Fecal Microbial Enterotypes Differentially Respond to a High-fat Diet Based on Sex in Fischer-344 Rats

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The gut microbiota interacts with the host gut environment, which is influenced by such factors as sex, age, and host diet. These factors induce changes in the microbial composition. The aim of this study was to identify differences in the gut microbiome of Fischer-344 (F344) rats fed a high-fat diet (HFD), depending on their age and sex. Fecal microbiomes from 6-, 31-, and 74-week-old, and 2-year-old both male and female rats (corresponding to 5-, 30-, 60-, and 80-year-old humans) were analyzed using 16S rRNA gene sequencing, phylogenetic investigation of communities by reconstruction of unobserved states, and enterotype (E) assessment. Moreover, the effect of an HFD on colonic epithelial cells was measured using real-time quantitative PCR. Alpha diversity decreased in the HFD group regardless of age and sex. Based on the enterotype clustering of the whole fecal microbiome, clusters from male rats were divided into E1 and E2 enterotypes, while clusters from female rats were divided into E1, E2, and E3 enterotypes. The female E3 group showed a significantly high abundance in the *Ruminococcus* genus and expression of *Tlr2* mRNA, which may reflect compensation to the HFD. Moreover, the female E3 group showed a lower ratio of opportunistic pathogenic strains to commensal strains compared to the female E2 group. Administration of an HFD influenced the rat fecal microbiota in all assessed age groups, which could be further differentiated by sex. In particular, female rats showed a compensatory enterotype response to an HFD compared to male rats.

Key Words Rats, Diet, high-fat, Microbiota, Aging, Sex

INTRODUCTION

The gut microbiota inhabits the gastrointestinal tract and interacts with the host gut environment. Various microorganisms colonize the gastrointestinal tract starting at birth, and their composition changes depending on the health status of the host [1,2]. In addition, gut microbiota ferments undigested food that man ingests and produces metabolites that can affect host health [3,4]. A high-fat diet (HFD) is associated with obesity and metabolic syndromes, which are major risk factors for cardiovascular disease and various types of cancer, such as prostate cancer [5]. Furthermore, the consumption of an HFD results in chronic systemic inflammation in the liver, adipose tissue, skeletal muscle, and intestine [6]. Also, dietary patterns affect the microbial diversity and the community structure in the gut microbiota [7,8]. Interestingly, a change in the host's diet, even for a short period of time, may alter the

microbiota, which persists for a few days. Indeed, short-term dietary changes even within 2 days alter the gut microbiota [3].

Apart from diet, aging also induces changes in the gut microbiome. Aging promotes physiological, genetic, immunological, and metabolic changes to occur throughout the body, thereby affecting the gut microbiota [9]. One of the examples of changes in the gut microbial composition is the *Clostridium difficile* infection, in which the rate of incidence is increased in elders [10,11]. As the host ages, the gut microbiota also slowly undergoes transitions from that of a younger person to that of an older person.

Recently, it has been shown that various health conditions are influenced by sex [12]. Similarly, sex differences in the gut microbiome have also been reported [13]. Analysis of the fecal bacterial community, comparing a rodent chow diet and oligofructose supplemented diet to rats, demonstrated that

sex differences in microbial metabolism affected inflammatory cytokine expression. Thus, increases in anti-inflammatory cytokine expression (e.g., interleukin [IL]-10) in females [14] and proinflammatory cytokine expression (e.g., IL-6) in males were observed [15]. Another study also reported that sex and hormonal changes affect the gut microbiome diversity and composition in 89 different inbred strains of mice [16].

The production of various metabolites from gut microorganisms, such as short-chain fatty acids (SCFAs like acetate, propionate, and butyrate) is one of the ways that the gut microbiome affects host health [3]. Among SCFAs, microbiome-derived butyrate has been reported to promote epithelial barrier function through the IL-10 receptor [17].

Previously, we reported the effect of an HFD on a young (6-week-old) and older (2-year-old) Fischer-344 (F344) rat gut microbiome, in both male and female rats [18]. The results suggested that sex influences the response of gut microbiota to an HFD, particularly in old age. We have also reported that the butyrate level in the cecum was significantly increased in 31-week-old rats among a cohort of 6-, 31-, and 74-week-old, and 2-year-old male F344 rats [19]. With this background, we hypothesized that 31- and 74-week-old rats might show a different microbiota pattern compared to 6-week-old and 2-year-old rats, depending on their sex, upon exposure to an HFD for 8 weeks. Therefore, the aim of this study was to identify differences in the gut microbiome of 6-, 31-, and 74-week-old and 2-year-old F344 rats fed an HFD based on the sex of the animals.

MATERIALS AND METHODS

Animals and diets

F344 rats, aged 6-, 31-, and 74-week, and 2-year-old were used in this study (OrientBio, Seongnam, Korea) (Table S1) [20,21]. Animals were housed in a specific-pathogen-free room at 23°C with a 12/12 h light/dark cycle and given unlimited access to food and water. The rats were divided into groups that were fed ad libitum, with two different commercial diets: a normal diet and an HFD (normal chow: 3.85 kcal/g; HFD: 5.24 kcal/g, 60% kcal derived from fat) (Product #D12492; Research Diets, Inc., New Brunswick, NJ, USA). Fecal samples were collected after 8-week administration of normal diet or HFD and stored at –80°C. The study was performed following the procedures in the Guide for the Care and Use of Laboratory Animals in Korea. All protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (Permission No. BA1506-178/027-01).

