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

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Abstract

Background: 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. However, the effect of insect feeding on the marmoset body has not been clearly characterized. Here, we examined the effect of enhanced insect feeding on the gut by analyzing the fecal microbiome and transcriptome of captive marmosets.

Methods: 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 fecal samples to evaluate the ecology and transcriptome of the microbiome, which were then compared among time points before (Pre), just after (Post), and two weeks after intervention (After) by total RNA sequencing (total RNA-seq).

Results: General health status was not changed throughout the experimental period in either group. Distinct changes were observed only in the microbiome of Group IF after the insect feeding treatment. A species of flagellate, Hypotrichomonas mariae , was identified for the first time in common marmosets, but only after enhanced insect feeding. The structure of the microbiome differed between the groups with and without insect feeding. There were significant changes in the transcriptome of Group IF only, and some of the changes in the transcriptome mirrored the fluctuations in the microbiome.

Conclusions: Enhanced insect feeding modified the distribution of the fecal microbiomes of the marmosets, which were suggested to interact with the transcriptomes. The rapid changes in the microbiome and transcriptome are deeply connected to the feeding ecology of marmosets in the wild. The results were informative for identifying the physiological impact of insect feeding and for detecting transcripts that are currently unidentifiable. The present study supports a feeding regimen for captive marmosets that includes insects to maintain the natural balance of symbiotic metabolism in the gut microbiome.

Background

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 diarrhea 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 diarrhea, anorexia, and anemia 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 etiology 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 impact of insects on the physiological functions of marmosets has not been clarified. In the present study, we aimed to determine the effect of insect feeding on captive marmosets by analyzing the microbiome and transcriptome extracted from fecal samples.

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

The total RNA-seq approach simultaneously describes microbial ecology and the transcriptome. It has been widely used in studies of marine ecology [17, 18], soil microbes [19, 20] and animal gut microbiomes [21, 22] to obtain information from all domains of microbial inhabitants, including *Eukaryote, 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 microbiome and transcriptome by comparing groups with and without insect feeding. The weekly weight and the daily fecal scores were recorded to monitor the general health status throughout the experimental period. RNA was extracted from fecal samples from different timepoints (before (Pre), just after (Post), and after (After) the experimental intervention), and DNA was then sequenced and annotated by a database to identify taxonomies. In human subjects, the microbiome was reported to dramatically modify microbiome diversity after a change in diet for five days [26]. Thus, insect feeding has a substantial impact on the physiological states of marmosets, who preferentially eat insects in wild habitats [12].

Results

General health condition

The fecal scores of the group with 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 Figure 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 Figure 1.

[Figure 1]

Whole microbial community of the marmoset gut in Group IF and group control (Group C): taxonomy, similarity, and diversity analyses.

Because the read counts of a sample from the After condition of Group IF were too low for unknown reasons, it was excluded from the further analyses. The total normalized read count data against the SSU rRNA sequences generated from the 17 fecal samples of the six common marmosets at the Pre, Post, and After conditions were 37966.78, with an average of 22.11 counts per sample (see Supplementary Table S1 for the normalized count data of the microbiome). The difference in read count data between Groups C and IF was not significant (t (913) = 1.08, p = 0.56, mean ± standard deviation (SD) for Group C: 22.36 ± 60.12; Group IF: 49.04 ± 67.32).

Phylum level. Figure 2 shows the relative abundance of the microbiome at the phylum level for each group across the conditions. *Actinobacteria* and *Firmicutes* were abundant in every sample of both groups. Table 1 shows the means and SDs of the normalized counts of the fecal microbiome at the phylum level for each group and the results of the comparison between sampling periods (Pre vs Post, Pre vs After, and Post vs After). Significant differences in *Actinobacteria* were found in both groups in the same comparisons (Pre vs Post and Pre vs After) with contrasting trends (decrease and increase in Groups C and IF, respectively). Except for *Actinobacteria*, the changes in Group C were in *Bacteroides* (Pre vs Post and Pre vs After) and *Proteobacteria* (Pre vs. Post). Differences in Group IF were in *Excavata* (Pre vs After), *Firmicutes* (Pre vs Post), and *Proteobacteria* (Pre vs After).

[Figure 2] [Table 1]

Genus level. Figure 3 shows the relative abundance of the microbiome at the genus level. *Bifidobacterium* (phylum *Actinobacteria*) and *Megamonas* (phylum *Firmicutes*) were more abundant than other genera in both groups. To determine the effects of insect feeding on the microbiome at the genus taxonomic level, the normalized counts of each sampling period were statistically compared using two factorial ANOVA and are summarized in Table 2. Significant changes specific to Group IF were detected in the genus categories *Prevotella* 1, *Prevotella* 2, *Prevotella* 9, and uncultured bacteria (phylum *Bacteroides*),

Hypotrichomonas (phylum *Excavata*), *Megamonas* and uncultured bacteria (phylum *Firmicutes*). *Prevotella* 1, *Prevotella* 2, and *Hypotrichomonas* significantly increased, while *Prevotella* 9, *Megamonas*, and uncultured bacteria significantly decreased. One species, "*Hypotrichomonas mariae*", was identified from the phylum *Excavata* (*Eukaryota* > *Excavata* > *Metamonada* > *Parabasalia* > *Hypotrichomonadea* > *Hypotrichomonas* > *Hypotrichomonas mariae*). In *Prevotella* 1, one species, "*Prevotella ruminicola*", was identified (*Bacteria* > *Bacteroidetes* > *Bacteroidia* > *Bacteroidales* > *Prevotellaceae* > *Prevotella* 1 > *Prevotella ruminicola*). No other species were identified.

[Figure 3] [Table 2]

To characterize the specific microbes that emerged from the experimental conditions, we used the linear discriminant analysis (LDA) effect size (LEfSe) analytical tool (see methods) on the normalized counts of multiple levels of taxonomy. An LDA score < 2 was set as the cut-off, and the histogram in Figure 4 (a) shows the microbiomes on various taxonomic levels ranked according to the effect size in each experimental condition. Figure 4 (b) shows the characterization of the microbiomes that emerged from the experimental conditions with taxonomic phylogenetic trees. Dominant microbes at the phylum level are indicated with different colors, with shading according to each experimental condition (i.e., the Pre, Post, and After periods of Groups C and IF). Dominant phylum taxonomies of the Pre, Post, and After conditions in Group IF were *Proteobacteria*, *Fusobacteria*, and *Excavata*, respectively, which were clearly distinct from the dominant phyla in the conditions in Group C. Dominant phyla of Group C at the Pre and Post periods were *Actinobacteria* and *Firmicutes*, respectively. Only one taxon in *Actinobacteria* was dominant in the After condition. The large overlap in *Firmicutes* between the Post condition of Group C and the Pre condition of Group IF and the small overlap in *Proteobacteria* between the Pre conditions of Groups C and IF depicted the common characteristics of the microbiome without insect feeding intervention.

