# Changes In The Gut Microbiota After Hepatitis C Virus Eradication

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# Abstract

The gut microbiota interacts with infectious diseases and affects host immunity. It has also been reported that liver disease is associated with changes in the gut microbiota. To investigate changes in the gut microbiota before and after eradication of hepatitis C virus (HCV) by direct-acting antiviral (DAA) treatment in patients with chronic hepatitis C (CHC), we investigated 42 samples from 14 CHC patients who received DAA therapy for HCV. Faecal samples were obtained before treatment (Pre), when treatment ended (EOT), and 24 weeks after treatment ended (Post24). The target V3–4 region of the 16S rRNA gene from faecal samples was amplified using the Illumina Miseq sequencing platform. The diversity of the gut microbiota did not differ significantly between Pre, EOT, and Post24. PCoA analysis showed that for each patient, the values at Pre, EOT, and Post 24 were concentrated within a small area. LEfSe analysis showed that the relative abundances of Faecalibacterium and Bacillus were increased at EOT and further increased at Post24, and these genera were significantly increased at Post24 compared to Pre. These results suggest that changes in the gut microbiota need to be considered as among the various effects on living organisms observed after HCV elimination.


# Introduction

About 71 million people worldwide were chronically infected with hepatitis C virus (HCV) in 2015[1], and most patients with HCV infection progress to cirrhosis, resulting in hepatocellular carcinoma (HCC). With the recent development of direct-acting antiviral (DAA) therapies, more than 95% of patients with chronic hepatitis C (CHC) are able to achieve HCV eradication, which suppresses the progression to cirrhosis and reduces the risk of developing HCC[2]. However, some individuals develop HCC even after HCV eradication; risk factors include advanced liver fibrosis, old age, male gender, diabetes, and high FIB-4 index at 24 weeks after the end of treatment[2–4]. Nonetheless, HCC occurs even in persons with none of these risk factors, indicating that other causes may be involved in carcinogenesis.

Cells in the human body are outnumbered 10- to 100-fold by microbial cells such as bacteria, fungi, and viruses; these include 100 trillion bacteria in the intestines[5,6]. The human gut microbiota stabilises within about 1 year after birth, and thereafter it is affected by factors such as diet, environment, antibiotics, and the use of proton pump inhibitors[7]. The development of next-generation sequencing (NGS) has made it possible to detect bacteria that are difficult to culture, and it has been reported that changes in the gut microbiota and associated pathologies contribute not only to digestive tract diseases but also to liver diseases and diseases of other organs. Recently, it was reported that the gut microbiota interacts with infectious diseases such as enterovirus, HIV, influenza, and hepatitis B, and affects host immunity[8]. It has also been found that liver disease is associated with changes in the gut microbiota[9]. Furthermore, increasing numbers of studies have shown that lipopolysaccharides, which are a component of the outer membrane of the cell wall of Gram-negative bacteria, flow from the portal vein to the liver, and increased levels of Toll-like receptor ligand promote inflammation and fibrosis in the liver[10–12]. Based on these facts, the gastrointestinal tract and liver are considered to have a close relationship, and constitute the

gut–liver axis[13,14]. Therefore, studies are underway to determine whether the gut microbiota is involved in the progression of liver damage and cirrhosis, as well as the development and promotion of HCC, but results thus far have been inconclusive.

It is possible, then, that there is an interaction between the gut microbiota and hepatitis C virus. A previous study compared cirrhotic patients with HCV and those in whom HCV had been eradicated[15]. However, there have been no prospective, longitudinal studies on changes in the gut microbiota before and 24 weeks after HCV eradication. Therefore, we conducted this study to elucidate changes in the gut microbiota before and after HCV eradication by DAA treatment in patients with CHC.