Fecal sample collection and metagenome sequencing

Bacterial genomic DNA was extracted from the frozen fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

DNA quantity and quality were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresed using 2% agarose gel. For the preparation of MiSeq library amplicons, the target gene, 16S rRNA V3-V4 region, was first amplified using the 341-F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and 805-R (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') primers. Secondly, the V3-V4 PCR amplicons were linked to the Illumina indices and adapters from Nextera® XT Index Kit (Illumina, San Diego, CA, USA). Short DNA fragments were eliminated using the FavorPrep™ DNA purification kit (Favorgen, Pingtung, Taiwan). To quantify the PCR amplicons, we used the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific). Sequencing was performed at Chunlab Inc. (Seoul, Korea) using the MiSeq system (Illumina).

Non-specific, non-target, and chimeric amplicons were removed in the quality control process to exclude reads with short lengths and low Q-values from the pre-filter. Using operational taxonomic unit (OTU) information (number of OTUs and sequences in each OTU), alpha diversity indices, such as abundance-based coverage estimator (ACE), Chao1, Jackknife, Shannon indices, Simpson indices, phylogenetic diversity, and Good's library coverage) were analyzed using EzBioCloud (Chunlab Inc.) (Table S2). To visualize sample differences, principal coordinates analysis was performed with unweighted UniFrac. Averaged taxonomic compositions (at phylum and family level) bar charts and selected taxa were created using GraphPad Prism (ver. 8.01; GraphPad Software, Inc., San Diego, CA, USA).

To predict the functional capabilities of the microbial community, we performed a phylogenetic investigation of communities by reconstruction of unobserved states.

Enterotype clustering and LEfSe analysis

The enterotype classification of the gut microbiota at the genus level was calculated using the R package "clusterSim" [22]. The optimal cluster number was determined by maximizing the value of the Calinski-Harabasz index [23].

Based on the relative taxonomic abundances, the taxonomic biomarker discovery and related statistical significance were assessed by the linear discriminant analysis effect size (LEfSe) method [24]. The criteria for conducting the LEfSe analysis were as follows: 1) an alpha value for the factorial Kruskal–Wallis H test between assigned taxa compared to that of the groups with less than 0.05, 2) an alpha value for the pairwise Wilcoxon test among the taxonomic compositions of less than 0.05, 3) a threshold of the logarithmic linear discriminant analysis (LDA) score for discriminative features less than 2.0, and 4) a multi-class analysis set as all-against-all. After this process, we simplified the LEfSe plot by 5) overlapped bacteria selection, 6) searched and classified the bacterial characteristics regarding reported papers: commensal strains
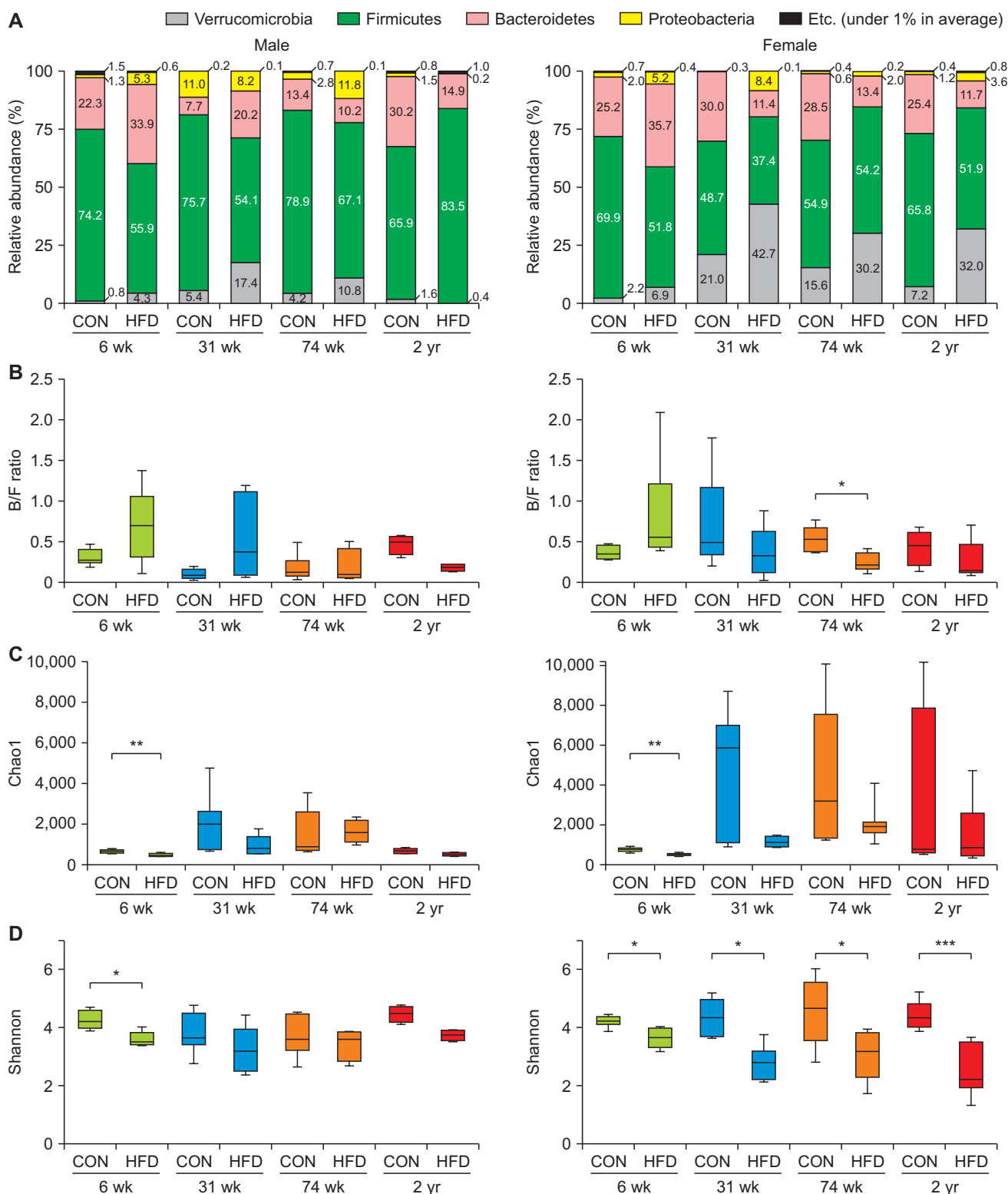
Figure 1. Comparison of age-associated variations in the fecal microbiomes in the male and female rats fed the control diet (CON) or high-fat diet (HFD). Fecal samples were collected and subjected to measurement of phylum composition (A), the Bacteroides to Firmicutes (B/F) ratio (B), Chao1 indicating the species richness (C), and Shannon indicating the diversity of species (D). Data are expressed as the median, Q1 (25%) and Q3 (75%). Whiskers show the minimum and maximum values. The statistical significance was determined by the Mann–Whitney test. CON, control diet; HFD, high-fat diet. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from the descending colon tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer, and the collected RNA was purified using an RNeasy Mini kit (Qiagen). Complementary DNA synthesis was performed using 1 μg of total RNA with the High-Capacity cDNA kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. RT-qPCR was performed in triplicate using a StepOnePlus™ Real-time PCR (Applied Biosystems, Waltham, MA, USA) with SYBR® Premix Ex Taq™ (TaKaRa Bio; Shiga, Japan) according to the manufacturer's instructions with designed primer (Table S3). The expression level of mRNA for the target genes was compared with endogenous control β-actin using the 2−ΔΔCt method.