[Figure 4]

To examine the similarity of the microbiome populations among the groups, we calculated the Jaccard index to create a distance matrix. In this case, all conditions of the two groups were divided into three categories according to the presence or absence of insect feeding. "NoIF" included all conditions from Group C and the Pre condition from Group IF, meaning that they did not receive insect feeding. "IF" and "AfterIF" corresponded to the Post and After conditions of Group IF, respectively. The left panels of Figure 5(a) show the pairwise comparisons of the distances between the groups, i.e., the distances among IF (top panel), AfterIF (middle), and NoIF (bottom). PERMANOVA revealed an overall significant difference (p = 0.0068), and post hoc comparisons detected a significant difference only between IF and NoIF (p = 0.0131). Figure 5(b) shows the PCoA using these distance data from three groups, with the red, black and green circles denoting the IF, AfterIF, and NoIF groups. In support of the statistical results above, IF was spatially separated from NoIF in these coordinate spaces.

[Figure 5]

To observe the changes in the diversity of the microbiome during the course of the experiment, the Shannon H', evenness, and dominance indices were calculated for each group, as shown in the left panels of Figure 6. Within Group C, a significant change was found in the Shannon H' index between Pre and Post (p = 0.012) and Pre and After (p = 0.011). Within Group IF, a significant change was found in the Shannon H' index between Pre and After (p = 0.001) and Post and After (p = 0.001). Among the groups, there were significant changes in the Shannon H' index of the Post condition (p = 0.005) and in the dominance index of the Post condition (p = 0.038).

[Figure 6]

Transcriptome analysis of Groups IF and C

Altogether, 45 different transcriptome IDs were counted (see Supplementary Figure S1 for the normalized counts of the transcriptomes in each sampling point for each group, and Supplementary Table S3 for normalized count data of transcripts). The difference between two sampling points (Pre vs Post, Pre vs After, and Post vs After) of the counts of each transcriptome was evaluated by two-way ANOVA, as indicated by the right columns of Supplementary Table S3. Assuming that treatment-relevant change (1) would occur after the Post period, (2) would stay at or return to the baseline after ending treatment, and (3) would be observed only in the IF group, the transcriptomes having these characteristics would be the most interesting to analyze. The transcriptomes with these characteristics were m.926, m.1046, m.1047, m.1331, and m.1346. Among them, m.926 and m.1346 were identified as functional genes of chloroplast. Others were uncharacterized genes without hits in databases. To observe the difference in the diversities of the transcriptomes, Shannon H', evenness, and dominance indices were calculated, similar to the microbiome analysis. As shown in the right column of Figure 6, none of the indices showed significant differences between the conditions in each group.

Relationship of the changes between the microbiome and transcriptome

Although most transcriptomes determined to be significant were not annotated in the database, their functional significance was evaluated by analyzing the similarity of changes between the microbiome and transcriptome and whether such changes closely interacted with each other according to the experimental conditions. Thus, we first performed correlation network analysis to identify the clusters with high correlations among the conditions via Pearson's product moment correlation coefficient (see methods) on the SSU rRNA and transcriptome data. Figure 7 shows the overview of the changes in the microbiome and transcriptome listed together, according to the experimental period (indicated on the bottom of the heatmap) in Groups C (left) and IF (right). Note that the microbiomes and transcriptomes that behaved similarly were near to each other in the heat map. They clustered into four groups (indicated by the trees on the right of the heatmap) according to the similarity of changes. In cluster 1, the increase specific to the Post and After periods of Group IF was shown in the lower part of the panel, but all changes were in the transcriptomes (from 1385 to m.1384). In cluster 2, a clear decrease specific to the Group IF Post and After periods was observed in the lower half of the panel (from m.1331 to *Pontibacillus*), whereas no specific change was shown in Group C. In cluster 3, an increase in the Post

and After periods of Group IF was shown in the upper part of the panel, including three transcriptomes, *Prevotella* 1, and *Hypotrichomonas*. In cluster 4, there was a decrease in the Post period and then an increase in the After period of Group IF, except for m.1398. This pattern of change was not the same as that in Group C.

[Figure 7]

Finally, to observe the direct correlation of the changes between the microbiome and transcriptome according to the conditions, we analyzed the autocorrelation using all datasets of the microbiome and transcriptome. Figure 8 shows the selected results of the autocorrelational analysis to visualize the direct comparison. Because the clusters of interest were found to be 2, 3, and 4 from the results in Figure 7, they are indicated by black, white, and gray circles, respectively. Note that there were high correlations (i.e., squares with brighter red) between the microbiomes and transcriptomes marked with clusters 2 and 3 on the lower right part of the panel. Additionally, there were negative correlations between the microbiomes marked with cluster 2 and transcriptomes marked with cluster 3, and vice versa.

[Figure 8]

Microbiome and transcriptome of the insects fed to the marmosets

To determine whether the species uniquely observed in the samples of Group IF after insect feeding (i.e., *Hypotrichomonas mariae* and *Prevotella ruminicola*) that changed in the microbiome and transcriptome were attributable to the insects fed to the marmosets, we analyzed the microbiome and transcriptome of the crickets and mealworms, which were from the same lot as those fed to the subjects, using the same protocol described in the methods (see Supplementary Table S4 and S5 for normalized count data of the microbiome and transcriptome, respectively). Neither "*Hypotrichomonas mariae*" nor "*Prevotella ruminicola*" was identified in the insect samples. None of the transcriptomes that differentially changed after insect feeding (i.e., m.926, m.1046, m.1047, m.1331, and m.1346) were also detected in the samples.

Discussion

The present study showed that in captive common marmosets, the treatment of enhanced insect intake for only one week modified the microbiome population in the gut, which interacted with the transcriptome simultaneously extracted from the fecal samples. Changes observed in the microbiome and transcriptome were not attributable to the insects themselves. The results indicated that diet change for seven days had an impact on the host microbiome, which was similar to the results from a study with human subjects who were given an animal-based diet for only five days [26]; these results suggest the importance of insect feeding, which is naturally observed in wild populations of common marmosets, and that providing insects to captive marmosets is an improved feeding regimen.

Impact on the microbiome

The abundant microbes at the phylum level in the marmosets in our colony were *Bacteroides* and *Firmicutes,* as shown in Figure 2, which was partially similar to the results obtained in another study showing that *Bacteroides* was the most common in both groups with and without WMS [14]. Significant changes that occurred only in Group IF were in *Excavata* and *Firmicutes.* In these phyla, the changes occurred just after intervention and did not return to the baseline level. Changes in *Proteobacteria* were observed in both Groups C and IF, and thus, it is not attributable to insect feeding.

