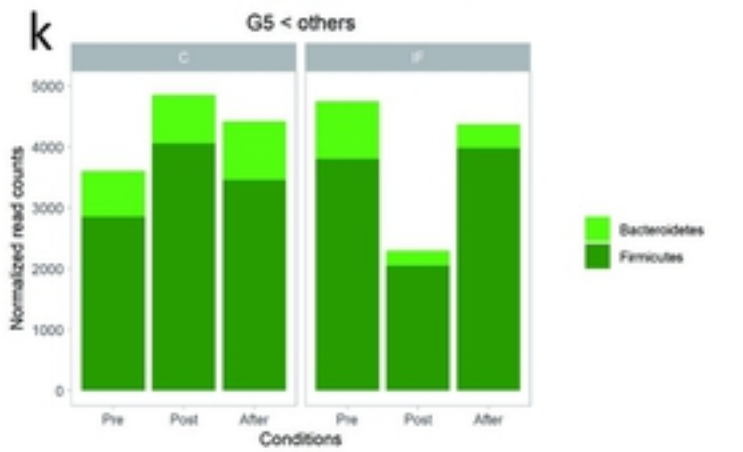


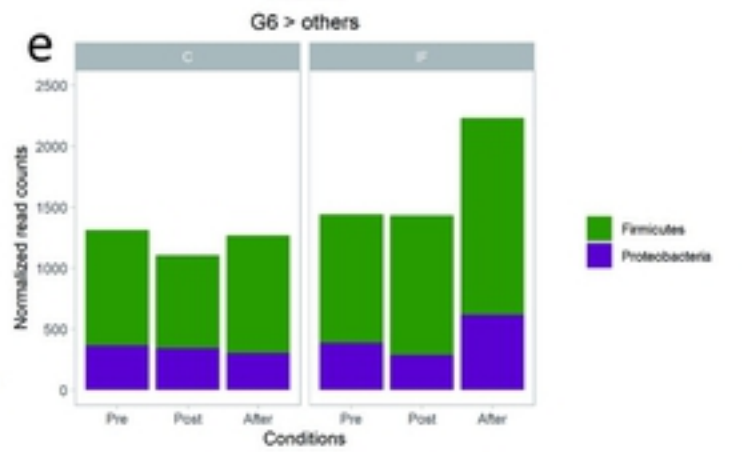


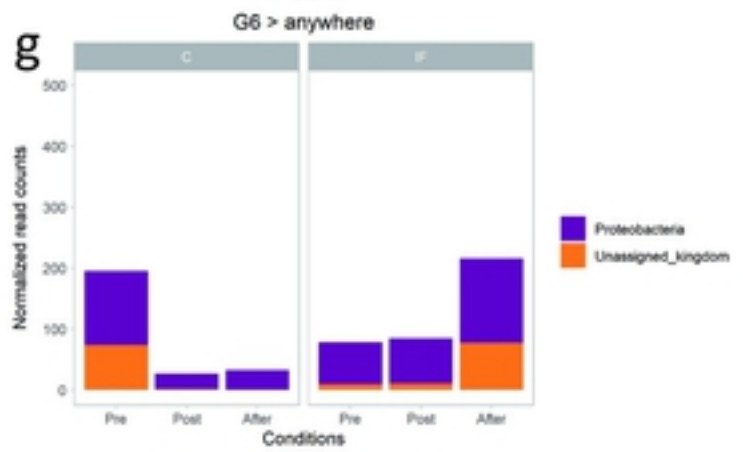