## Results

# Biochemical changes

Table 1 shows the patient backgrounds at the start of DAA treatment. All patients had CHC, with a median body mass index of 22.0 kg/$m^2$. There were two patients with diabetes, but their blood glucose control was within the normal range. The median alanine aminotransferase (ALT) value was 22.5 IU/L, and there were no cases of chronic active hepatitis with high ALT fluctuations. Liver biopsy was not performed, but the median FIB-4 index [age × AST/platelet count ($10^9$ /L) × √ALT] was 2.6. One of the factors of FIB-4 index was age and patient was 67.0 years of median age.

FIB-4 was not high considering age, this suggesting that fibrosis had not progressed significantly. Eleven patients had HCV genotype 1b, two had serotype 1, and one had genotype 2b.

Table 1
Patient Characteristics

| | n = 14 |
|---|---|
| Gender (Male/Female) | 2/12 |
| Age (years) | 67.0 (57.3–75.8) |
| BMI (kg/$m^2$) | 22.0 (19.9–24.6) |
| Glucose (mg/dL) | 94.5 (89.8–112.3) |
| Total cholesterol (mg/dL) | 157.5 (138.3–188.3) |
| Albumin (g/dl) | 4.2 (3.7–4.4) |
| Total bilirubin (mg/dL) | 0.6 (0.6–0.9) |
| AST (IU/L) | 26.5 (23.8–48.8) |
| ALT (IU/L) | 22.5 (15.8–49.3) |
| γ-glutamyl transpeptidase (IU/L) | 18.0 (13.0–31.8) |
| Platelet count (×$10^4$/$mm^3$) | 17.2 (13.5–22.2) |
| Prothrombin time (%) | 97.2 (79.7–105.9) |
| Genotype (1b/1/2b) | 11/2/1 |
| HCVRNA (Log IU/mL) | 6.3 (5.3–6.8) |
| FIB-4 index | 2.6 (1.1–4.1) |
| PPI (present/absent) | 1/13 |
| DM (present/absent) | 2/12 |

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; PPI, proton pump inhibitor; DM, diabetes mellitus

Biochemical test showed a decrease in AST after the start of treatment; specifically, the level was significantly lower at both EOT and Post24 than that at Pre (Fig. 1A). In addition, the ALT level at EOT was nonsignificantly lower than that at Pre, but the ALT level at Post24 was significantly lower than that at Pre (Fig. 1B). Gamma-glutamyl transpeptidase (γGTP) was slightly increased at both EOT and decreased at Post24 compared to Pre, but neither difference was significant (Figs. 1C). Total cholesterol and albumin were slightly increased at both EOT and Post24 compared to Pre, but neither difference was significant (Figs. 1D, E). These results indicate that liver inflammation was reduced after treatment, but there was no significant improvement in liver reserve. The blood glucose levels at EOT and Post24 were not significantly different from that at Pre (Fig. 1G), and BMI also did not change (Fig. 1F).

# Changes in gut microbiota

Alpha diversity indicating species abundance showed no significant differences between Pre, EOT, and Post24 using the Chao1, Observed, and Shannon Index methods (Fig. 2A, B, C). Similarly, there were no significant differences between Pre, EOT, and Post24 in beta diversity (Supplementary Fig. 1A, B). In the plan view of the PCoA analysis, the Pre, EOT, and Post24 coordinate plots for each patient were located in the same vicinity as each other (Supplementary Fig. 2).

At the phylum level, the results for the Pre, EOT, and Post24 time points are shown for Cases 1 to 14 (Supplementary Fig. 3). In Case 5, Firmacutes was dominant, but in the other cases Bacteroidetes was dominant. In the average of all cases, the proportions of Bacteroidetes and *Fusobacteria* decreased according to Pre, EOT, and Post24 (Supplementary Fig. 4, green scores), and the proportion of Firmicutes and Verrucomicrobia increased (Supplementary Fig. 4, red scores).

Furthermore, when we examined the average of all cases at the genus level, the proportions of the genera *Bacteroides*, *Phascolarctobacterium*, and *Fusobacterium* decreased progressively from Pre to EOT to Post24 (Fig. 3, green numbers), while those of the genera *Lachnospira*, *Faecalibacterium*, *Oscillospira*, and *Akkermansia* increased (Fig. 3, red numbers).