We observed that at the genus level, Bifidobacterium was relatively abundant in both groups, which was consistent with the results of a previous study [15], partly due to the shared complete marmoset food in common with our colony. In the phylum *Bacteroides,* Group IF-specific changes were observed in the genus "*Prevotella",*, and only "*Prevotella ruminicola"* was identified in "*Prevotella* 1" in Table 2 at the species level. This species utilizes noncellulosic polysaccharides as energy sources [27] and was not identified in the insect samples. Considering that common marmosets are highly dependent on gums and exudates from wild trees [12] and utilizing the products fermented by the gut microbiome for energy resources, it is interesting to confirm the interaction between two important food resources for common marmosets, indicating that once fed insects, the microbiome related to polysaccharide fermentation increased and remained even after ending the intervention.

Two different analyses, LEfSe and PCoA, confirmed that microbiome populations changing with and without insect feeding were differentially localized. Some overlaps between Group C and the Pre condition of Group IF in Figure 4 (b) validated that the microbiome of marmosets without insect feeding were similar in population. The PCoA in Figure 5 (b) confirmed the same results, and a plot of AfterIF (i.e., the After condition of Group IF) was more similar to IF than to NoIF. This provides a visualization of the changes in the populations of some microbiomes after insect feeding and the stabilization of these populations at the same level after two weeks.

Possible symbiotic relationships with flagellates

The change in the phylum *Excavata* was attributable to one species, "*Hypotrichomonas mariae",*, belonging to the genus *Hypotrichomonas.* This species was reported for the first time only recently in samples of the large intestine of northern greater galago (*Otolemur garnettii)* living in southeastern Africa [28]. The species did not come from the insect samples. Thus, this is the first report of the detection of *Hypotrichomonas mariae* in the feces of common marmosets, who originally lived in South America. Ceza et al. [28] also argued that because the hosts of *Hypotrichomonas* spp., including *H. mariae,* showed no sign of disease, the genus is harmless and a host-specific commensal to each species. *H. mariae* belongs to the order *Parabasalia,* and most of the species of this order are parasitic flagellates, such as *Trichomonas* spp., which are frequently detected in healthy nonhuman primates, including common marmosets [29], and *Pentatrichomonas hominis,* which has been detected in the feces of captive common marmosets [30]. Although they are rarely associated with clinical signs or pathology, these species may become a cause of diarrhea if host animals have problems in their immune system. The fact that the species appeared just after insect feeding suggested the possibility that *Trichomonas*

spp. play roles in balancing the microbiome in marmosets' guts. Future studies are needed to clarify the functional impacts and the dynamics of the microbiome caused by the increase in flagellates in common marmosets.

Comparison of these results with the results from animal-based diets

A study examining the effects of an animal-based diet on the microbiome in dogs reported that feces became firm and that the Shannon H' index increased after raw beef was added to commercial food for 14 days [31]. Chickens fed *Tenebrio molitor* larvae for 54 days showed an increase in *Firmicutes* and a decrease in *Bacteroides* at the phylum level [32]. Although there was no increase in Shannon H' after insect feeding, the decrease in the Shannon H' index and increase in dominance observed after ending the insect feeding suggested that insect feeding impacted the microbiome population in Group IF. Whether the short intervention period (7 days) was responsible for the absence of an increase in diversity can be determined by a study with a longer period of intervention.

Correlational change between the microbiome and transcriptome

Although significant changes were observed in some transcriptomes in Group IF, the information acquired from the database was not sufficient to speculate the functional relationships between the microbiomes and transcriptomes. For example, m.926 and m.1346 were identified as functional genes of chloroplast, but they were correlated with the microbiomes that did not show differential changes according to the experimental conditions (cluster 1 in Figure 7). In contrast, however, some microbiomes and transcriptomes showed similar changes and thus were categorized into the same clusters (clusters 2 and 3 in Figure 7). Additionally, the functional significance of the transcriptomes could be inferred by the microbiomes that showed similar changes across the experimental conditions, as shown in Figure 8. Thus, our analysis using total RNA-seq, which enabled us to concurrently detect the dynamics of the microbiome and transcriptome, was effective in searching for functional genes that are currently unidentifiable after establishing a metagenomic database of transcriptomes with a full-length cDNA library.

Limitations and future perspectives of the study

We expected that if the insect feeding for a week had any impact on the gut microbiome and gene expression, there should be changes observed only in Group IF after the intervention. Family members were compared in the two groups because they were thought to share similar microbiomes because families are social units in common marmosets, and social units share microbiome communities in primates [33]. As Table 1 and Table 2 show, however, there are many changes in the normalized counts observed in both groups and in only Group C. Because there was no systematic, visible reason for the change in Group C, sampling feces from several points in each experimental period may reduce the influence of temporal events.

In the present study, we used frozen crickets and giant mealworms instead of live ones. 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]. The present study showed that feeding common marmosets insects changes their physiological status by balancing the microbiome to modulate metabolites. Consideration must be given to decide 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 a high-calcium insect 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 change in the fecal microbiome 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 microbiome and transcriptome of captive common marmosets. The total RNA-seq method was used to analyze the microbiome and transcriptome 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 microbiome via food sources and suggest that the feeding ecology in wild habitats is an important key to developing food regimens appropriate for the microbiome of common marmosets.

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. Mean weight was 460g, with a range from 374g to 499g. 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 olfactory accessible to each other. The case was in a breeding room on a 12- hour light-dark cycle, maintained at 28° and 50% of the temperature and humidity, respectively. According to this housing condition, they 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 post-mortem 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 sardine. 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 feces or diarrhea. 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 treatment of insect feeding, 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 [34].

Procedure

Experimental design. The family was divided into two experimental groups, each of three subjects. One group (Group IF, consisted of three offspring females) was fed one cricket and one giant mealworm per day for seven continuous days. The other group (Group C, consisted 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 fecal 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 reservation. To analyze the microbiome and the transcriptome of the feces from the marmosets, fresh samples were collected from the floor of the breeding cage. To collect the fresh feces, we waited for defecations early in the morning (8:30–10:00 AM) when they usually frequently defecated. Three pieces of feces were collected from each group, and the feces 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 feces 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 the cDNA construction processes.

Fecal and insect RNA extraction, sequencing, and taxonomic annotation. Fecal RNA was purified by using the RNeasy PowerMicrobiome kit (Qiagen, Hilden, Germany). The kit was operated with an automatic system, QIAcube (Qiagen, Hilden, Germany), according to the protocol (RNA_RNeasyPowerMicrobiome_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. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers DRA008965 and DRA008966 for the marmoset and insect microbiomes, respectively.

