Original Article

Title

Effects of fetal growth restriction on postnatal gut microbiota in a rat model

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Conflicts of Interest and Source of Funding

None declared

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Author Roles

H.S. and I.S. contributed to the conception and design of this study. Y.A. and I.S. acquired fecal samples. Y.A., K.A., and K.T. performed the statistical analysis, interpreted the data, and drafted the manuscript. H.S. and T.S. critically revised the manuscript for important

intellectual content. All authors have read and approved the final manuscript.

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Abstract

Objective: Fetal growth restriction (FGR) indicates increased risks of lifestyle-related diseases in adulthood. Previous studies showed the association between human gut dysbiosis and various diseases. However, reports examining the relationship between FGR and gut microbiota are scarce. Herein, we hypothesized that FGR may cause gut dysbiosis and analyzed the gut microbiota in a FGR rat model by restricting maternal protein intake during pregnancy.

Methods: The FGR group was developed by feeding pregnant Sprague Dawley rats a diet containing 7% protein until birth. Control rats were fed 21% protein. Fecal samples of 2–11-week-old pups were collected weekly. DNA was extracted from each sample and subjected to PCR amplification and sequencing. Additionally, short-chain fatty acids in the cecum were analyzed at two weeks of age, when there were differences in the occupancy of the gut microbiota.

Results: Comparative analysis of the gut microbiota showed differences only at two weeks of age. *Verrucomicrobia* was significantly more abundant in the control group ($q < 0.1$), whereas pathogenic bacteria, including *Enterococcus* and *Enterobacteriaceae*, tended to increase in the FGR group. The abundance of acetic and butyric acid-producing bacteria also differed between groups. Acetic acid in the cecum was considerably

decreased in the FGR group, while butyric acid was increased compared to that in the control group.

Conclusions: Normalizing the alteration of FGR on postnatal gut microbiota may have beneficial effects for the host, since the FGR group caused gut dysbiosis.

What is known

- Fetal growth restriction indicates increased lifestyle-related disease risk in adulthood.
- Dysbiosis is associated with various diseases, including obesity and diabetes.
- The mother-child environment and factors including the conception period and delivery and lactation methods influence the postnatal gut microbiota.

What is new

- The FGR group exhibited differences in fecal microbiota and changes in short-chain fatty acid concentration of the cecum compared to the control group in the early postnatal period.
- In fecal microbiota, pathogenic bacteria, including *Enterococcus* and *Enterobacteriaceae*, tended to increase in the early postnatal FGR group, while *Akkermansia* decreased.

1. INTRODUCTION

In recent years, the prevalence of low birth weight infants (< 2,500 g) has remained high among full-term babies (1), accounting for approximately 9.4% of all births recorded in 2019 (2). One of the plausible reasons of low weight during the birth can be attributed to fetal growth restriction (FGR), which is caused by undernutrition of pregnant women (resulting from the personal desire to lose weight and insufficient nutritional intake) (3), decreased placental blood flow, and placental insufficiency due to increased gestational age. Approximately one-fourth of young Japanese women are "thin," with body mass index (BMI) of less than 18.5 kg/m^2, where the mean total caloric intake remained below 1,600 kcal/day during pregnancy (4). Regarding FGR, the Developmental Origins of Health and Disease (DOHaD) theory has recently been proposed, which indicates that various environmental factors during development from the embryonic period to infancy influence the risk of lifestyle-related diseases in adulthood and beyond (5).

Contrastingly, many studies have shown that human gut dysbiosis is associated with various diseases, including obesity and diabetes (6-8). The human gut microbiota forms a complex ecosystem that interacts with the metabolic and immune activities of the host and is closely related to normal physiological functions, health, and disease (9). Short-chain fatty acids, including acetic acid, propionic acid, and butyric acid, are metabolites

of intestinal bacteria that maintain the intestinal barrier function and act intracellularly as direct signals. The gut microbiota of infants, which changes considerably in the early postnatal period, is intricately related to factors such as maternal stress during pregnancy, obesity, mode of delivery, mode of lactation, and drug administration (10). However, reports examining the relationship between FGR and gut microbiota are scarce.

In this study, we hypothesized that FGR may cause gut dysbiosis and analyzed the gut microbiota in a rat model of FGR by restricting maternal protein in pregnancy. Additionally, based on the results, short-chain fatty acids measurement in the cecum was performed.