In the multiple comparisons by LEfSe, there was no significant difference in specific gut microbiota between Pre and EOT or between EOT and Post24. However, compared with Pre, Post24 Ruminococcaceae at the family level and *Faecalibacterium* at the genus level were significantly increased. Bacillales at the order level, Bacillaceae at the family level and *Bacillus* at the genus level were significantly increased at Post24 (Figs. 4A and 4B). When the relative abundances of *Faecalibacterium* and *Bacillus* were compared between Pre, EOT, and Post24, these genera were increased at EOT and further increased at Post24, and the relative abundances of *Faecalibacterium* and *Bacillus* were significantly increased at Post24 compared to Pre (Fig. 1H, 1I).

# Discussion

In this study, there was no significant difference between Pre, EOT, and Post24 in terms of either the alpha or beta diversity of the gut microbiome, and dysbiosis of the gut microbiome did not vary between the three time points. A previous study also found that cirrhotic patients treated with DAAs showed no significant difference according to whether or not HCV had been eradicated, though it was not a longitudinal analysis[16]. Similarly, dysbiosis of the gut microbiome in CHC patients did not show a significant difference before DAA treatment compared with 3 months after virus eradication[17]. In PCoA analysis of individual patients, the Pre, EOT, and Post24 values were relatively close to each other, and the change of diversity was relatively small.

In our study, LEfSe analysis showed that numbers of the *Bacillus* and *Faecalibacterium* genera were increased at Post24 compared to Pre.

It has been reported that *Faecalibacterium prausnitzii* is decreased when dysbiosis is present in the digestive tract, such as in Crohn's disease, and *Faecalibacterium prausnitzii* is a butyric acid–producing

bacterium that has anti-inflammatory effects[18]. Butyric acid has been reported to metabolise Foxp3 + Treg cells and suppress inflammation in humans[19]. It has also been shown that T cells are exhausted in persistent viral infections such as CHC and HIV[20]. Immune status has been shown to be normalised by HCV elimination[21]. Based on these facts, it is possible that *Faecalibacterium prausnitzii* recovered original status along with the recovery of the immune status in the process of HCV elimination. In this regard, further studies, including analyses of immunity, are needed in the future.

Probiotics containing the genus *Bacillus* have been reported to eliminate *Staphylococcus aureus* in the intestines as well as to increase its colonisation in the nares[22]. It has been suggested that HCV elimination increases the numbers of *Bacillus* spp. and may thereby reduce infections with bacteria such as *S. aureus*; this process may be related to reverse immunosuppression induced by HCV eradication, similar to the aforementioned increase in *Faecalibacterium prausnitzii*. Further studies on this topic are warranted.

The reason that *Faecalibacterium prausnitzii* and *Bacillus* genera showed no significant increase at EOT compared to Pre is that although all 14 patients were virus negative at EOT, eight patients showed negative results 4 weeks before EOT, and changes in the intestinal flora may not have been completed by EOT. By Post24, at least 24 weeks had passed since the virus was eliminated; thus, it was thought that this time point adequately reflected the extent of changes in the gut microbiota.

The effects of HCV eradication on the gut microbiota in liver cirrhosis were previously reported. *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus* decreased after treatment, but intestinal permeability and inflammation did not change[15]. The present study excluded patients with cirrhosis and examined only those with CHC. Therefore, the *Clostridiales* genus did not decrease and the *Streptococcus* and *Lactobacillus* genera did not increase, while the *Bacillus* and *Faecalibacterium prausnitzii* genera increased. The study mentioned above suggested that virus control in patients with liver cirrhosis may alter the gut microbiota associated with improved liver reserve and liver fibrosis. By contrast, our study examined only patients with chronic hepatitis in order to assess the direct effects of virus control. Compared with the aforementioned study that included patients with advanced liver fibrosis or cirrhosis, our study did not demonstrate significantly improved hepatic reserve function, indicating a direct effect of changes in the gut microbiome after eradication of HCV.