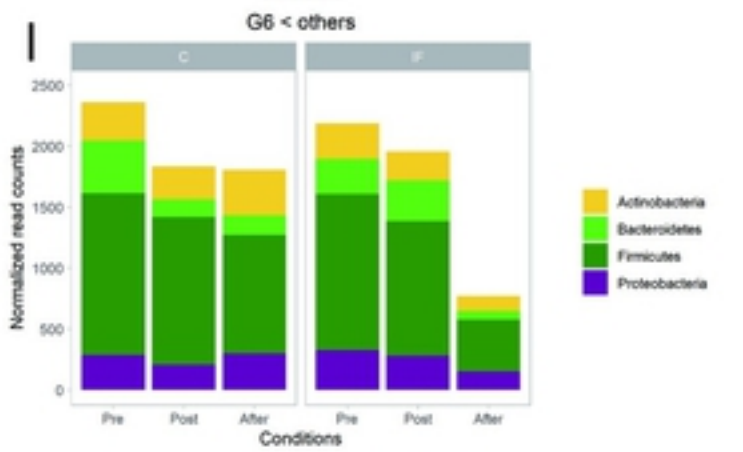


Figure8

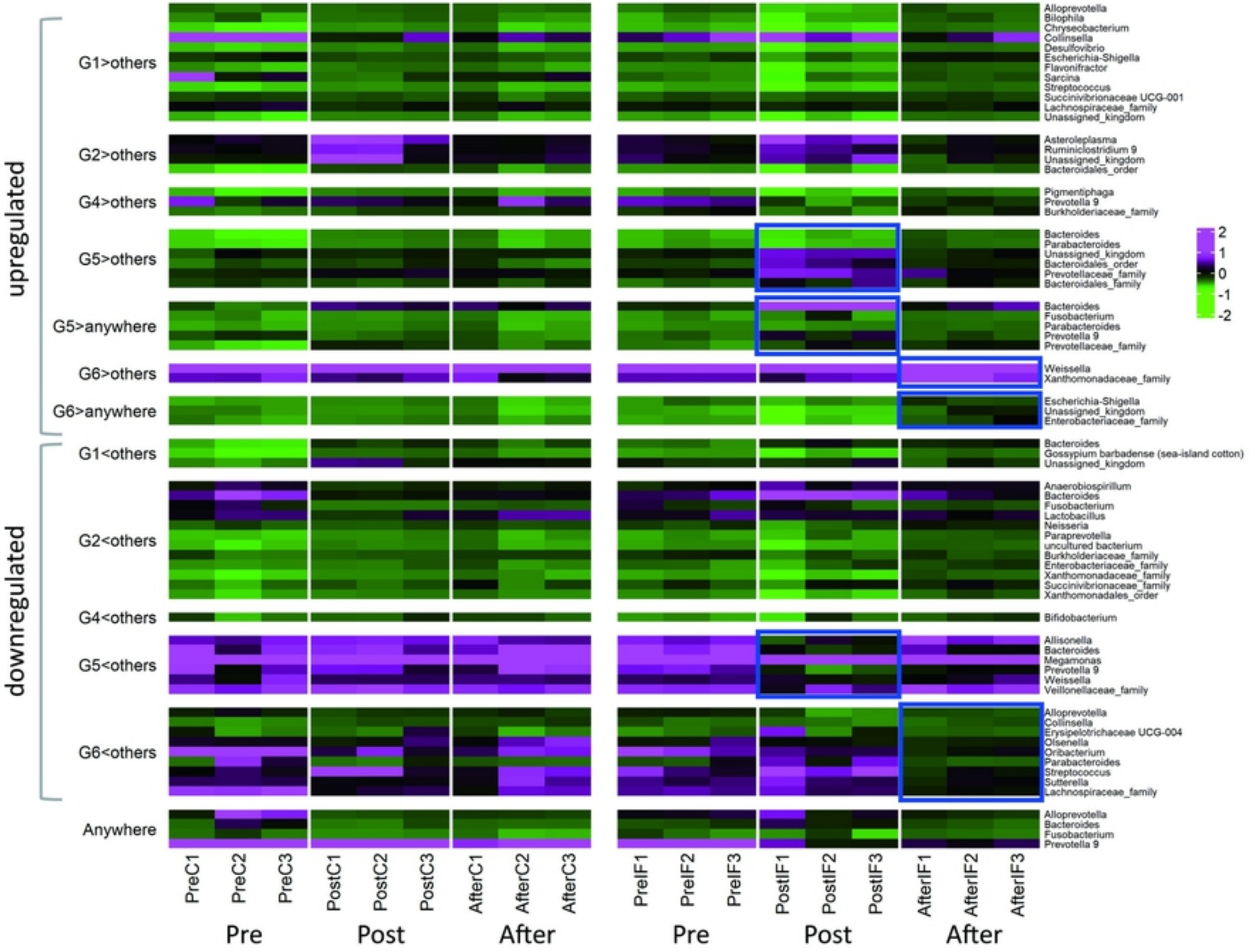