2. METHODS

2.1. Animals and experimental designs

Four eight-week-old Sprague Dawley rats (two males and two females) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and housed in individual cages in the same room with free access to food and water at 24–25°C, 60% relative humidity, and 12:12 h light/dark cycle at Juntendo University Animal Facility (Tokyo, Japan). The experiment did not begin immediately; rather, the rats, who were 11 weeks old, were mated three weeks after they had been given time to acclimate to their

environment. Pregnant rats were fed either a regular diet containing 21% protein (control group) or an isocaloric diet containing 7% protein (FGR group) from the day pregnancy was confirmed until delivery. Protein and food restriction in rodents are widely used to model prenatal undernutrition in pregnancy (11). The diets with different compositions (Table S1) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Each group consisted of one pregnant rat. After delivery, each mother rat was fed a normal diet for 21 d of lactation. The pups were weaned at 21 d of age; thereafter, two pups were housed in each cage and fed a normal diet. At one week of age, the number of pups was adjusted to four males each in the FGR and control groups. Since several studies have suggested that males are more susceptible to early-life adversity because of a strategy to prioritize growth (12), female rats were preferentially excluded from this study. Their weight was measured at birth and at 1, 3, 4, 8, and 12 weeks of age. Fecal samples were collected from four male pups in each of the FGR and control groups weekly between 2–11 weeks of age. Each pup was temporarily separated from its mother and spontaneously excreted feces were collected. Fresh samples were frozen at -80°C until analysis.

Additional experiments were performed after the gut microbiota analysis. By restricting maternal protein in pregnancy using the abovementioned method, an additional sub-group of the FGR group was created with a corresponding control group. Their weight was

measured at birth. Short-chain fatty acids of the cecum, but not of the feces, were measured in four male pups in each of the FGR and control groups at two weeks of age when there were differences in the occupancy of the gut microbiota. The cecum and the proximal colon are the principal sites of colonic fermentation in rodents. Considering that short-chain fatty acids are absorbed in the colon and that sufficient sample volumes were collected, the content of the cecum was in the target of this study. Fresh samples were frozen at -80°C until analysis.

This study was approved by the Animal Experiment Committee of Juntendo University, Japan (Animal Experiment Protocol Approval No. 2022257) and strictly followed the recommendations for laboratory animals of Juntendo University.

2.2. Microbiota analysis

PCR amplification and DNA sequencing of the V3–V4 region of the bacterial 16S rRNA gene was performed using the Illumina MiSeq instrument (Illumina, San Diego, CA, USA), as described previously (13). After removing sequences that are consistent with data from the Genome Reference Consortium Human Build 38 (GRCh38) and phiX reads from the raw Illumina paired-end reads, the sequences were analyzed using the QIIME2 software package version 2017.10 (https://qiime2.org/). Potential chimeric sequences

were removed using DADA2 (14), followed by trimming 30 and 90 bases at the 3′ region of the forward and reverse reads, respectively. Taxonomical classification was performed using a naïve Bayes classifier trained on the Greengenes13.8 with a 99% threshold of full-length sequences of operational taxonomic units. QIIME2 software was used for alpha diversities and Bray-Curtis principal coordinate analysis (PCoA).

2.3. Short-chain fatty acids measurement

The concentrations of short-chain fatty acids (acetic acid, propionic acid, butyric acid, and other minor compounds, such as iso-butyric acid, n-valeric acid, iso-valeric acid, and n-caproic acid) were measured using gas chromatography (GC) (GC-FID (7890 B; Agilent Technologies, USA) after extraction. Briefly, 100 mg of the sample was transferred into bead tubes, mixed with 9x 0.5% phosphoric acid solution, and then heat-treated (85°C, 15 min). After centrifugation (18,400 ×*g*, 10 min), the supernatant was transferred to a new tube, mixed with an equal volume of ethyl acetate, and centrifuged again (18,400 ×*g*, 10 min). The ethyl acetate layer was transferred to a vial, and the internal standard (4-methyl valeric acid) was added as described previously (15).

2.4. Statistical analysis

GraphPad Prism version 9.4.1 was used for statistical analysis. Differences in body weight and short-chain fatty acids between the FGR and control groups were confirmed using the unpaired t-test. Significant differences were considered at $p < 0.05$.

Intergroup difference of microbiota was analyzed using ALDEx2 (16), and significant differences were considered at $q < 0.1$. A permutational multivariate analysis of variance (PERMANOVA) test for Bray-Curtis distances was used for multivariate analysis to test the variation in microbiota composition explained by each factor.