It was reported that dysbiosis of the gut microbiome worsened as patients progressed from chronic hepatitis to liver cirrhosis, intestinal *Clostridiales* decreased, and *Streptococcus* and *Lactobacillus* increased with the progression of hepatitis C from chronic hepatitis to cirrhosis[23]. Our study examined only patients with CHC, and therefore the above changes in the gut microbiome did not occur.

One limitation of this study is the lack of healthy controls, due to the difficulty in accumulating healthy people with the same median age (67 years) as the patients with CHC. For this reason, it was not possible to determine whether the gut microbiota was similar in CHC patients before treatment and in healthy people. Further studies are needed in this regard.

In this study, we observed changes in the gut microbiota due to HCV control in CHC. The diversity of the gut microbiota did not change significantly in CHC patients after HCV eradication. However, the relative abundance of *Bacillus* and *Faecalibacterium prausnitzii* was significantly increased in patients at 24 weeks after treatment (Post24) compared to those before treatment (Pre). This suggests that changes in the gut microbiota after HCV elimination have secondary effects on living organisms that should be considered.

## Methods

# Patients and samples

This prospective longitudinal study was conducted in patients with CHC who were treated with DAAs at the Department of Gastroenterology and Hepatology of Nagoya University Hospital. Patients were given a sufficient explanation of the study then asked to provide written consent to participate. This protocol was approved by the Ethics Committee of Nagoya University School of Medicine (2016 – 0428) and was conducted in accordance with the Declaration of Helsinki (1975).

DAAs were administered for the treatment of hepatitis C, and stool samples were collected before DAA treatment (Pre), at the end of treatment (EOT), and at 24 weeks after the end of treatment (Post24). Blood was collected every 4 weeks from the start of treatment, and biochemical tests and serum were stored at -80°C.

Informed consent was obtained from all patients treated with DAAs. Three of them received steroids, three had a history of treatment for HCC, and one was excluded because of cirrhosis. Finally, 14 patients with CHC were examined in this study.

# Microbiota analysis

All procedures were performed according to the protocol of the Human Microbiome Project. DNA was extracted from patient stool using the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany), and the DNA was purified to 5 ng/μl. Amplicon PCR specifically targeted the V3–4 region of 16S rRNA (forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3 ', reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' 95°C). Initial denaturation was performed at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and final elongation at 72°C for 5 minutes. The Amplicon PCR product was 550 bp in length and was cleaned up with AMPureXP beads (Beckman Coulter, Brea, CA, USA). Next, a Nextera XT index kit (Illumina, San Diego, CA, USA) was used for index PCR. Initial denaturation was conducted at 95°C for 3 minutes, followed by 8 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and final elongation at 72°C for 5 minutes. Subsequently, the DNA was purified using the same beads described above. After a 4 nM cDNA library was created from each sample, a 20% spike of Phix was added and paired-end reads were performed with 600 cycles of 2 × 300 reads using

MiSeq Reagent Kit V3 (Illumina) at a final concentration of 8 pM. Sequences were determined using the Illumina Miseq sequencer (Illumina). Quantitative Insights into Microbial Ecology (QIIME1.9.1) software (http://qiime.org)was used for the subsequent analysis of 16S rRNA gene sequence data and Greengenes 13_8 was used as an operational taxonomic unit (OTU) database[24] After the chimaera check, the OTU was determined and a secondary analysis was performed using linear discriminant analysis of effect size (LEfSe), a Greengenes13_8 database provided by the Galaxy module (http://huttenhower.sph.harvard.edu/galaxy/) that explains differences between groups by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance, and the Microbiome Analyst (https://www.microbiomeanalyst.ca) for comprehensive statistical, functional, and meta-analysis of microbiome data[25].