Statistical analysis

Statistical calculations, except for those on the pyrosequencing data, were performed using PASW Statistics version 18.0.0 (SPSS Inc., Released 2009; PASW Statistics for Windows, Version 18.0.; SPSS Inc., Chicago, IL, USA). The groups were compared using the Mann-Whitney U-test (also known as the Wilcoxon rank-sum test). A P-value < 0.05 was considered to be statistically significant.

RESULTS

Compositional changes depend on sex, age, and diet

The daily calorie intake and the gained body weight during the 8-week HFD feeding period were reported in our previous study [25]. The weight gain was decreased with age both in male and female rats. When we compared taxonomic compositional differences between the HFD-fed group and the normal diet group at the phylum level (Fig. 1), Bacteroidetes decreased in the HFD-fed group in male (2-year-old, P = 0.021) and female rats (31-week-old, P = 0.016; 74-week-old, P = 0.015; 2-year-old, P = 0.010). In the case of Firmicutes, the 6-week-old male (P = 0.037) and female (P = 0.006) HFD-fed groups showed decreased relative abundance compared to the normal diet group. Verrucomicrobia increased in 6-week- (P = 0.004) and 31-week-old (P = 0.031) male rats and 6-week-old (P = 0.016) and 2-year (P = 0.008) female rats (Fig. 1A). In terms of the Bacteroidetes to Firmicutes (B/F) ratio, the 74-week-old female group showed a decreased ratio in the HFD-fed group compared to the normal diet group (Fig. 1B). In contrast, the Chao1 index, which is an alpha-diversity index indicating species richness, was decreased in the 6-week-old male (P = 0.004) and female (P = 0.006) HFD-fed groups compared to the normal diet-fed group (Fig. 1C). Moreover, the Shannon diversity index, an indicator of diversity in species, decreased in all female HFD-fed groups

(6-week-old, P = 0.010; 31-week-old, P = 0.010; 74-week-old, P = 0.020; 2-year-old, P < 0.001) compared to the normal diet-fed group (Fig. 1D).

Taxonomic difference between the HFD-fed group and normal diet group

At the genus level, the *Pseudoflavonifractor* showed a decreasing tendency in all the HFD-fed groups and significantly decreased in the 74-week-old (P = 0.002) and 2-year-old (P = 0.041) female HFD-fed groups compared to the normal diet group (Fig. 2A). *Akkermansia muciniphila* species showed a significant increase in the 6-week-old (P = 0.004) and 31-week-old (P = 0.031) male groups and 6-week-old (P = 0.016) and 2-year-old (P = 0.008) female groups (Fig. 2B). *Phascolarctobacterium faecium* species significantly increased in the HFD-fed groups of 6-week-old (P = 0.002), 31-week-old (P = 0.001), and 74-week-old (P = 0.029) males, and 6-week-old (P = 0.022) and 31-week-old (P = 0.002) females compared to the normal diet group (Fig. 2C). Contrarily, the number of unknown species PAC001125_s was significantly increased in the 2-year-old male HFD-fed group (P = 0.021) (Fig. 2D). The result of LEfSe analysis also showed a similar tendency (Figure S1). Except for the 2-year-old male group, *Phascolarctobacterium faecium* species increased in HFD fed male group, and *Akkermansia muciniphila* species increased in HFD fed groups of 6-week and 2-year-old female and 31-week-old male rats. However, it was hard to find any pattern differences between normal diet and HFD fed groups except *Phascolarctobacterium faecium* and *Akkermansia muciniphila* species.