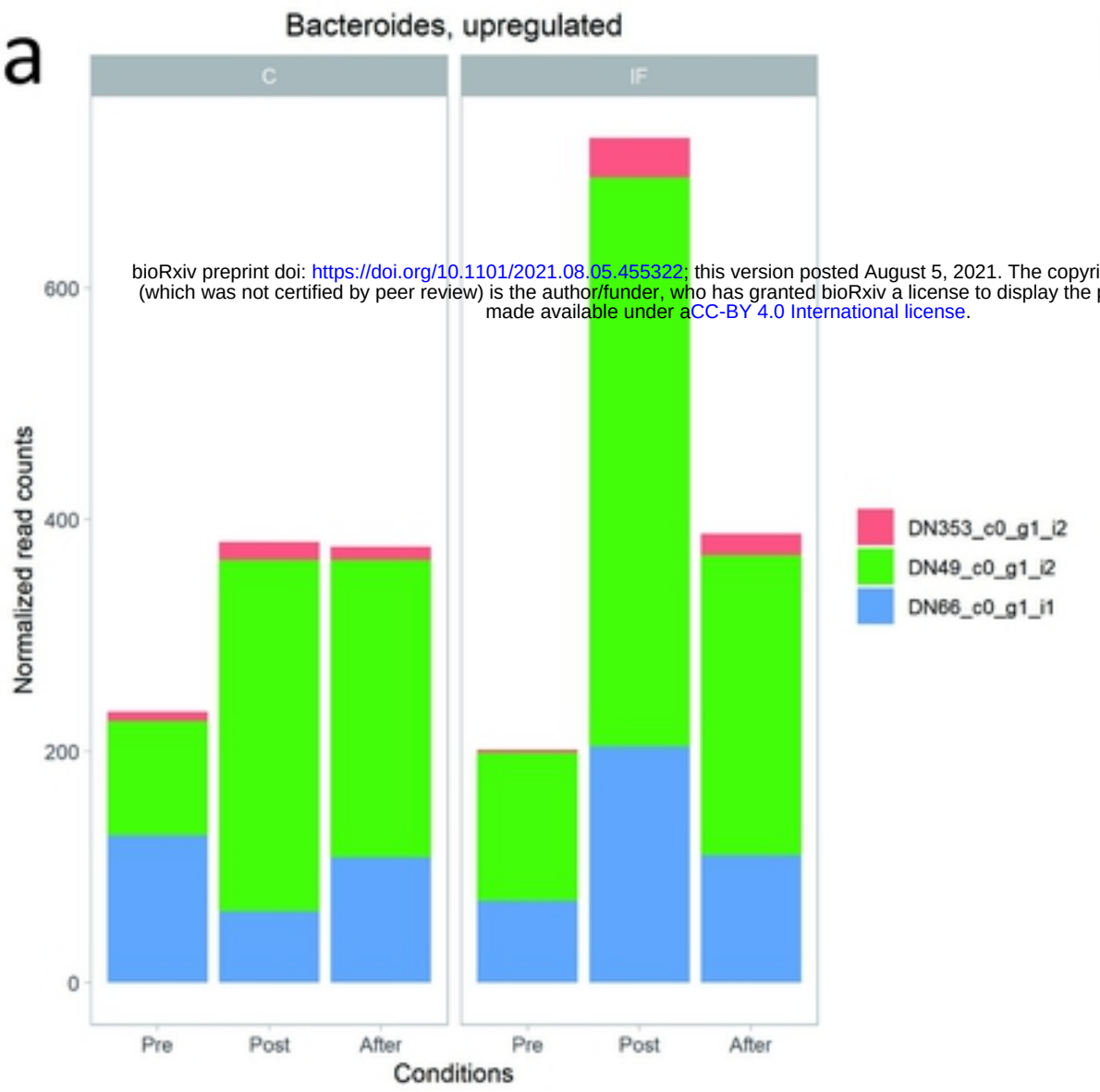
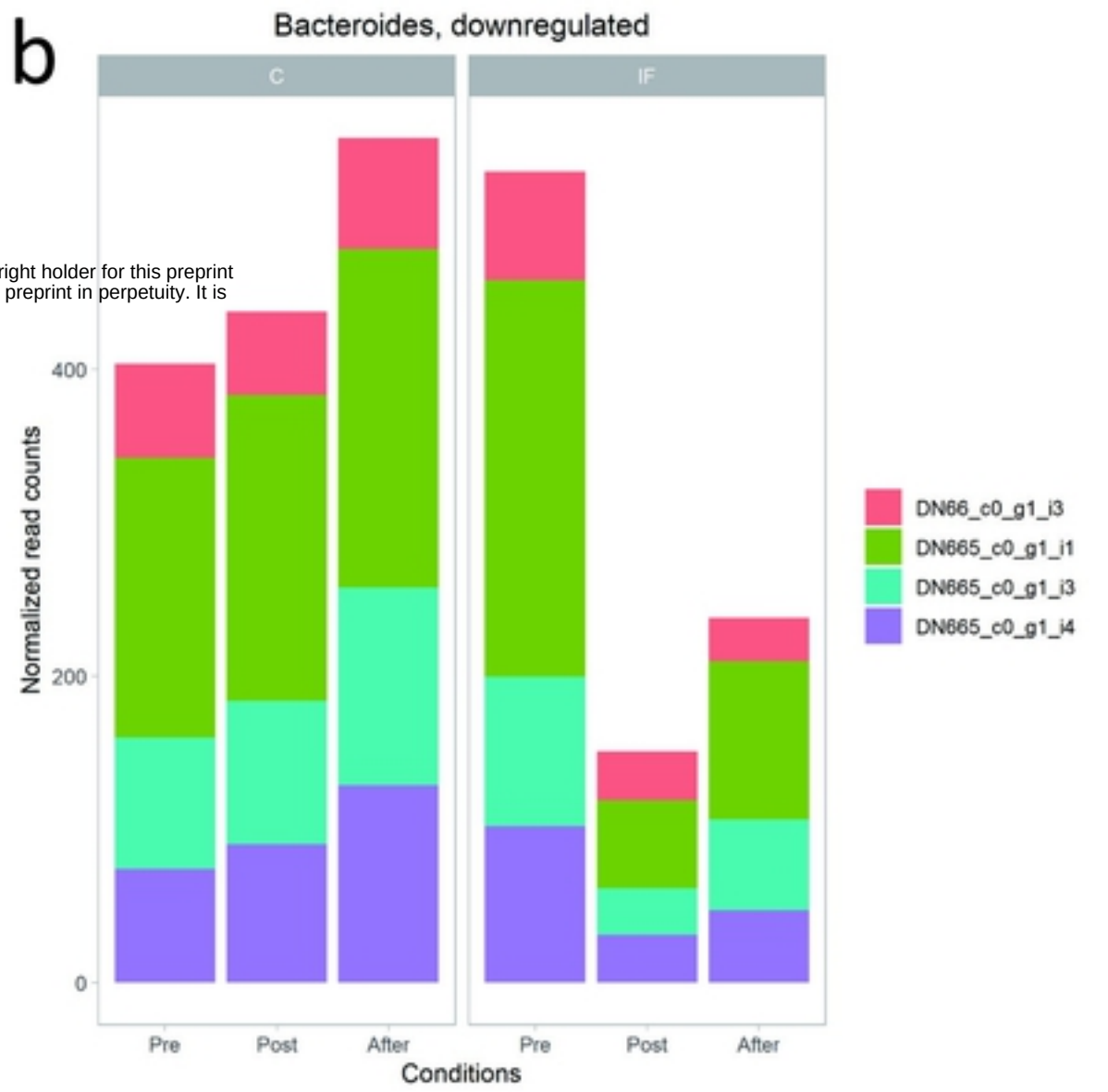
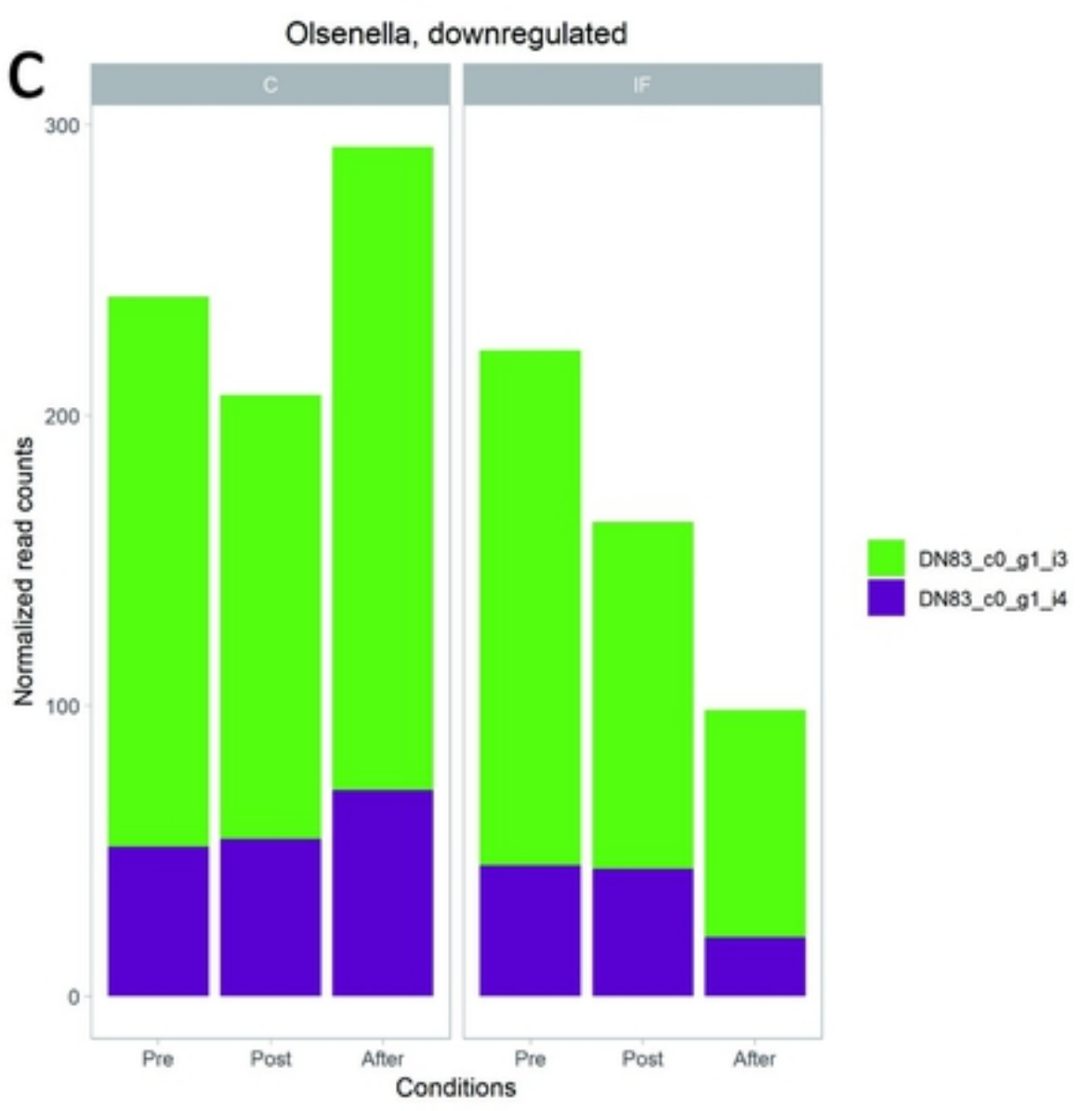