# Statistical analysis

The categorical chi-square test or Fisher's exact test was used when comparing two groups. In the case of a continuous variable, Student's t-test or the Mann-Whitney U test was performed as necessary. The alpha diversity of the gut microbiota, which indicates species richness, was analysed by the Chao1, Observed, and Shannon methods, and beta diversity was analysed by principal coordinates analysis (PCoA).

# Abbreviations

HCV, hepatitis C virus; HCC, hepatocellular carcinoma; DAA, direct-acting antiviral; CHC, chronic hepatitis C; NGS, next-generation sequencing; EOT, end of treatment; OUT, operational taxonomic unit; LEfSe, linear discriminant analysis of effect size; PCoA, principal coordinates analysis; AST, alanine aminotransferase; ALT, alanine aminotransferase

# Declarations

**Conflict of interest:** The authors declare that there is no conflict of interest for this study.

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### Author contributions

TH conceived the concept and designed the study; TH, MI, KY, TI, YI, TK acquired the data; TH, KY, TT analyzed and interpreted the data; TH wrote the manuscript; MI, KY, MN, HK, RM, TI, YH, MF contributed to revise the manuscript for important intellectual content; MI, MF supervised the manuscript.

All authors approved the final version of the manuscript.

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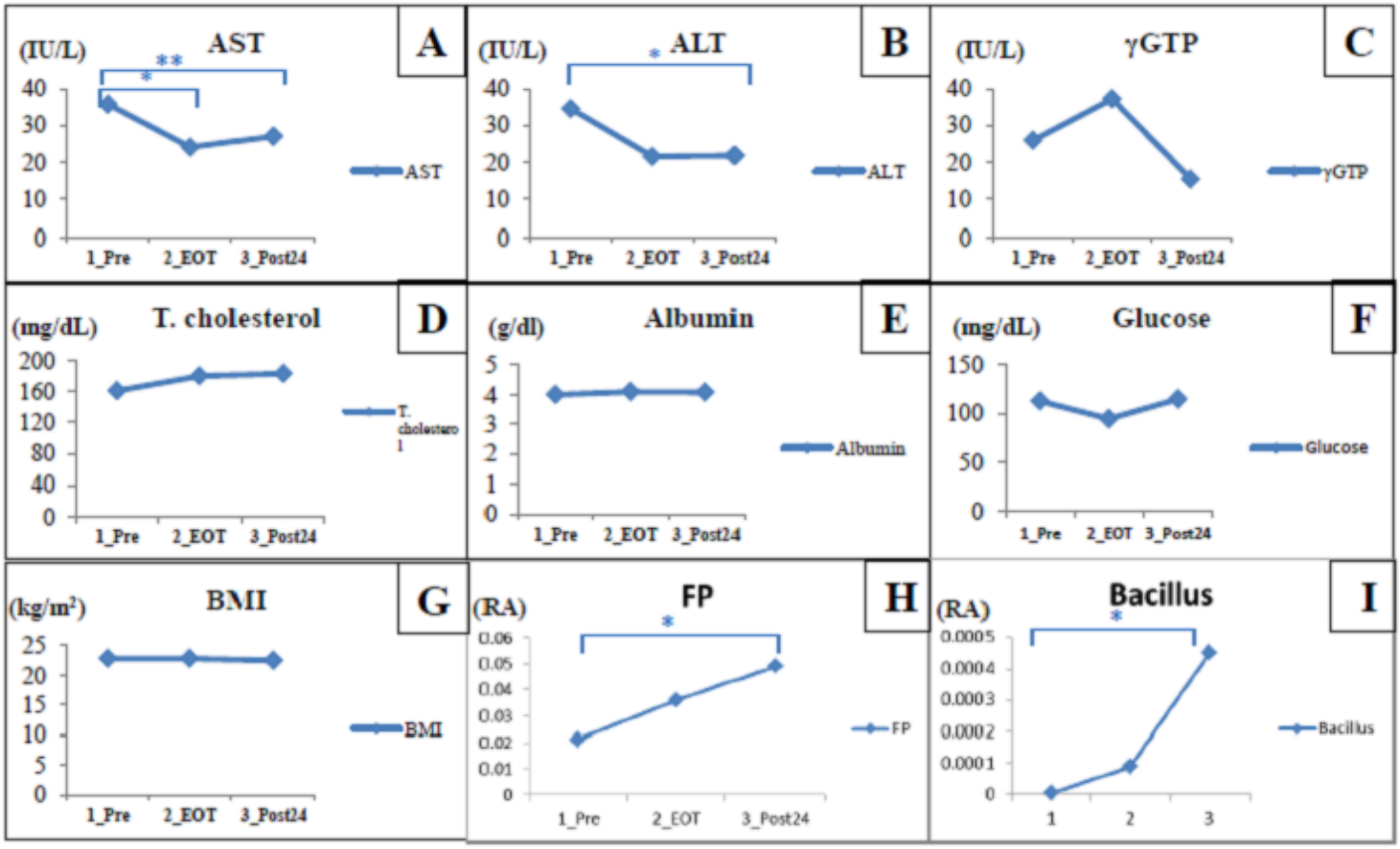
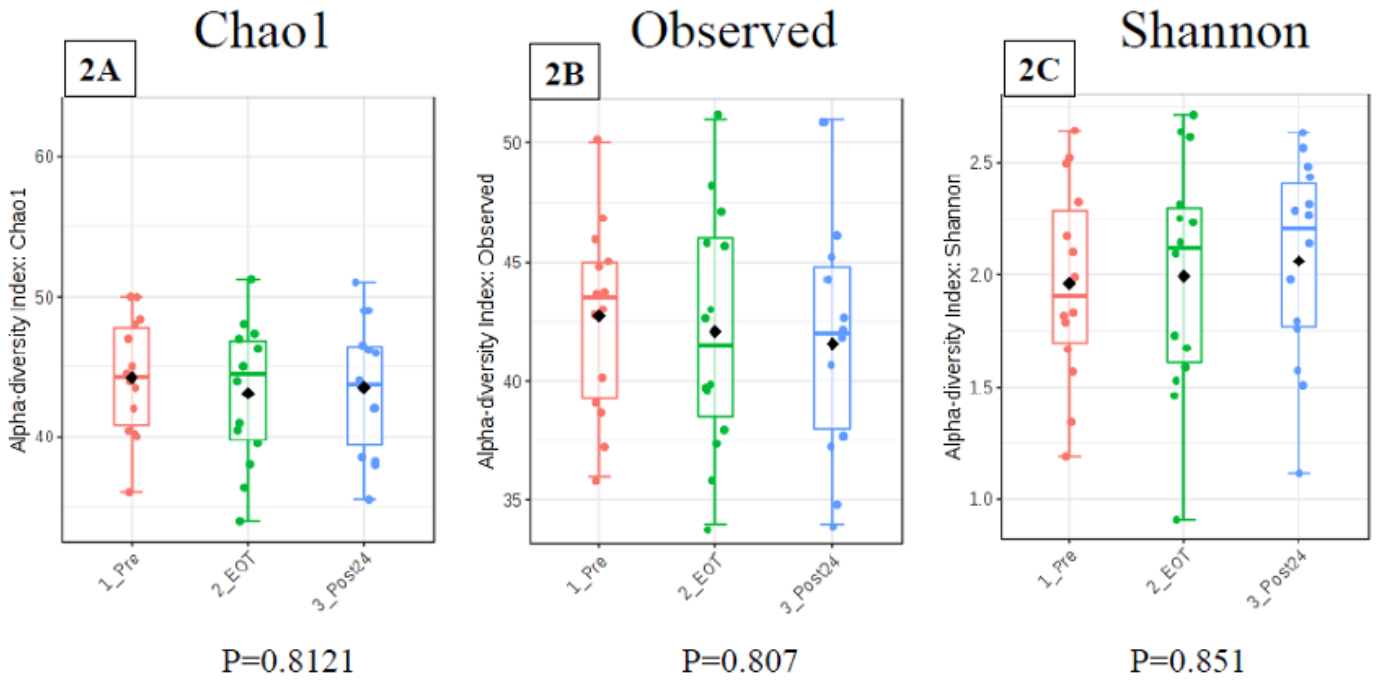
# Figures