3. RESULTS

3.1. Development of the FGR model

The mean birth weight ± SD of the FGR group (5.25 ± 0.32 g, n = 13) was lower than that of the control group (6.64 ± 0.36 g, n = 10) with statistical significance ($p < 0.05$), confirming that the median birth weight of the FGR group (5.30 g) was lower than the 10th percentile distribution of the birth weight of the control group (6.03 g) (Figure S1). At 12 weeks of age, the mean birth weight in the FGR group was significantly lower than that in the control group ($p < 0.05$). However, the percentage of birth weight (×100%) remained higher in the FGR group than in the control group, indicating a gradual increase (Figure S2). At 12 weeks of age, the percentage of birth weight in the FGR group was

significantly higher than that in the control group ($p < 0.05$).

3.2. Analysis of gut microbiota in feces

We analyzed the fecal microbiota of four rats in each group. At the phylum level, from 3 to 12 weeks of age, *Firmicutes,* followed by *Bacteroidetes,* were the most prevalent in FGR and control groups. However, the FGR group at two weeks of age had significantly more *Proteobacteria* than the control group of similar age ($q < 0.1$), whereas the control group had more *Verrucomicrobia* (Figure 1-a). The ALDEx2 results showed statistical significance only at two weeks. At the genus level, *Clostridiales*;f_;g_, *Clostridium*, *Eubacterium*, and *Allobaculum* were significantly more abundant in the control group ($q < 0.1$), whereas *Blautia* and *Enterococcus* were more abundant in the FGR group. *Akkermansia* and *Enterobacteriaceae*, which belong to *Verrucomicrobia*, and *Proteobacteria*, which differed at the phylum level, were not significantly different at the genus level but tended to be more abundant in the control and FGR groups, respectively (Figure 1-b). Principal coordinate analysis (PCoA) of the fecal microbiota showed that the balance of the gut microbiota was generally different, as well as in the early postnatal period. However, the tendency for the microbiota to stabilize with age was similar between the two groups (Figure 2). The alpha diversity of the flora increased with age,

but no difference was observed between the two groups (Figure 3).

3.3. Analysis of short-chain fatty acids in the cecum

In the additional sub-group of the FGR group, the mean birth weight ± SD of the FGR group (5.94 ± 0.39 g, n = 7) was lower than that of the control group (8.41 ± 0.47 g, n = 14) with statistical significance ($p < 0.05$). Short-chain fatty acid concentrations in the cecum were measured in four male pups of each group at two weeks of age. The mean acetic acid ± SD of the FGR group (12.40 ± 0.76 μmol/g, n = 4) was significantly lower than that of the control group (13.83 ± 0.55 μmol/g, n = 4) ($p < 0.05$), whereas the mean butyric acid ± SD of the FGR group (2.73 ± 0.30 μmol/g, n = 4) was significantly higher than that of the control group (2.13 ± 0.34 μmol/g, n = 4) ($p < 0.05$). Propionic acid levels tended to decrease in the FGR group, although the difference was not statistically significant. No differences were found in other minor short-chain fatty acids (Figure S2).

4. DISCUSSION

The gut microbiota of newborns, especially preterm infants, is more susceptible to external factors than that of adults due to the developmental stage. Therefore, the combined effects of external factors cause differences in postnatal changes in the

individual microbiota (17). Due to various difficulties in examining the human gut microbiota until adulthood, we analyzed the gut microbiota in the FGR rat model with gestational maternal protein restriction and compared it with that of the control group. This study showed that the gut microbiota in the early postnatal feces of the FGR group differed from that of the control group before weaning and stabilized after feed intake started. Pathogenic bacteria, such as *Enterococcus* and *Enterobacteriaceae*, tended to increase in the early postnatal FGR group. In addition, *Akkermansia*, known to contribute to maintaining the mucin layer and reducing obesity and hyperglycemia, was more abundant in the control group than in the FGR group.

It would have been desirable to collect the first stool sample around one week after birth. This might have helped us obtained even more robust results, since from 8 to 14 days after birth, rat pups not only suckle but may also eat solid food from the scraps in the bedding. However, since we temporarily separated the pups from their mothers and collected spontaneously excreted stool samples, we decided to collect the samples at one-week pre-weaning to ensure enough samples and to avoid excessive stress on the mother rats. In a previous study comparing the gut microbiota in the FGR and normal rats, on day 12 after birth, the total number of bacteria and population size of some individual strains (*Bifidobacterium* sp., bacteria from clostridial clusters IV and XIVa) were

decreased in pups with FGR compared to those in control pups (18). Although the present alteration in the FGR group differs from that in the previous report, this result in the postnatal gut microbiota obtained one-week pre-weaning indicated that the changes in the FGR group were unfavorable in terms of pathogenicity and benefits.