Group C
Group IF
upregulated
downregulated
G1>others
G2>others
G4>others
G5>others
G5>anywhere
G6>others
G6>anywhere
G1<others
G2<others
G4<others
G5<others
G6<others
Anywhere
Alloprevotella
Bilophila
Chryseobacterium
Collinsella
Desulfovibrio
Escherichia-Shigella
Flavonifractor
Sarcina
Streptococcus
Succinivibrionaceae UCG-001
Lachnospiraceae_family
Unassigned_kingdom
Asteroleplasma
Ruminiclostridium 9
Unassigned_kingdom
Bacteroidales_order
Pigmentiphaga
Prevotella 9
Burkholderiaceae_family
Bacteroides
Parabacteroides
Unassigned_kingdom
Bacteroidales_order
Prevotellaceae_family
Bacteroidales_family
Bacteroides
Fusobacterium
Parabacteroides
Prevotella 9
Prevotellaceae_family
Weissella
Xanthomonadaceae_family
Escherichia-Shigella
Unassigned_kingdom
Enterobacteriaceae_family
Bacteroides
Gossypium barbadense (sea-island cotton)
Unassigned_kingdom
Anaerobiospirillum
Bacteroides
Fusobacterium
Lactobacillus
Neisseria
Paraprevotella
uncultured bacterium
Burkholderiaceae_family
Enterobacteriaceae_family
Xanthomonadaceae_family
Succinivibrionaceae_family
Xanthomonadales_order
Bifidobacterium
Allisonella
Bacteroides
Megamonas
Prevotella 9
Weissella
Veillonellaceae_family
Alloprevotella
Collinsella
Erysipelotrichaceae UCG-004
Olsenella
Oribacterium
Parabacteroides
Streptococcus
Sutterella
Lachnospiraceae_family
Alloprevotella
Bacteroides
Fusobacterium
Prevotella 9
2
1
0
-1
-2
PreC1
PreC2
PreC3
PostC1
PostC2
PostC3
AfterC1
AfterC2
AfterC3
PreIF1
PreIF2
PreIF3
PostIF1
PostIF2
PostIF3
AfterIF1
AfterIF2
AfterIF3
Pre
Post
After
Pre
Post
After