Enterotypes of microbiomes

The principal coordinates analysis plot (Figure S2) was separated by diet while sex-and age-related separation did not appear. Therefore, we performed enterotype clustering for each sex using the Calinski-Harabasz index (Figure S3). As the Calinski-Harabasz index was maximized at two with males and three with females, enterotypes were separated into Enterotype1 (E1) and Enterotype2 (E2) in males (Figure S3; Fig. 3A), while females were separated into Enterotype1 (E1), Enterotype2 (E2), and Enterotype3 (E3) (Figure S3; Fig. 3B). Male E1 was composed of all normal diet groups and three of the 2-year-old HFD-fed group. The rest of the rats in the HFD-fed group were included in E2 (Table 1). Female E1 was composed of four of the 6-week-old, six of the 31-week-old, four of the 74-week-old, and five of the 2-year-old normal diet groups. Female E2 was composed of one of the 6-week-old normal diet group, six of the 6-week-old, one of the 31-week-old, five of the 74-week-old, and five of the 2-year-old HFD-fed groups. Female E3 was composed of one of the 6-week-old, one of the 74-week-old, and one of the 2-year-old normal diet groups, as well as five of the 31-week-old, six of the 74-week-old, and five of the 2-year-old HFD-fed groups (Table 1).
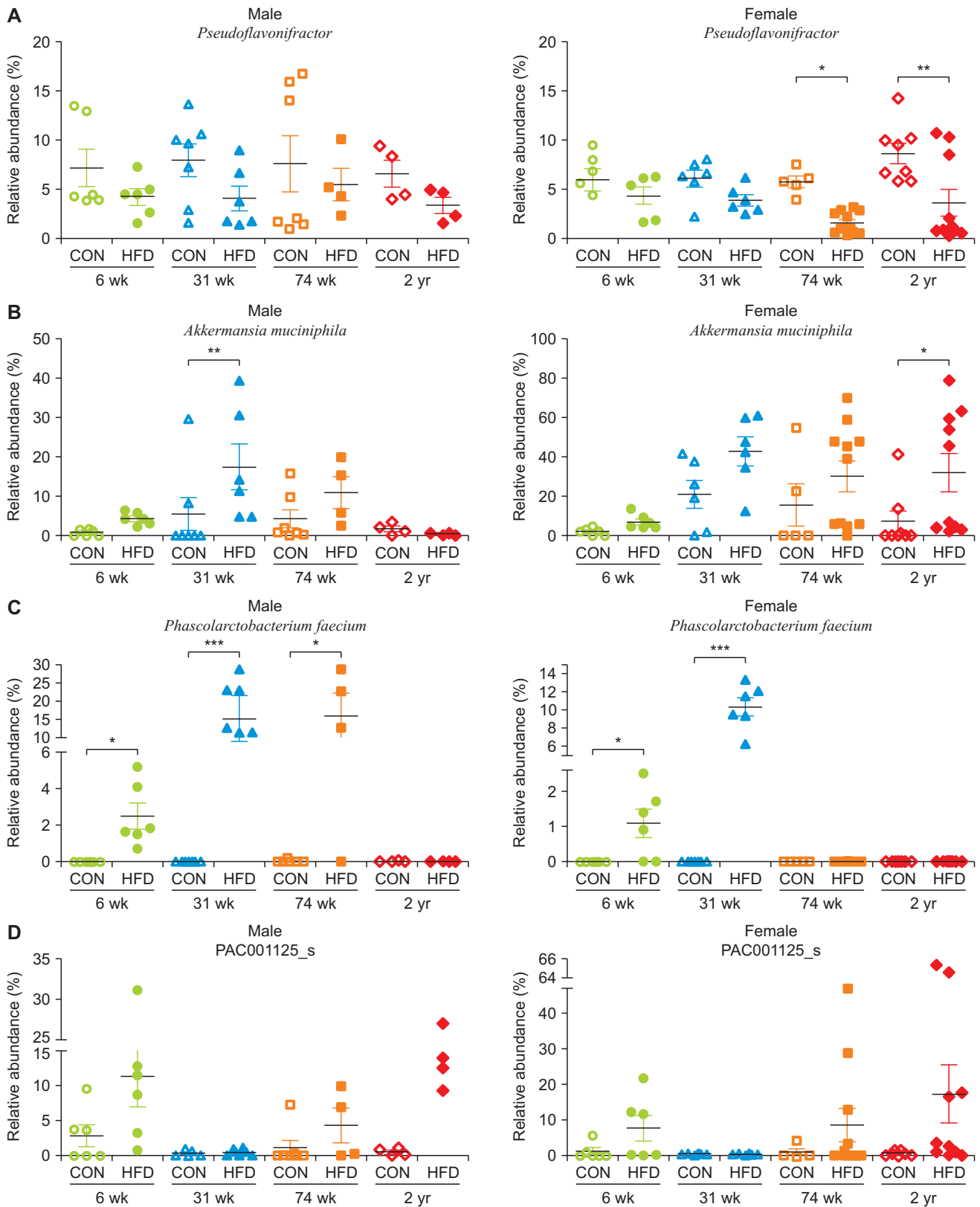
Figure 2. Alterations in the abundance ratio of fecal microbial species depending on age and diet. The relative abundance of *Pseudoflavonifractor* (A), *Akkermansia muciniphila* (B), *Phascolarctobacterium faecium* (C), and *PAC001125_s* (D). Collection of fecal samples and their microbiome analysis was conducted as described in "MATERIALS AND METHODS". The statistical significance was determined by the Mann–Whitney test; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
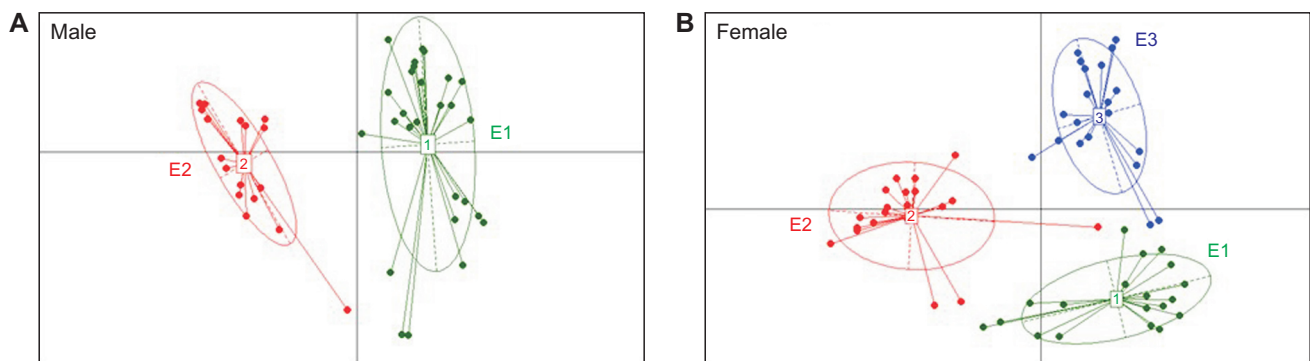
Figure 3. Enterotype-like clustering analysis in the fecal samples of male and female rats fed control (CON) or high-fat diet (HFD). There are two fecal enterotypes (E1 and E2) in males (A) and three enterotypes (E1, E2, E3) in female rats (B). The optimal cluster number was determined by maximizing the Calinski-Harabasz index value (Figure S2).