The current results indicated that, among bacteria producing acetic acid and butyric acid, the genera *Eubacterium* and *Clostridium* were more abundant in the control group than in the FGR group, while the genus *Blautia*, which is reported to be significantly and inversely correlated with visceral fat in humans ($p < 0.05$ for men and $p < 0.01$ for women) (19), was more common in the FGR group. The short-chain fatty acid content in the rat cecum was measured at two weeks of age. Acetic acid was decreased considerably in the FGR group, while butyric acid increased compared to the control group. The higher relative proportion of short-chain fatty acid producers in the control group can explain this change in acetic acid level. However, it is difficult to explain the increase in butyric acid level in the FGR group. In humans, a previous report showed that administering *Bifidobacterium breve* as a beneficial probiotic during the neonatal period significantly decreased fecal butyric acid concentrations ($p < 0.05$). In addition, the ratio of acetic acid to total short-chain fatty acids was significantly increased in immature infants compared to those in the control groups ($p < 0.05$) (20). Butyric acid has anti-inflammatory and

proinflammatory properties and beneficial effects on the intestinal tract in adults. In infants, butyric acid increases interleukin-8 secretion in enterocytes induced by inflammatory stimuli. Interleukin-8 functions as a potent chemotactic factor for neutrophils, which induces inflammation. Therefore, the results of the decreased acetic acid and increased butyric acid obtained one-week pre-weaning in the FGR group could be unfavorable for the host.

In humans, the postnatal gut microbiota is influenced by the mother-child environment, such as the period of conception, method of delivery, and method of lactation. In terms of delivery method, the transmission of maternal *Bacteroides* strains and high-level colonization by opportunistic pathogens are reported to be associated with the hospital environment in babies delivered by cesarean section (21). Moreover, the relative occupancy of the gut microbiota in breast- and formula-fed infants was different (22). Based on these findings, the present study further revealed that changes in the gut microbiota occur in FGR rats in the same mother-child environment, except for the difference in maternal protein restriction during pregnancy.

This study has some limitations. The composition of breast milk after delivery was not confirmed, and the effects of oligosaccharides and lactoferrin as prebiotics were not explored. Additionally, FGR is not only caused by low nutrition during pregnancy but

also by factors such as low body weight before pregnancy, maternal smoking, and decreased blood flow to the placenta; thus, many factors come into play in clinical practice. Therefore, the present results were obtained in a maternal low-protein nutrition model during pregnancy, and the possibility that other factors may combine to produce different results should be considered. Moreover, this study included only rats, and the types of bacteria living in rats are different from those living in humans. Among the bacteria that showed significant differences in this study, some, such as *Allobaculum*, were frequently detected in rats, while others, such as *Blautia* and *Enterococcus*, were also frequently detected in humans. Therefore, it may be difficult to apply the results of this study to humans, but the difference in microbiota between the control group and the FGR group is a significant result despite the difference in hosts.

FGR is considered as a risk factor causing lifestyle-related diseases in adulthood, such as hypertension, cardiovascular disease, obesity, insulin resistance, diabetes, and metabolic syndrome (23, 24). However, in the present study, the long-term effects of changes in the gut microbiota in the FGR group have not been studied. Based on the previous studies and the current results, we speculate that early postnatal dysbiosis in FGR offspring may represent a future risk for lifestyle-related diseases (25), and further studies are needed to investigate the direct correlation of FGR and these diseases. Normalizing the alteration

of FGR on early postnatal gut microbiota may have beneficial effects for the host.

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Figure and Table legends

Figure 1-a: Fecal microbiota composition in samples at the phylum level of the fetal growth restriction (n = 4) and control (n = 4) groups. Relative abundances are shown on the vertical axis and weeks on the horizontal axis.

Figure 1-b: Fecal microbiota composition in samples at the genus level of the fetal growth restriction (n = 4) and control (n = 4) groups. Genus levels with significant differences in relative abundance at two weeks of age, and *Akkermansia* and *Enterobacteriaceae*;g_ are shown in box-and-whisker plots. * q-value < 0.1 was considered as a significant difference.