We used a total RNA-seq analysis method based on the pipeline of All-RNA-Information sequencing ("ARIseq" [16]) for analyzing both rRNA and mRNA profiles to identify the taxonomy of the microbiome and to search for their functions.

The obtained raw paired-end reads were trimmed by using Trimmomatic–0.35 [35] with seed mismatch settings: palindrome clip: simple clip threshold = 5:30:7, minimum read length of 100 bp, head crop of 6 bp and 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 [36] with local mode default condition as "best-hit" analysis. The data were transformed to BAM format for expression analysis. Mapped reads were counted by using eXpress [37] 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 [38].

To analyze the metatranscriptome, paired-end reads were assembled by using the Trinity v2.4.0 program package [39] with paired-end mode default setting. Open reading frames (ORFs) and the encoded protein sequences were predicted using the 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 [40] (https://trinotate.github.io/). The obtained functional annotations were combined with read count data by R.

The obtained read count data were normalized according to Love et al. [41] using the TCC package in R. Additionally, SSU rRNA reads or ORFs with less than 10 mapped reads in total from all samples in the original count data were excluded by R.

Data analysis

The general health condition of the subjects was evaluated by the weight and the fecal score. The weight was measured once during each period of the experiment (i.e., Pre, Post, and After) as a part of the weekly physical examination performed by our laboratory. The fecal scores were measured daily by visual inspection of the fecal shape into three levels (partially adopted from [42]): 3 corresponded to normal feces (solid, with little liquid), 2 corresponded to loose feces (globules with liquid but still formed), and 1 corresponded to diarrhea (mostly globules, a large amount of liquid, and partially muddy).

The effects of the treatments (insect feeding vs control) and the sampling periods (Pre, Post, and After) on the microbiome were statistically analyzed using 2-way ANOVA with the post hoc comparison corrected by the Bonferroni method. To evaluate the diversity of the microbes in the fecal samples, the Shannon H' index was calculated at the genus level by using the equation H' = -Σpilog2pi, where pi = ni/N. Evenness was also calculated using the equation J' = H'/H'_{max}, where H' is the Shannon H' index and H'_{max} is the possible maximum value of H' (all species evenly distributed). Dominance was calculated by D = Σ(ni/n)^2, where ni is the number of individuals of taxon i. These indices were calculated by using the software package PAST (ver. 3.23, [43]).

For the microbiomes showing significant differential changes according to the experimental conditions revealed by ANOVA, correlation network analysis was performed based on Pearson's product moment correlation coefficient matrix by using the R package. The normalized read count datasets for the SSU rRNA, transcriptome and mixed data were used for calculation. The obtained network was detected with a threshold ≧ 0.5 and visualized by Gephi with OpenOrd algorithm [44, 45]. The cluster members were sampled from the datasets based on a correlation network, which was used to draw a heatmap with hierarchical clustering by using the ComplexHeatmap package [46] in R.

To visualize the altered microbes under the specific experimental conditions and their phylogenetic relationships, we used the LEfSe program package [47] to make a cladogram (Figure 4) by following the instructions published online (https://huttenhower.sph.harvard.edu/galaxy/). We applied a LEfSe threshold of 2, an alpha value of 0.1 for the ANOVA test, and an alpha value of 0.1 for the Wilcoxon test.

The distance between the groups was calculated for detecting similarities using the Jaccard index and was tested statistically by PERMANOVA using the QIIME 2 plugin "diversity core-metrics" and "diversity beta-group-significance" [48]. For "diversity core-metrics", "–p-sampling depth" was set at 500. For "diversity beta-group-significance", "—p-permutations" was set at 10000. In this analysis, the samples were divided into three groups: PostIF, AfterIF, and NoIF. PostIF and AfterIF corresponded to the Post and After conditions of Group IF. NoIF included all conditions from Group C and the Pre condition of Group IF. The distance matrix between the groups was also used for calculating PCoA using the "cmdscale" function in R. Then, the results were plotted using the dimensions between 1 and 2, 2 and 3, and 1 and 3.

List Of Abbreviations

ANOVA: analysis of variance

Group C: group control.

Group IF: group insect feeding

LDA: linear discriminant analysis

LEfSe: linear discriminant analysis effect size

MWS: marmoset wasting syndrome

ORF: open reading frame

PCoA: principal coordinate analysis

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. A 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

A. I. and Y. Y. are directors of RIKÆNALYSIS Corporation (RIKEN Venture Company). The other authors declare no competing financial interests.