Figure9







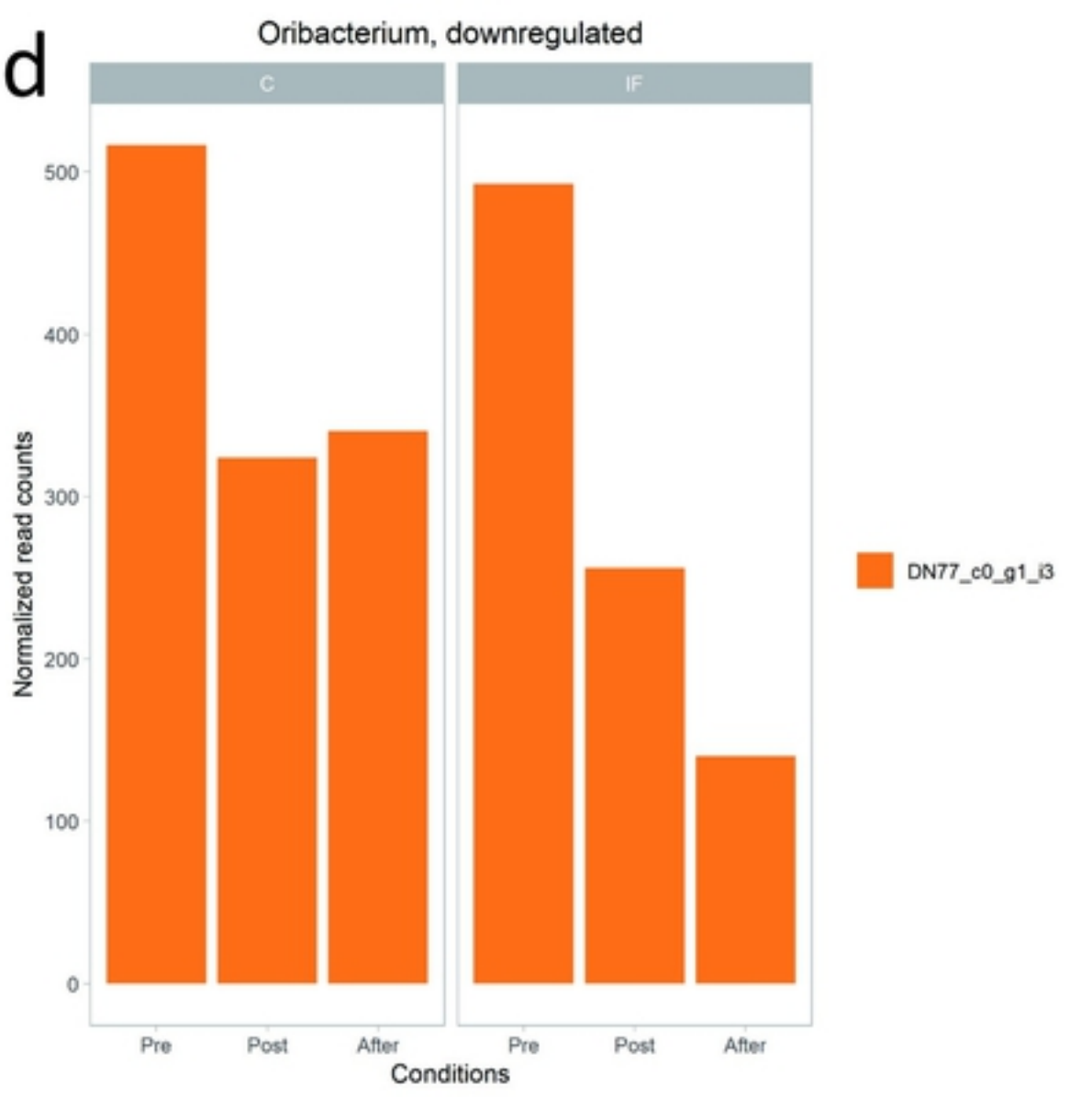
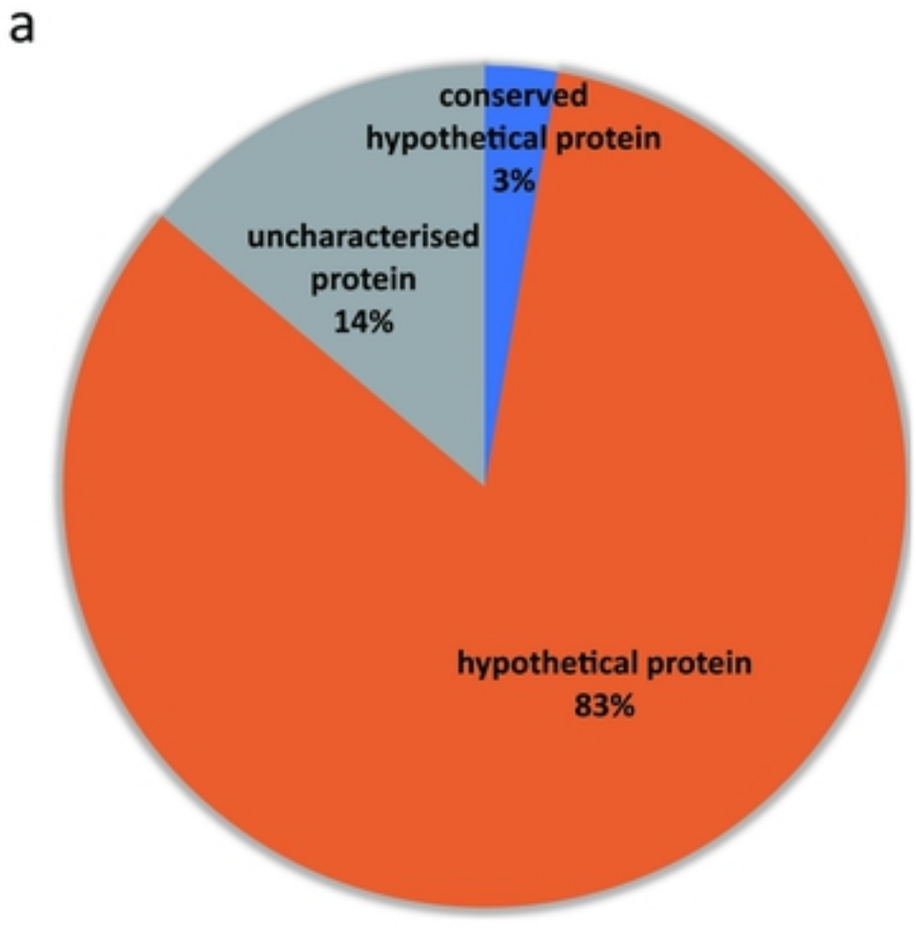
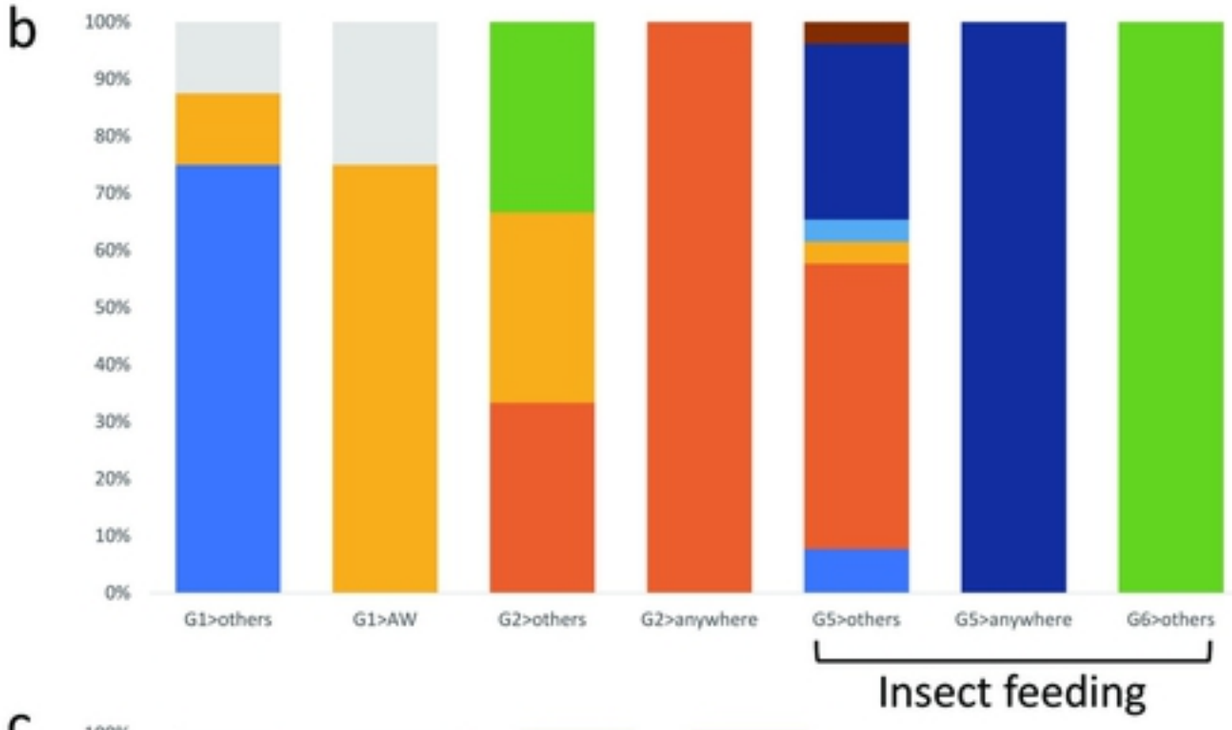


Figure10


a
conserved hypothetical protein 3%
uncharacterised protein 14%
hypothetical protein 83%


b
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G1>others
G1>AW
G2>others
G2>anywhere
G5>others
G5>anywhere
G6>others
Insect feeding