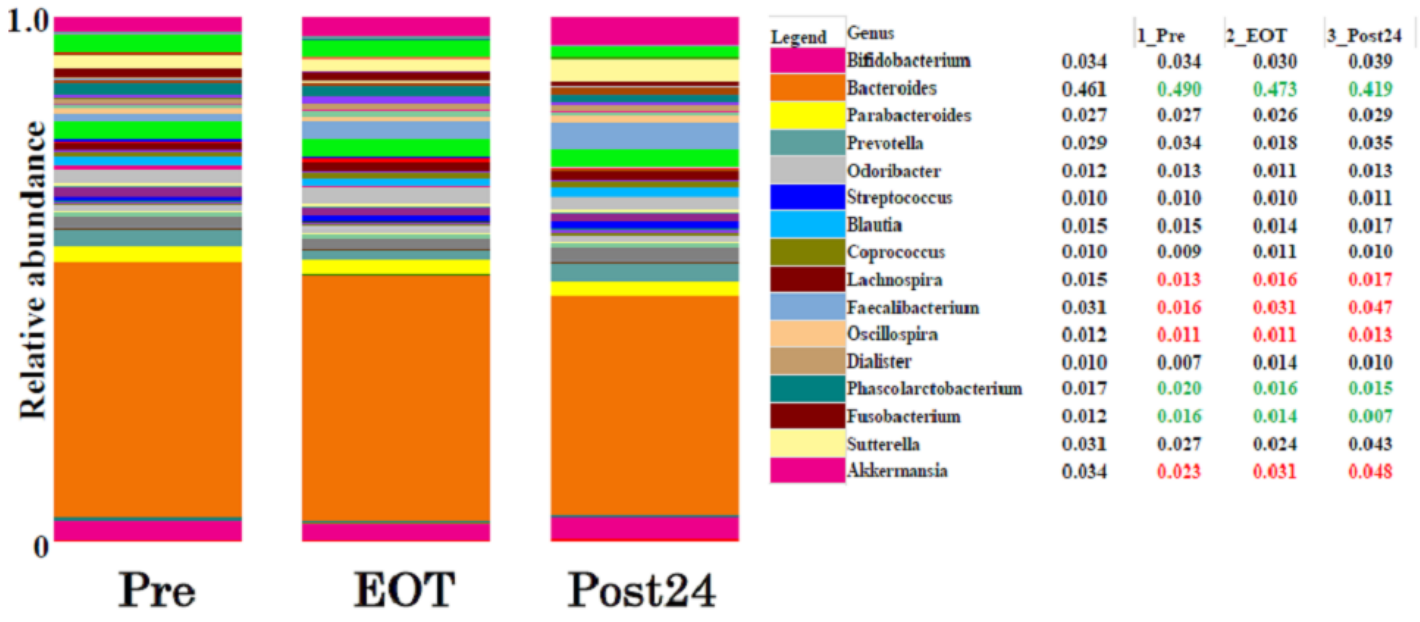

## Figure 1

Changes in the gut microbiota and biochemical test results. Comparison between Pre and EOT, Pre and Post24. (A) AST, aspartate aminotransferase; (B) ALT, alanine aminotransferase; (C) γGTP, gamma-glutamyl transpeptidase; (D) Total cholesterol, (E) Albumin, (F) Glucose, (G) BMI, body mass index; (H) FP, Faecalibacterium prausnitzii; (I) Bacillus. * P<0.05, ** P<0.01 Mann-Whitney U test between two points. RA, Relative Abundance.