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Authors' contributions

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

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N/A

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Figures

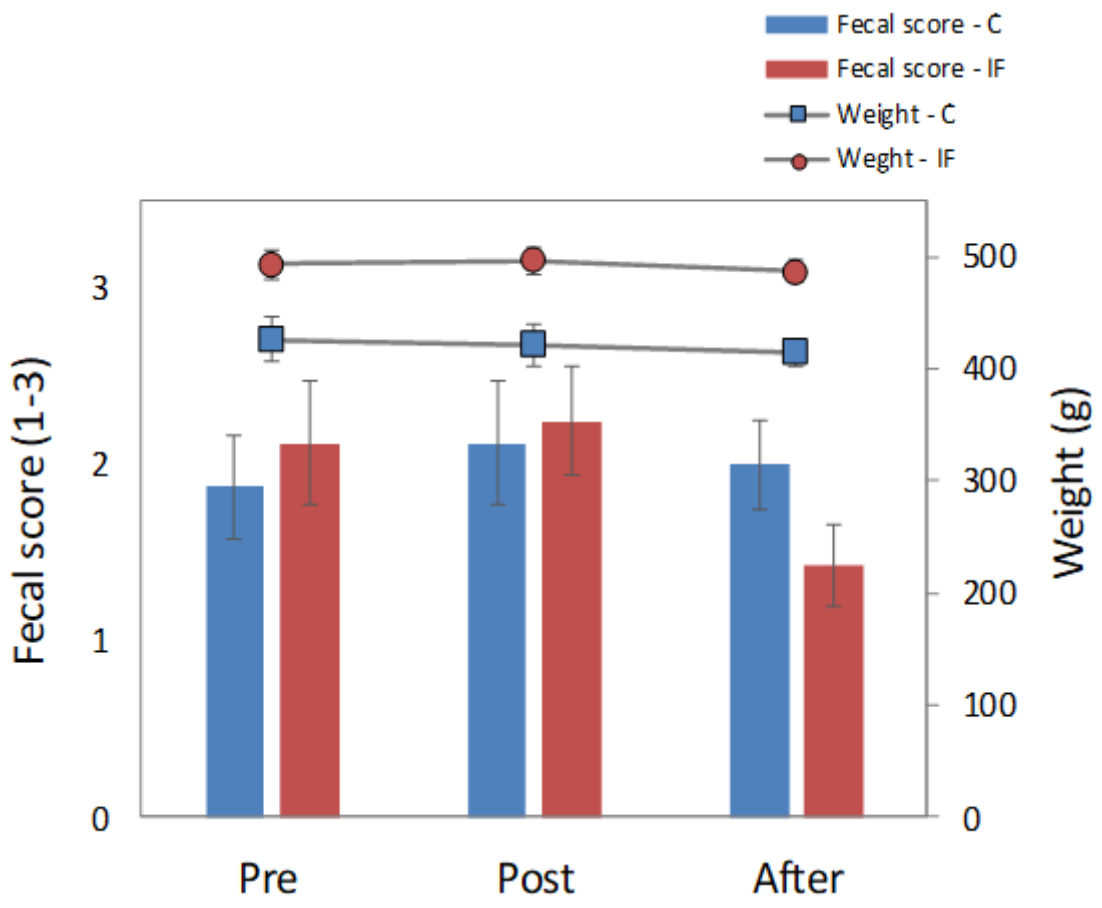

Figure 1

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

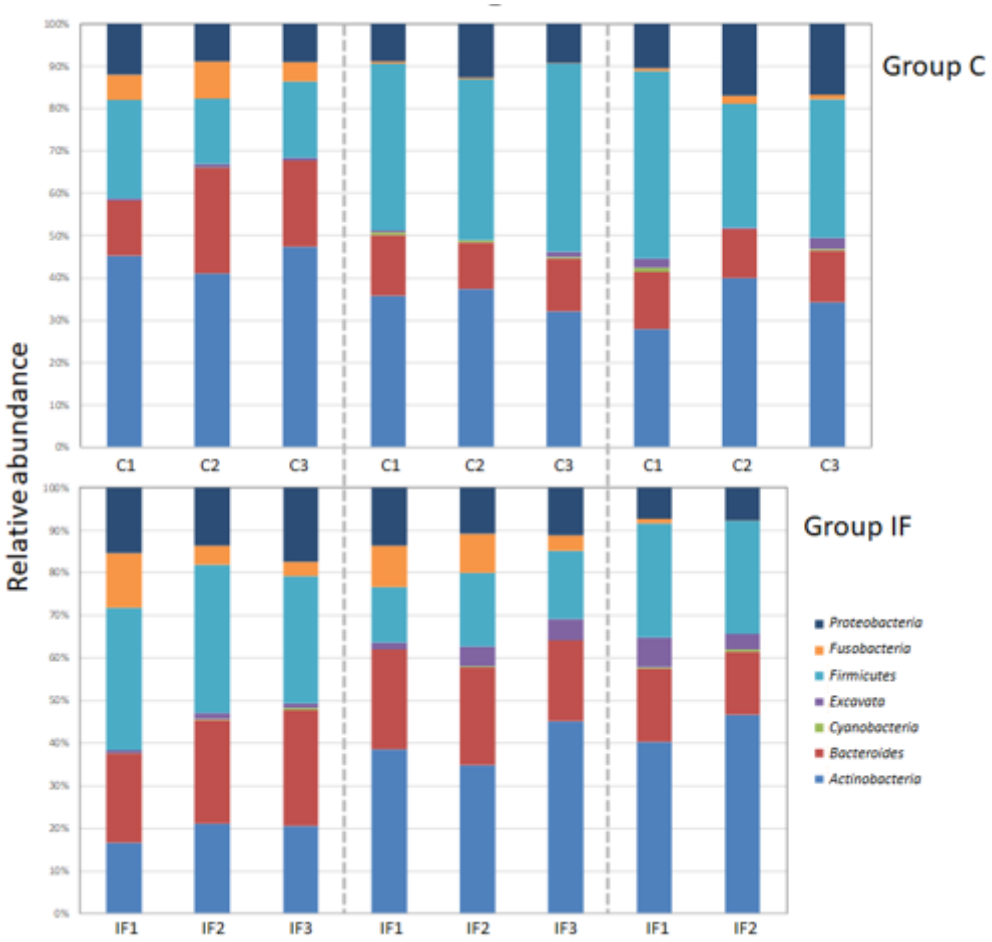

Figure 2

Relative abundance of microbes at the phylum level in the Pre, Post, and After periods for Groups C (upper panel) and IF (lower panel).