Figure 2: Bray–Curtis principal coordinate analysis (PCoA) of the fecal microbiota. UniFrac PCoA of fecal microbiota in samples collected from the fetal growth restriction (FGR) (n = 4) and control (n = 4) groups. Two-group distances were calculated based on the presence or absence and relative abundance of the observed bacterial taxa, respectively. Closer plots in the PCoA figure indicate more similar microbiota composition. The percentage of variation explained by the principal coordinates (PC) is indicated on the axes. The round markers represent the control group, and the square

markers represent the FGR group.

Figure 3: Alpha-diversity indices. The solid line and round markers represent the control (n = 4) group, and the dotted line and square markers represent the fetal growth restriction (n = 4) group.

Supplementary Figure S1: Birth weights of the fetal growth restriction (FGR) (n = 13) and control (n = 10) groups (A). * $p < 0.05$ FGR group vs. control group (t-test).

Supplementary Figure S2: Weight of FGR and control groups from birth to 12 weeks of age (A). The graph is plotted as logarithmic Y axis. Data shown are averages of each group (n = 4 male pups) after one week of age.

% of birth weight ($\times 100\%$) of FGR and control groups from birth to 12 weeks of age (B). The percentage change from birth at each day of weight measurement is shown. * $p < 0.05$ FGR group vs. control group (t-test).

Supplementary Figure S3: Short-chain fatty acid concentration in the cecum of the fetal growth restriction (FGR) (n = 4) and control (n = 4) groups at two weeks of age. Data are

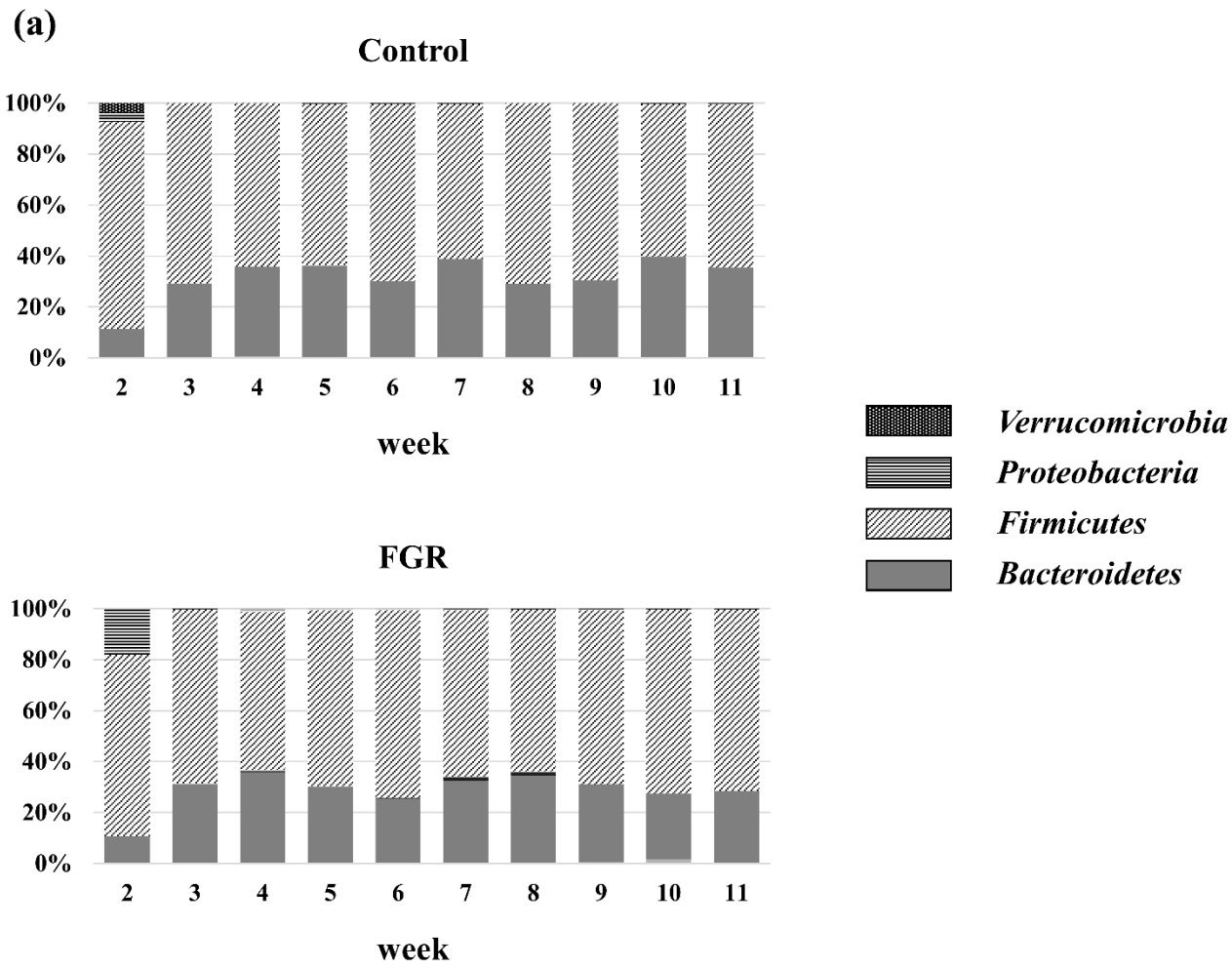
shown in box plot and expressed in micromoles of each short-chain fatty acid per gram of cecum content. * $p < 0.05$ FGR group vs. control group (t-test).