Table 1. The predominant species in each enterotype of male and female rat samples shown in Figure 3

Enterotype	Diet	6 wk	31 wk	74 wk	2 yr	Dominant bacteria species
Male						
E1	CON	6	7	7	4	PAC001490_s (4.0%), EU622773_s (3.7%), PAC002478_s (3.2%)
	HF	0	0	0	3	
E2	CON	0	0	0	0	*Phascolarctobacterium faecium* (11.2%), *Akkermansia muciniphila* (10.2%),
	HF	6	6	4	1	*Bacteroides sartorii* (9.8%)
Female						
E1	CON	4	6	4	5	*Akkermansia muciniphila* (10.8%), EU622773_s (6.7%), *Bacteroides sartorii* (3.5%)
	HF	0	0	0	0	
E2	CON	1	0	0	2	*Akkermansia muciniphila* (53.1%), JAGT_s group (6.4%), *Bacteroides sartorii* (4.3%)
	HF	6	1	5	5	
E3	CON	1	0	1	1	PAC001125_s (16.2%), *Bacteroides sartorii* (9.6%), PAC002478_s (5.1%)
	HF	0	5	6	5	

Values are presented as number of rats. E, enterotype; CON, control diet; HF, high-fat.

Compositional feature of the enterotypes

We plotted the taxonomic composition of each enterotype, and observed clear differences at the phylum and genus levels (Fig. 4). In the case of males, E2 showed an increase in population of Verrucomicrobia ($P < 0.001$), Proteobacteria ($P = 0.002$), and Bacteroidetes ($P = 0.197$) phyla, while Firmicutes phyla decreased significantly ($P = 0.004$) compared to the composition of E1 (Fig. 4A). Furthermore, *Bacteroides* genus were increased significantly in E2 and *Prevotella* genus increased significantly in E1, both of which belong to Bacteroidetes phyla (Fig. 4B). In enterotypes from females, Proteobacteria phyla increased significantly in E2 (E1 vs. E2, $P < 0.001$; E2 vs. E3, $P = 0.001$), whereas E3 showed a significant decrease in Firmicutes phyla (E1 vs. E3, $P < 0.001$; E2 vs. E3, $P < 0.001$) and Bacteroidetes phyla (E1 vs. E3, $P < 0.001$; E2 vs. E3, $P = 0.006$), and a significant increase in Verrucomicrobia phyla (E1 vs. E3, $P < 0.001$; E2 vs. E3, $P < 0.001$) (Fig. 4C). Furthermore, *Bacteroides* genus was increased significantly in E2 (E1 vs. E2, $P = 0.002$; E2 vs. E3, $P = 0.001$), *Prevotella* genus increased significantly in E1 (E1 vs. E2, $P < 0.001$; E1 vs. E3, $P < 0.001$), and *Ruminococcus* increased significantly in E3 (E1 vs. E3, $P = 0.005$; E2 vs. E3,

$P = 0.008$) (Fig. 4D).