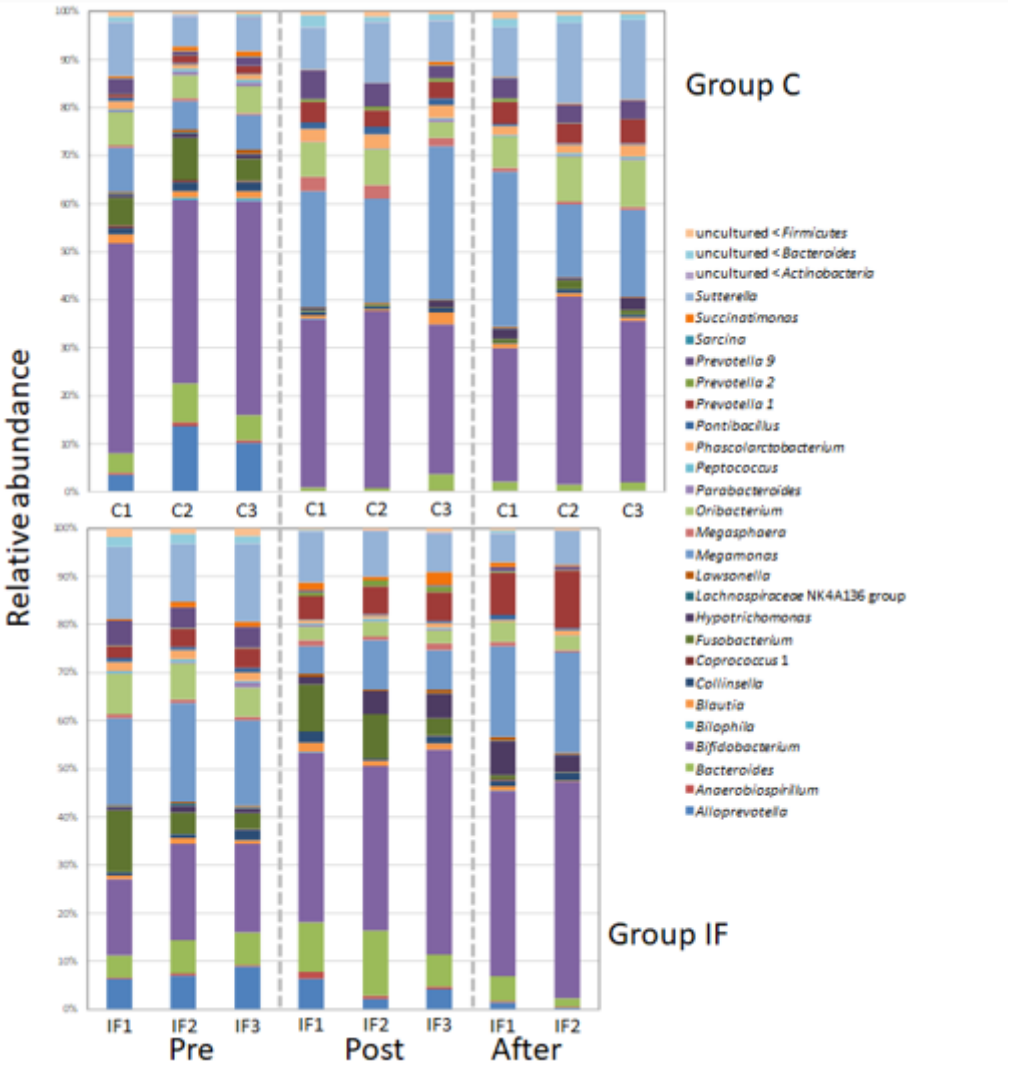

Figure 3

Relative abundance of microbes at the genus level in the Pre, Post, and After periods for Groups C (upper panel) and IF (lower panel).

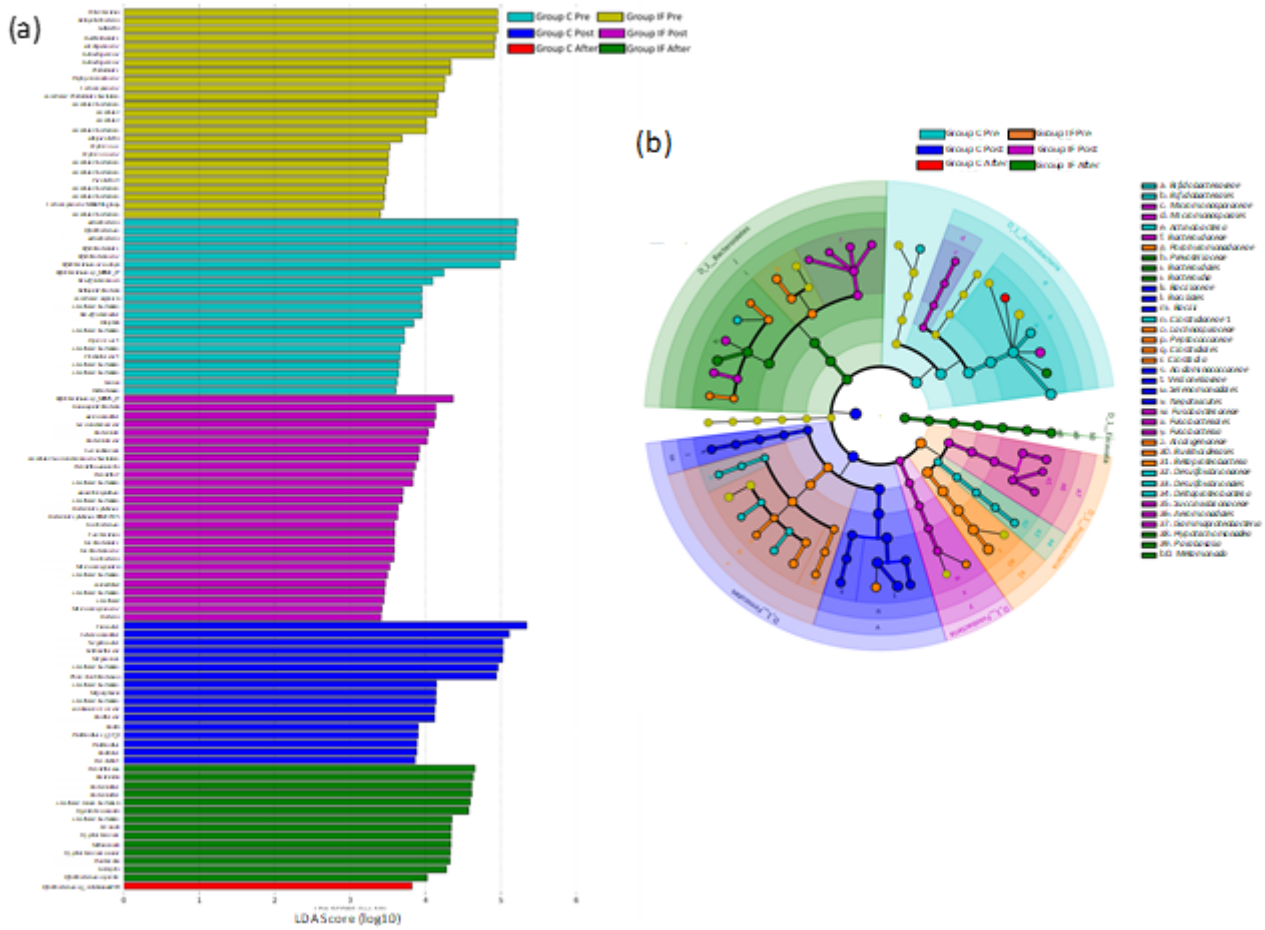

Figure 4

LEfSe characterization of the dominant microbial taxa according to the experimental conditions of each group. (a) Ranked lists of microbes by effect size for Groups C and IF in all three conditions. (b) Cladogram based on the ranked list in (a) to visualize the relationships between the experimental conditions and the phylogenetic relationships among the microbes. (This figure has also been copied to a supplemental file for better readability.)