Supplementary Table S1: Comparative feed compositions

Supplementary Table S2: Significant difference test between groups at the Phylum level (ALDEx2)

Supplementary Table S3: Significant difference test between groups at the Genus level (ALDEx2)

Figure. 1-a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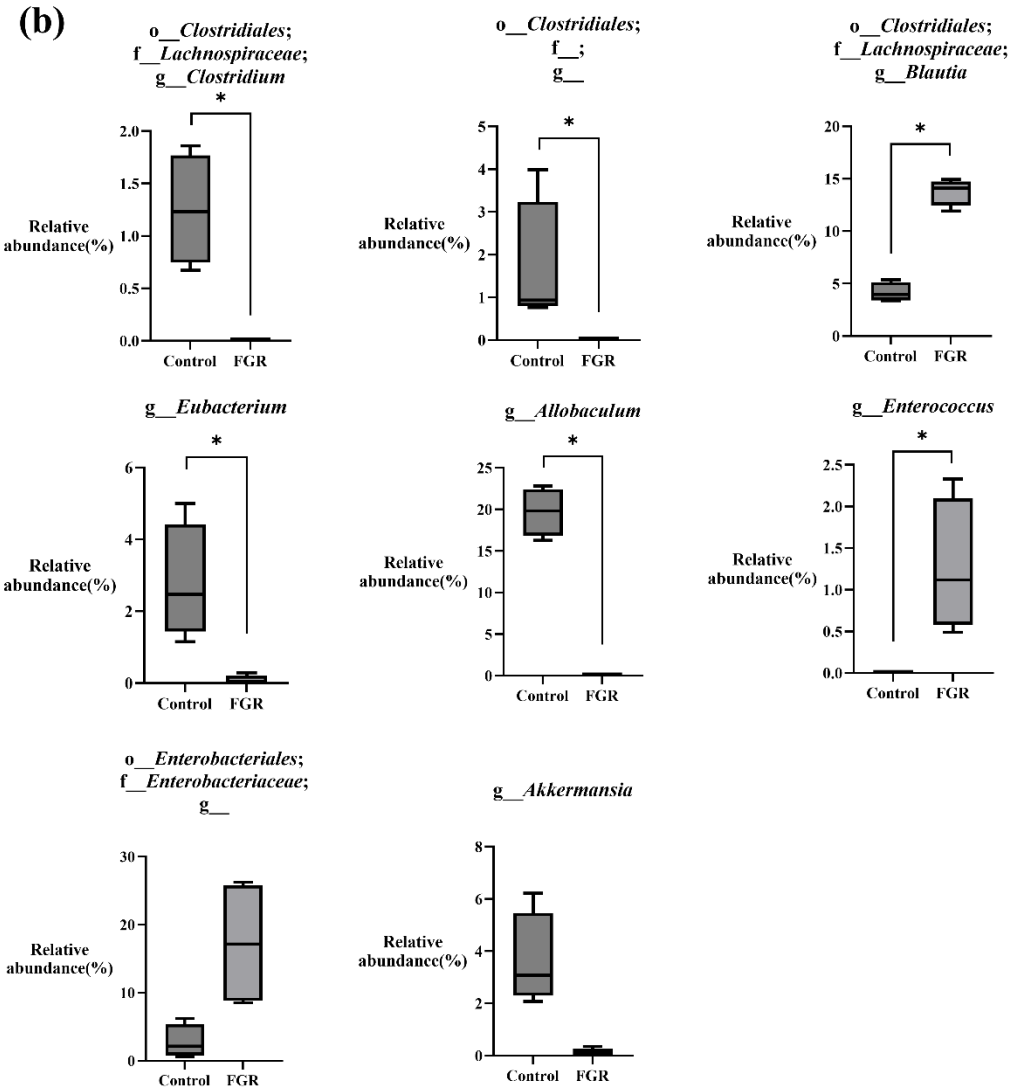
Proteobacteria
Firmicutes
Bacteroidetes