Characteristics of the enterotypes

The results from the LEfSe analysis (Fig. 5) indicated that the microbiomes of male E1 and female E1 were dominated by *Monoglobus pectinilyticus* and *Acutalibacter muris* species compared to male E2 or female E2 and female E3 (Fig. 5A-5C). In contrast, the microbiome of female E2 was dominated by *Clostridium leptum*, *Desulfovibrio fairfieldensis*, *Bacteroides thetaiotaomicron*, and *Romboutsia timonensis* species compared to female E1 and E3, which are known as opportunistic pathogenic strains (Fig. 5B and 5D). In female E3, *Akkermansia muciniphila* was the dominant species compared to female E1 and E2, which are known as commensal strains (Fig. 5C and 5D). From the difference in abundance between each enterotype, based on the LEfSe analysis, commensal strains and opportunistic pathogenic strains were categorized in accordance with previous studies, and their ratios were compared (Fig. 5E). Male E1 and female E1 dominated strains were all commensal bacteria. Meanwhile, female E2 had higher opportunistic pathogenic strains compared to female E3. Upon assessment of mRNA expression
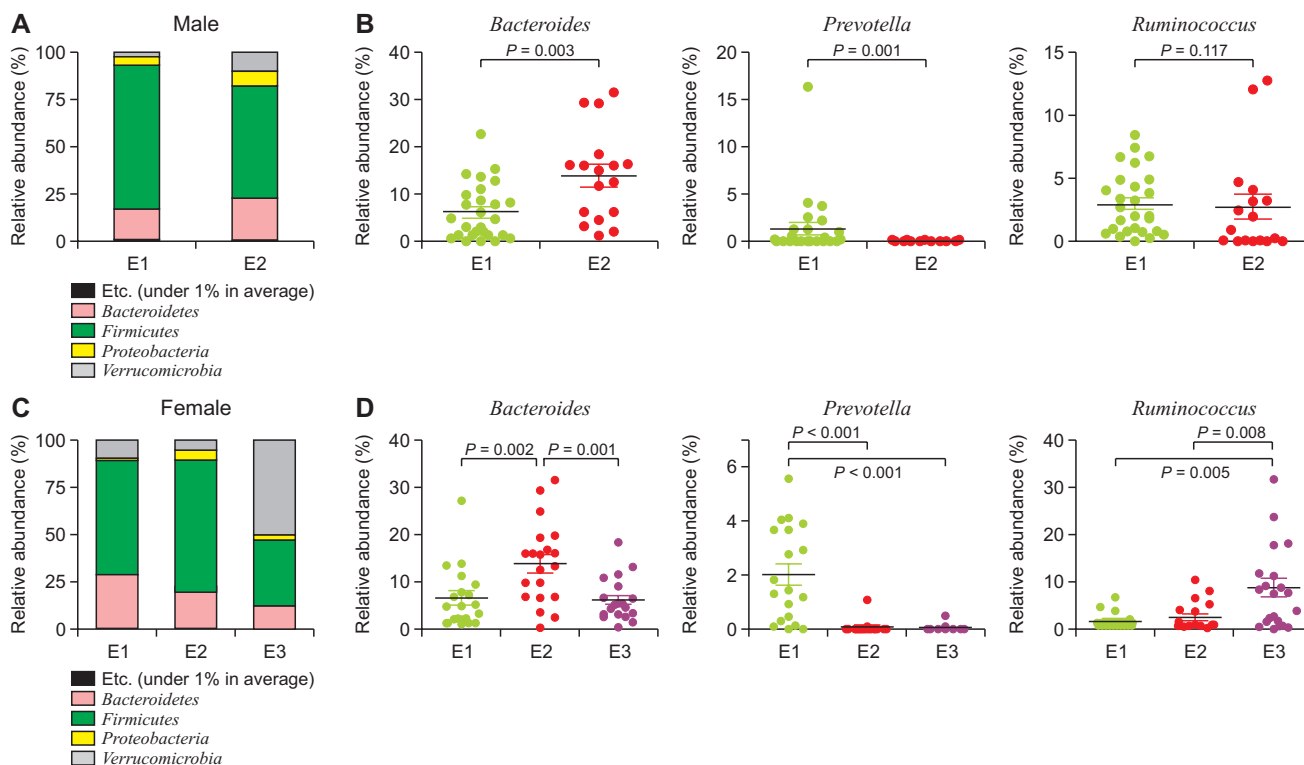
Figure 4. Comparisons of relative abundance of representative fecal microbial enterotypes. Fecal samples from the male (A, B) and female (C, D) rats from control or high-fat diet-fed were collected and subjected to analysis of microbial enterotypes at the phylum level (A, C) and 3 representative human enterotypes (*Bacteroides*, *Prevotella*, and *Ruminococcus*) genera. The statistical significance was calculated by the Mann–Whitney test.

levels using RT-qPCR, male E1 was found to be significantly higher for *Mucin 2* (*Muc2*) (*P* < 0.001) and *Occludin* (*Ocln*) (*P* = 0.034) compared to male E2 (Fig. 6A). In addition, female E1 was significantly higher for *Muc2* (E1 vs. E2, *P* = 0.002; E1 vs. E3, *P* = 0.001) and *Ocln* (E1 vs. E2, *P* = 0.018; E1 vs. E3, *P* = 0.022) compared to female E2 and E3. Moreover, female E3 was significantly higher for *Toll-like receptor 2* (*Tlr2*) compared to female E2 (*P* = 0.010) (Fig. 6B).

DISCUSSION

In our present study, it was found that an HFD influenced fecal microbiota at all ages. Interestingly, the microbiota differed by sex, in a way that female rats showed a compensatory enterotype response to an HFD. In addition, an HFD lowered alpha diversity regardless of age and sex, suggesting that long-term administration of an HFD leads to intestinal microbial homeostasis. In agreement with a previous report [26], we observed an increase in the ratio of opportunistic pathogenic to commensal strains in the HFD groups, suggesting that an HFD not only destroys the diversity but also increases the chances of colonizing opportunistic strains.

The human gut microbiota mostly consists of two major phyla, Bacteroidetes and Firmicutes, which account for more

than 90% of the total population [27]. Since the relationship between the two major phyla, expressed as the B/F ratio, is related to several pathological conditions, this ratio has been used as an eventual biomarker [27]. Numerous studies have supported that higher F/B ratios are associated with a healthy state [28,29], and lower B/F ratios are associated with obesity [30] and colorectal cancer [31,32]. Some studies have demonstrated that the gut microbiomes of obese animals and humans exhibit lower B/F ratios compared to normal-weight individuals [28,31,32]. In our previous study, Figure 1B, the B/F ratio lowered in 2-year-old male and female rats, suggesting the old age vulnerable to obesity and colorectal cancer compared to young age.