## Figure 2

Alpha diversity between Pre, EOT, and Post24 using Chao1 (2A), Observed (2B), Shannon (2C).




## Figure 3

Relative abundance of bacteria is presented at the phylum level. Differences in the microbiota between Pre, EOT, and Post24. Relative abundance of bacteria is presented at the genus level.

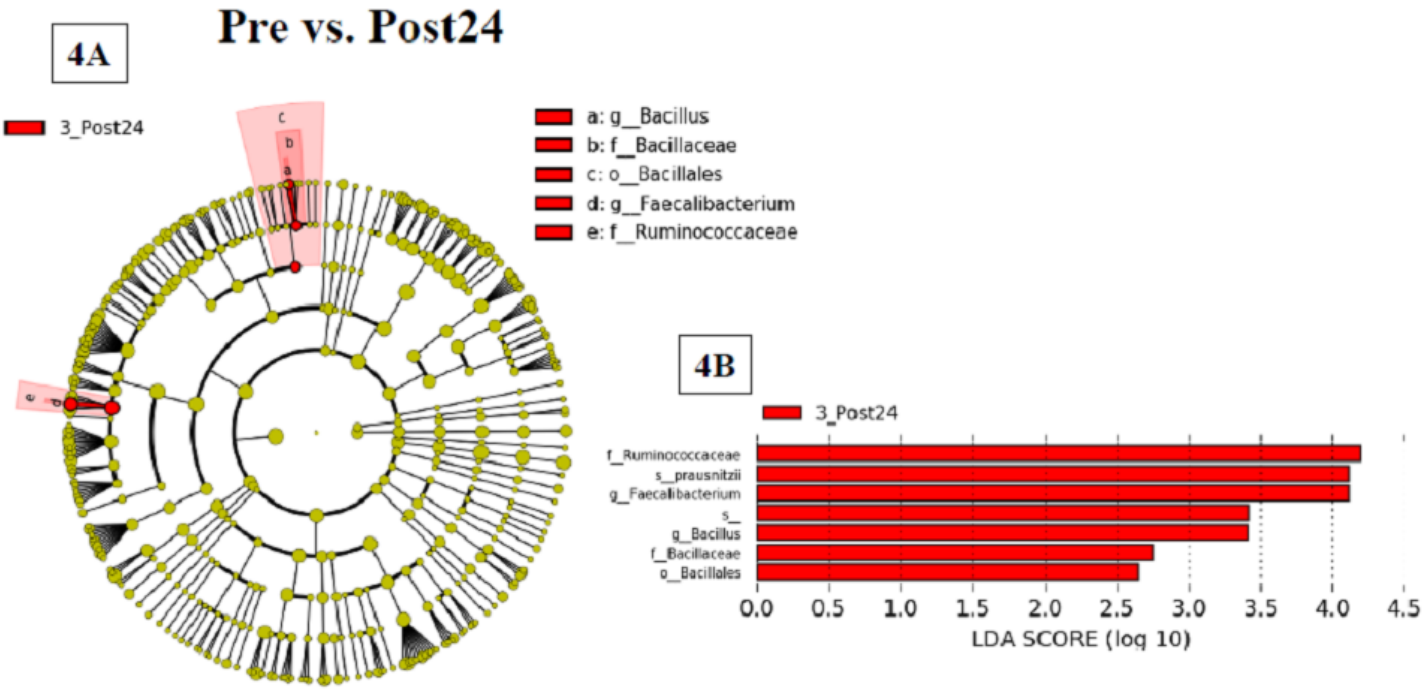



**Figure 4**

Differences in the microbiota between Pre and Post24. 4 (A) Cladogram plotted from linear discriminant analysis of effect size (LEfSe) analysis showing the taxonomic levels. In the circle graph, the objects from the center of the circumference show phylum, class, order (o), family (f), genes (g), speicies, respectively. (B) LEfSe plot showing enriched microbiome associated with Post24 (red).

## Supplementary Files

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