Figure. 1-b


(b)
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f__Lachnospiraceae;
g__Clostridium
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f__Lachnospiraceae;
g__Blautia
g__Eubacterium
g__Allobaculum
g__Enterococcus
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Relative abundance(%)
Control
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Figure. 2

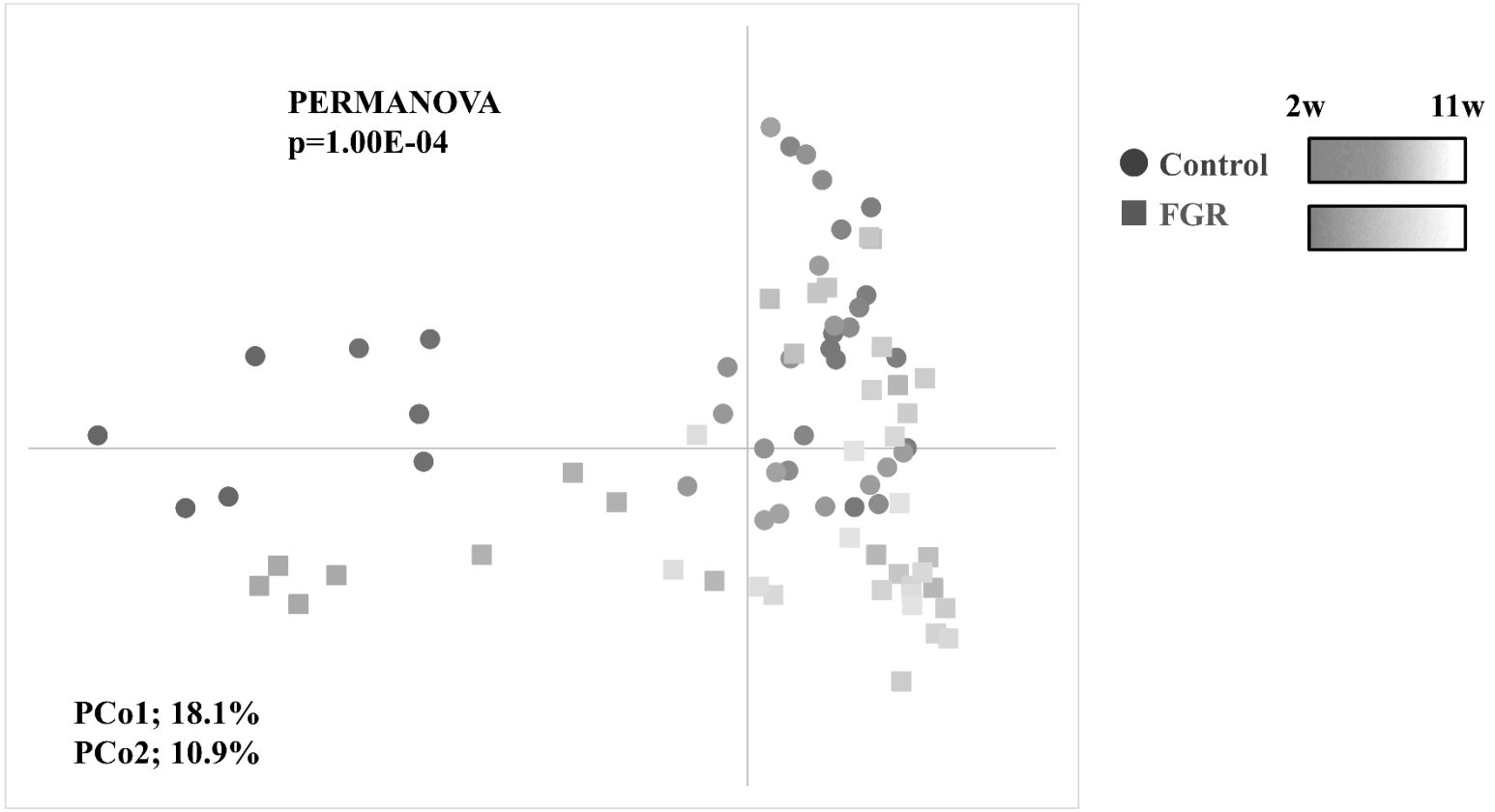
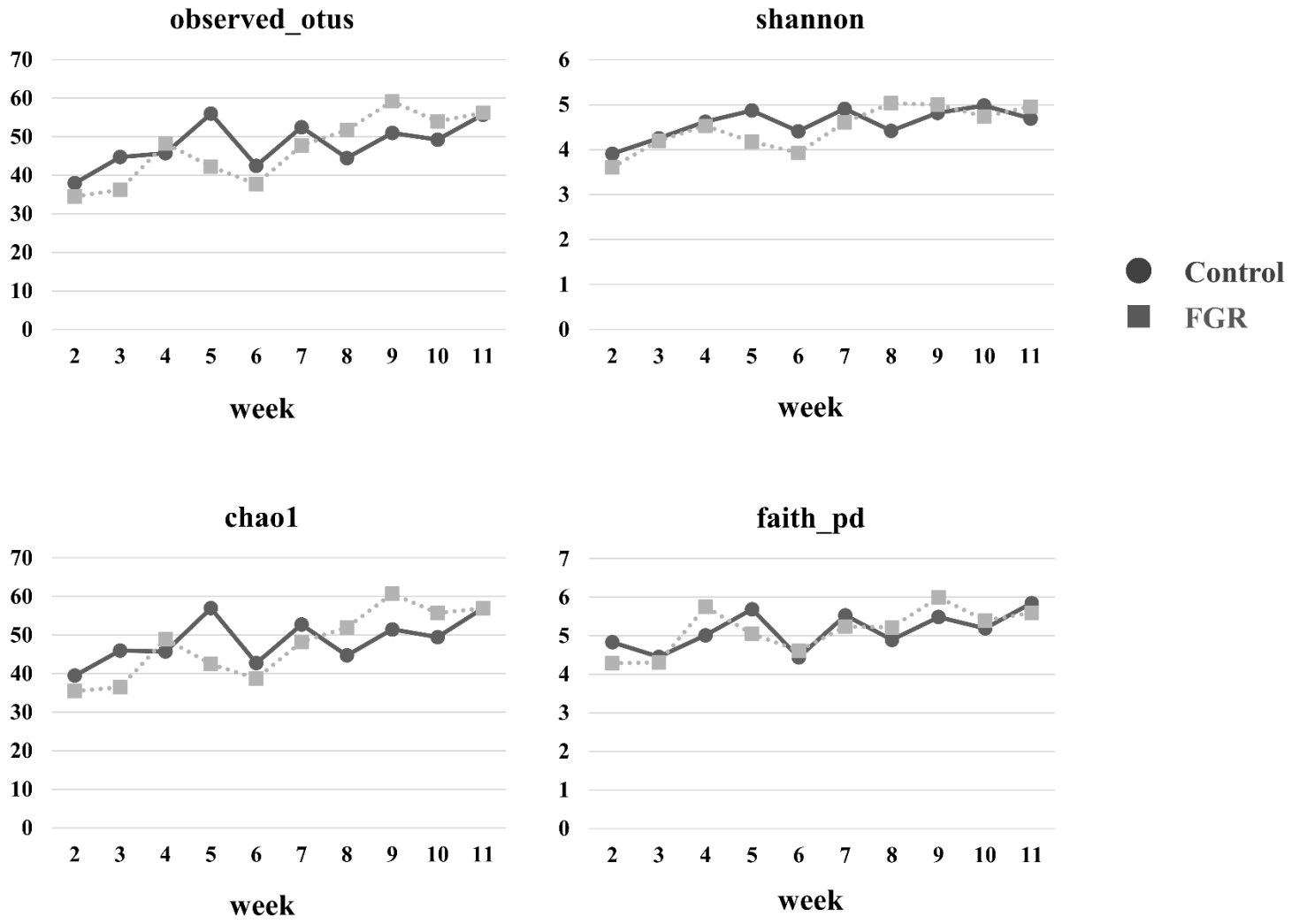


Figure. 3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