Sex differences influence not only the gut microbial composition but also the response to a change in host diet. In this study, the enterotypes in male and female rats were categorized differently. In the case of males, there were two enterotypes, E1 and E2. The male E1 included all normal diet groups regardless of their age and some 2-year-old HFD-fed male rats, while the male E2 included the rest of the male rats in the HFD-fed groups. Similarly, in females, there were three enterotypes. The female E1 included almost all normal diet groups regardless of their age, and female E2 and E3 included the rest of the HFD-fed groups.
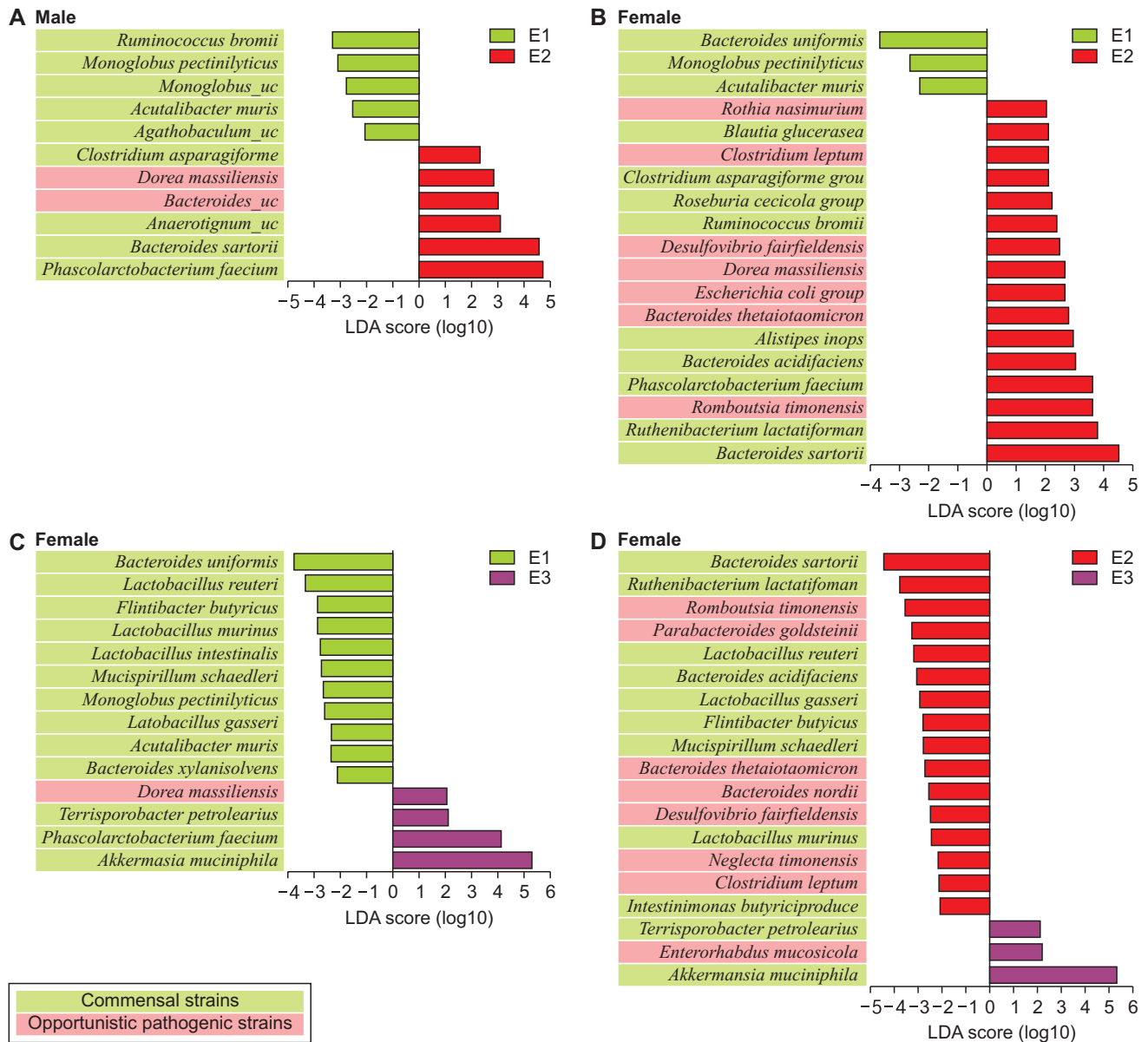
Figure 5. The compositional alteration in the fecal microbiota of male and female rats. Bar plots of species with significant differences in abundance with linear effect size (LEfSe) analysis in male (A) and female (B-D) rats. Linear discriminant analysis (LDA) scores of species showed increased species in E1 (green), E2 (red), and E3 (purple) enterotypes. The ratio of opportunistic pathogenic to commensal strains was calculated and listed in (E).
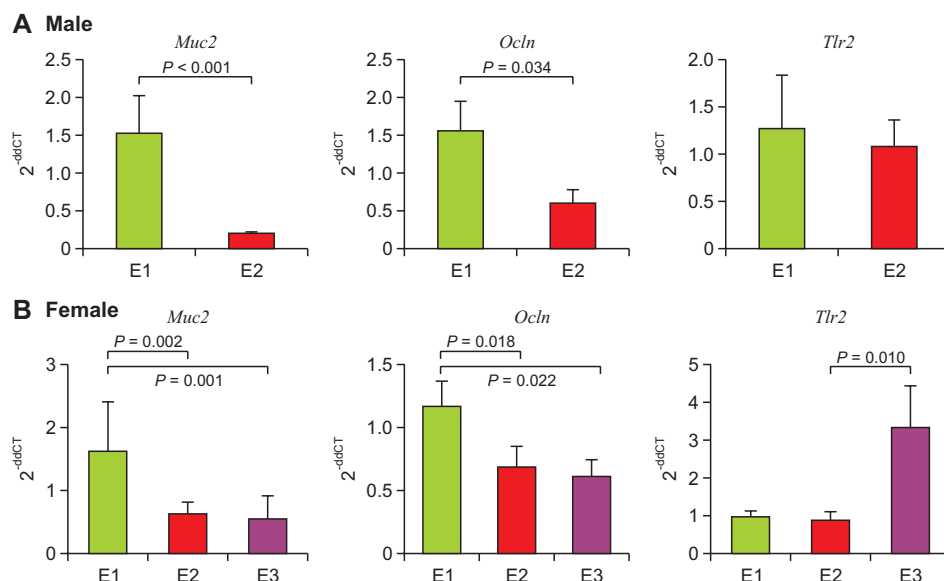
Figure 6. Differential host mRNA expression of *Muc2*, *Ocln*, and *Tlr2* in male and female enterotypes. The host colon mRNA expression of corresponding genes in the fecal microbiota of male (A) and female (B) rats was measured by Real-time quantitative PCR (RT-qPCR) as described in MATERIALS AND METHODS. The statistical significance was calculated by the Mann–Whitney test. *Muc2*, *Mucin 2*; *Ocln*, *Occludin*; *Tlr2*, *Toll-like receptor 2*.