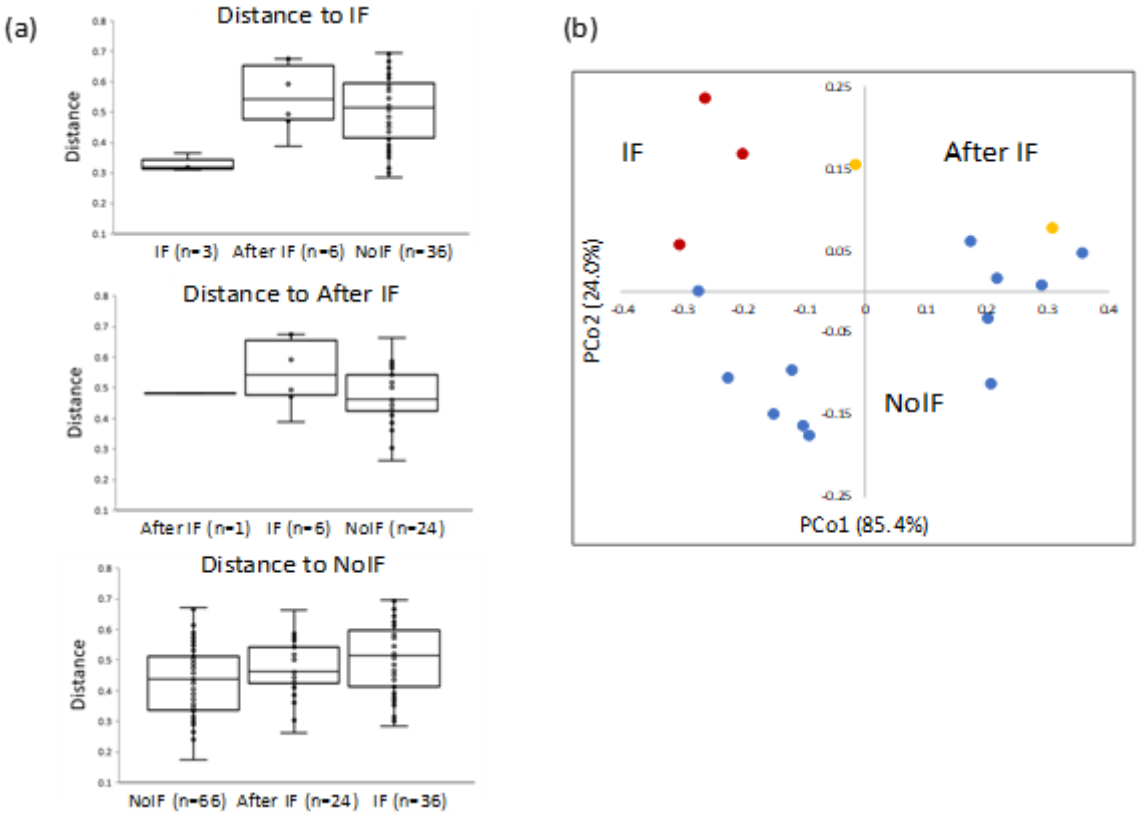

Figure 5

(a) Pairwise comparisons of the distances as calculated by the Jaccard index: distance to IF (top), AfterIF (middle), and NoIF (bottom). (b) PCoA of the distances among the groups. Red: IF; yellow: AfterIF; blue NoIF.

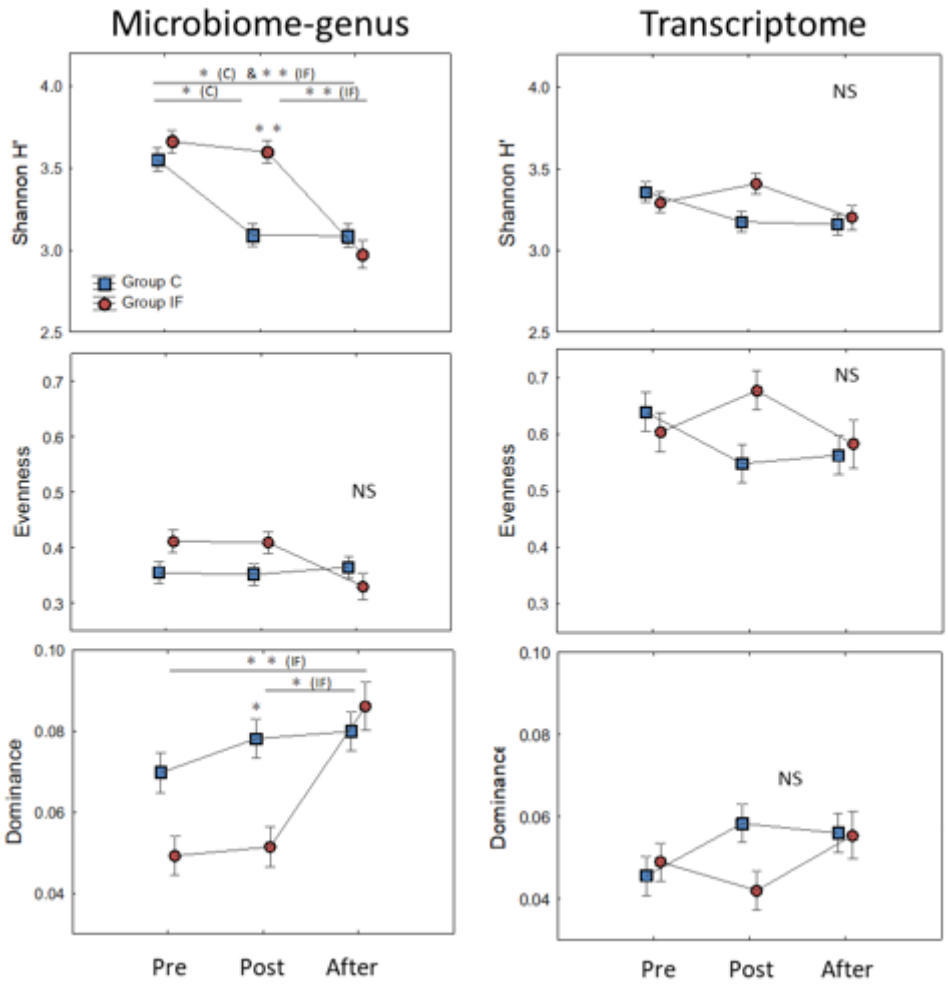

Figure 6

Shannon (H'), evenness, and dominance diversity indices of the microbiome at the genus level (left panels) and of the transcriptome (right panels) in Groups C (blue squares) and IF (red circles). Significant differences between the conditions of each group are depicted by asterisks with horizontal bars above the graph, whereas those between groups are depicted by asterisks above the plots. *: $p < 0.05$, **: $p < 0.01$.