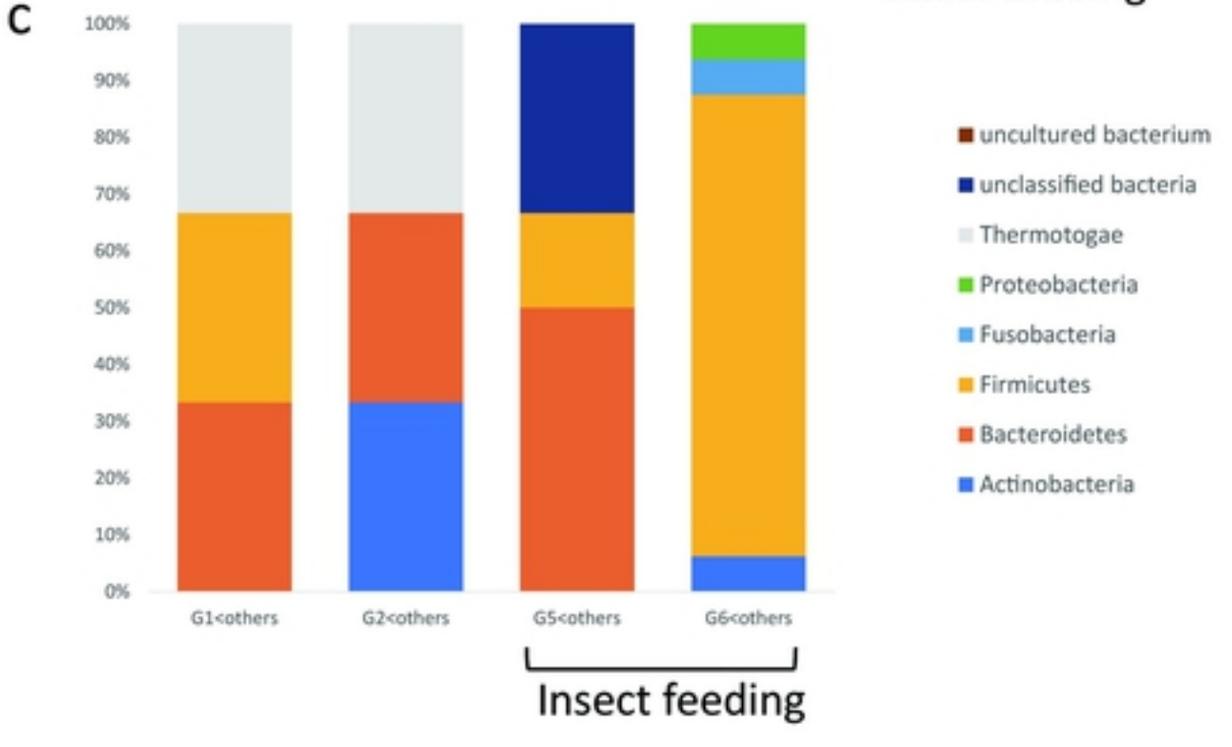

c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G1<others
G2<others
G5<others
G6<others
Insect feeding
uncultured bacterium
unclassified bacteria
Thermotogae
Proteobacteria
Fusobacteria
Firmicutes
Bacteroidetes
Actinobacteria