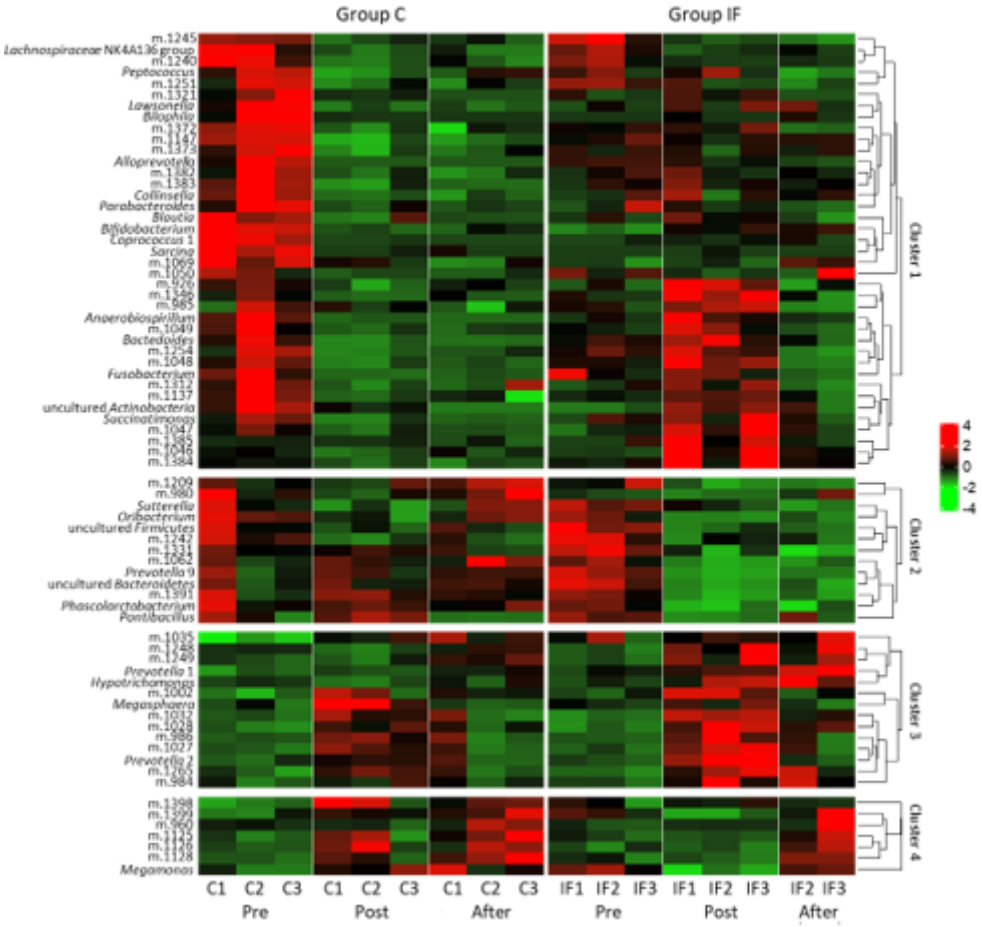

Figure 7

Heatmap of the correlation network analysis between the SSU rRNA data of the microbiome and the transcriptome differentially clustered into four clusters for each group. Hierarchical clustering enabled items from the SSU rRNA and transcriptome data to be listed together, where items with similar characteristics were listed close to each other. Increases and decreases are shown in red and green, respectively.

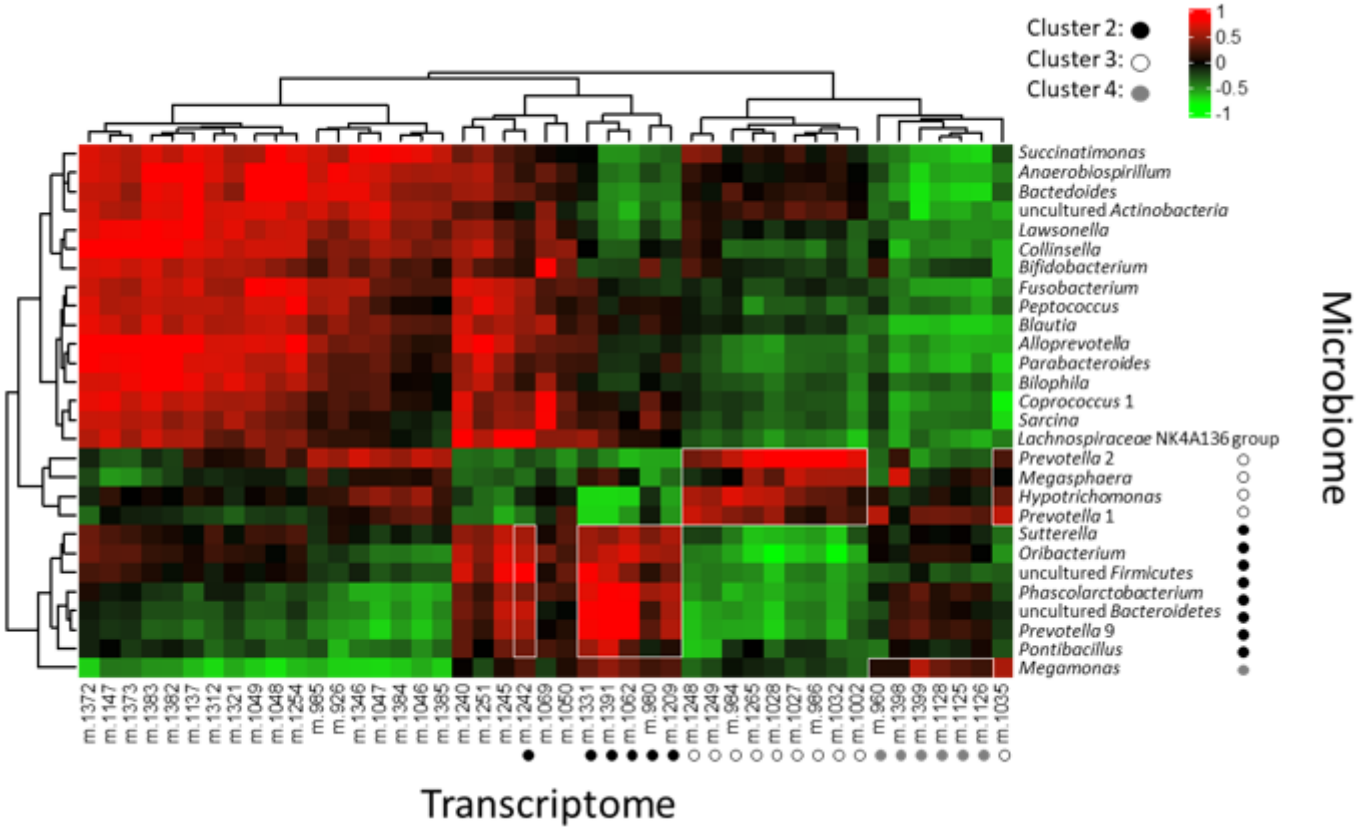

Figure 8

Correlational network between the microbiome and transcriptome. Three clusters that show covariant changes among conditions are depicted in this figure with circles (cluster 2: black; cluster 3: white; cluster 4: gray) next to the microbiome or transcriptome IDs. Correlations from the same clusters are marked with white rectangles on the heatmap.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SITableS5TranscriptomeAnnotationInsect.xlsx
- NC3RsARRIVEGuidelinesChecklistYumikoYamazaki.pdf
- Tables.docx
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- SITableS3MeanNormTranscriptome.docx
- SITableS4rRNANormAnnotationInsect.xlsx
- SIFigureS1NormTranscriptome.docx
- Figure4.docx
- SITableS2TranscriptomeAnnotation.xlsx