Figure11

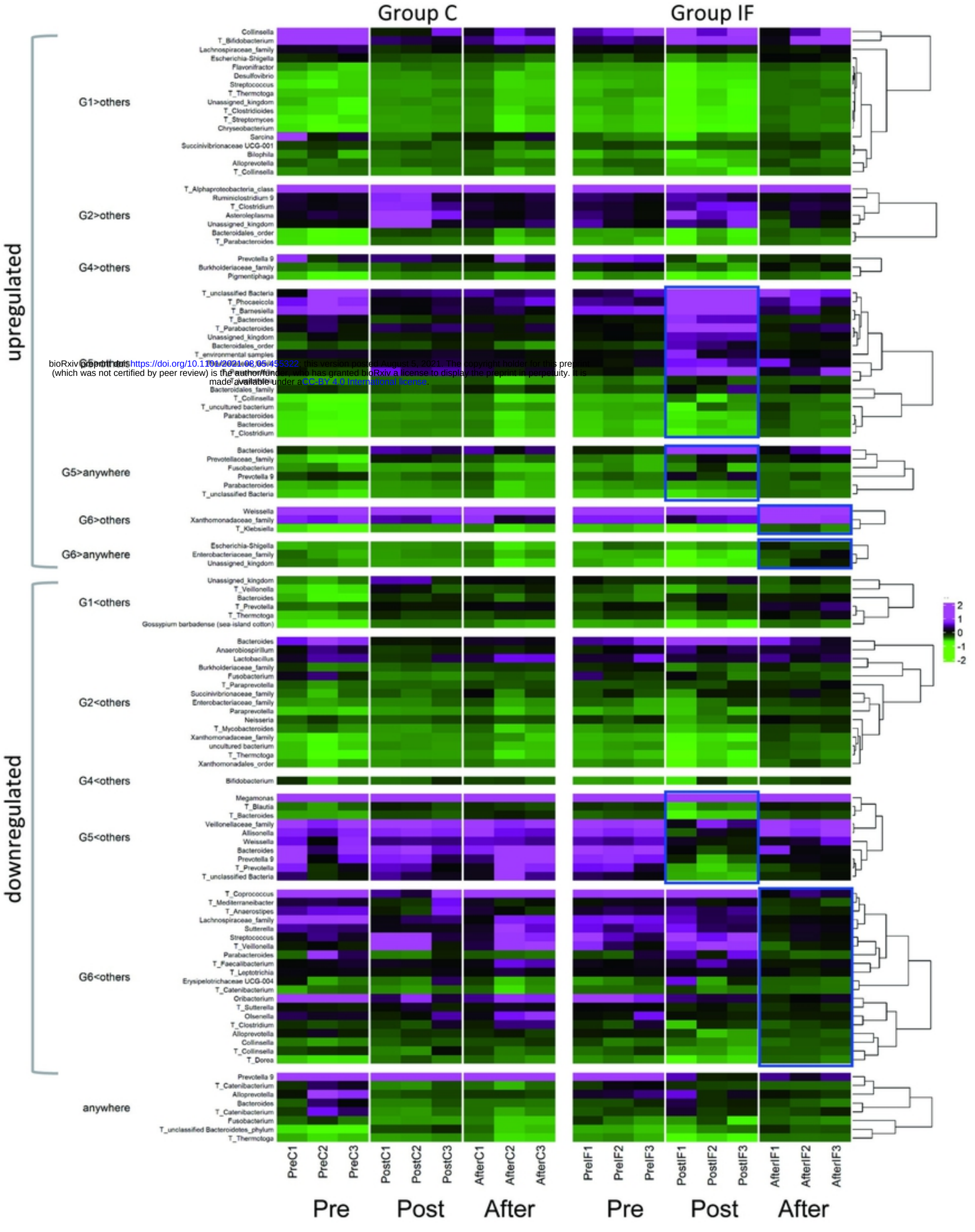


Figure12

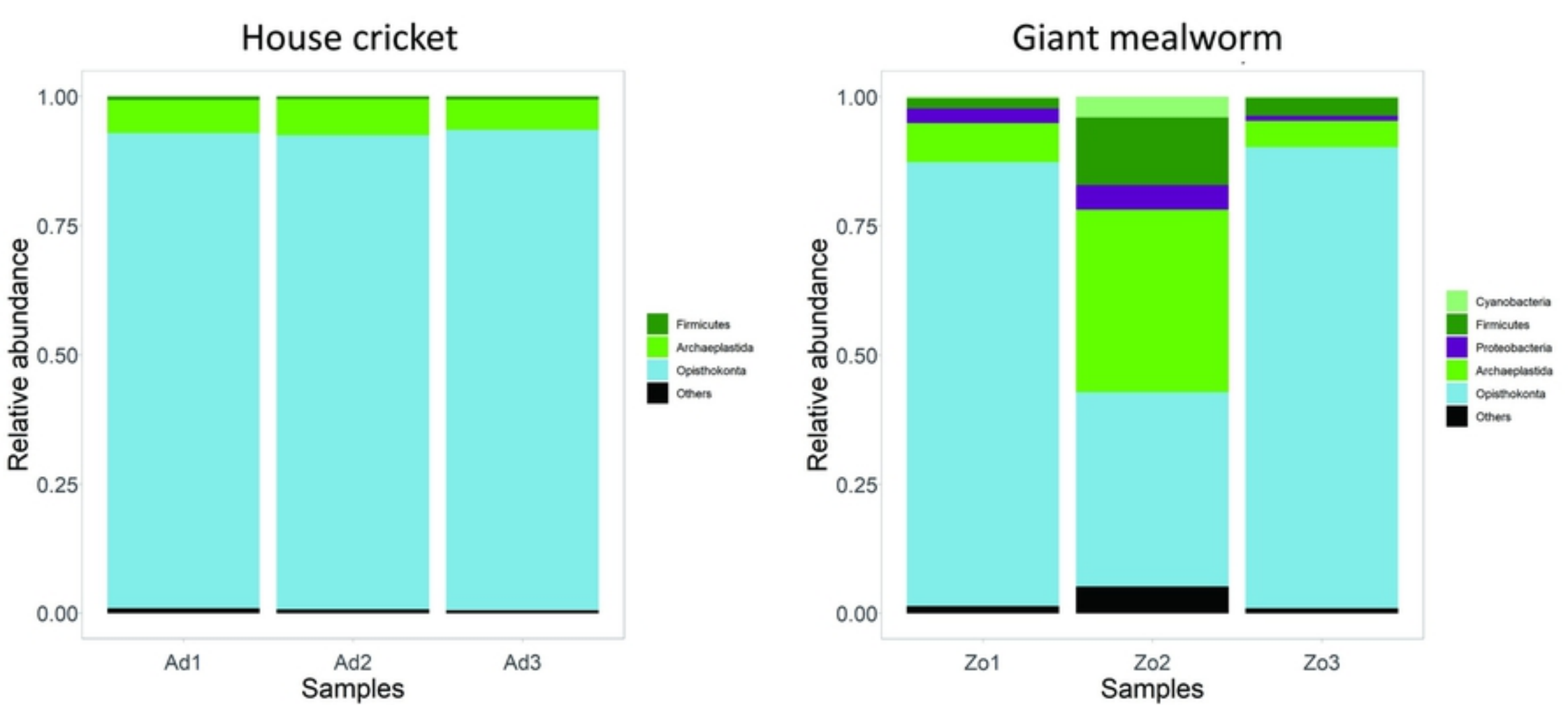

House cricket
Giant mealworm
Relative abundance
1.00
0.75
0.50
0.25
0.00
Ad1
Ad2
Ad3
Samples
Zo1
Zo2
Zo3
Cyanobacteria
Firmicutes
Proteobacteria
Archaeplastida
Opisthokonta
Others

Figure13