When we compared their relative abundance at the genus level, they were clearly classified into three types; male E1 and female E1 belong to the *Prevotella* type, male E2 and female E2 belong to the *Bacteroides* type, and female E3 belongs to the *Ruminococcus* type. These three taxa were reported as the dominant gut taxa contributing to the clustering of enterotypes in humans and represent the host's gut characteristics. For example, the fecal microbial community in humans who consume protein and animal fat for a long period is dominated by the *Bacteroides* genus, while *Prevotella* is dominant in long-term consumers of carbohydrates. In the case of the *Ruminococcus* genus, although it did not differentiate well from the *Bacteroides* and *Prevotella* genus, the *Akkermansia muciniphila* strain in their microbial community is well known. Similar to our data, male E2 and female E2 types, including most of the HFD-fed group, appear to be of the *Bacteroides* type in humans. In the case of the female E3 group, the high relative abundance of *Akkermansia muciniphila* may be close to the *Ruminococcus* type in humans [22,33,34].

Animal microbiota studies had been criticized because of low relevance between animal and human microbiota [35]. Previously we reported that sex differences influence the response of the gut microbiome to an HFD, particularly in the 2-year-old age group but not in the 6-week-old F344 rats [17]. In the present study, we extended this analysis to the middle-aged group of 31- and 74- week-old rats in addition to the 6-week-old and the 2-year-old groups. In addition, we also performed an enterotype analysis, which uncovered a difference present in all age groups.

Moreover, when we analyzed mRNA expression of host colon depending on enterotypes, the female E3 group showed significantly higher mRNA expression of *Tlr2* compared to the female E2 group. Toll-like receptor 2 (TLR2) is a membrane receptor related to the immune system, recognizing pathogens, and activating innate immunity. It is known that intestinal epithelial TLR2 critically controls mucosal inflammation by directly preserving tight junction-associated barrier integrity [36], and the mechanism of barrier protective function is related to TLR2-mediated IL-10 production [16]. Moreover, the *Ruminococcus* genus, which is significantly high in the female E3 group, is well known to be categorized as a butyrate-producing bacteria [37] and influences IL-10 production [13]. In this context, we assume that the female E3 group represents the compensation ability of females to an HFD.

Akkermansia muciniphila is mucin-utilizing bacteria that colonize the mucin layer [38]. There was a report on the increase of *Akkermansia* in the cecal area of the HFD fed compared to the low-fat diet-fed rats [39]. *Akkermansia muciniphila* harbors all genes encoding enzymes involved in the citric acid cycle, which is important for fatty acid metabolism, while many bacteria species seem to lack genes for the whole cycle [40]. Therefore, we consider that the administration of fatty acid (HFD groups) contributes to colonization of *Akkermansia muciniphila* dominant in HFD groups.

This study has several limitations. The enterotype of normal mice with a homogeneous diet cannot reflect normal human gut microbiota due to fundamental differences in the diet pattern. It would be difficult to apply these results to human gut microbiota. However, it is nearly impossible to perform this kind of study regarding the effect of HFD on the gut microbiota in human beings. Therefore, we chose the rat model. As the aim of this study was to identify differences in the gut microbiome under a controlled diet in rats, we tried to cluster the HFD group only (Figure S1). However, we could not find consistent results. Thus, we compared the effect of HFD on gut microbiota by sex and age by enterotyping.

Also, there was no significant difference in the middle-age

group of rats. From the results of our previous reports, only the older male group was influenced by an HFD, while younger males and females were unaffected [18]. Thus, we expected gradual microbial changes in the middle-age (31- and 74-week-old) group of rats. However, since it was impossible to follow every time point of aging, there was a huge time gap among different aged rats. However, we carried out this study in rats at 31 weeks of age and kept them under steady conditions with the corresponding diets until they became 74-week and 2-year-old. Therefore, we assume that the microbial changes may appear sharply in old age. Another limitation was the small number of 2-year-old male rats as the average life expectancy of a rat is nearly 2 years, and most rats naturally die by this time because of senescence [41,42]. In particular, older males tended to die earlier than females, and hence supplementing 2-year-old male rats was difficult. We assume that the lack of a compensation mechanism in older males, as observed in the female E3 enterotype, might account for the short life span of male rats.

Another limitation of this study is that the E3 enterotype appeared in some female rats even though the same HFD was given to the inbred rat strain. During the experiments, like the case of the male, we need to supply the enough number of 74-week and 2-year-old female rats to meet to analyze statistical significance properly. We consider that the old-aged female rats possess different microbiota initially because of the different batch, provoking manifestation of heterogenous enterotype, E3.

In the previous study, we found that aging itself affects the rodent's colon motility [42]. Another study has reported that HFD causes constipation in mice [43]. Moreover, some studies reported that constipation caused by HFD feeding is related to dysbiosis in colon mucus [44,45]. Therefore, the change of microbiota in our study may be associated with both aging and HFD. Furthermore, we could measure fecal consistency and amount to reveal the relation in constipation between aging and HFD.

In conclusion, HFD influenced fecal microbiota at all ages, regardless of sex, decreasing alpha and beta diversity. However, the shape of the fecal microbiota differed by sex in terms of enterotype clustering. In particular, female rats developed a compensatory enterotype response to an HFD.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

The raw unprocessed gene datasets of 16S rRNA, which were generated during the current study, are available with the NCBI Sequence Read Archive (SRA accession number: SRR 12052376~12052436), https://www.ncbi.nlm.nih.gov/sra/PRJNA640490. The processed data generated and analyzed during this study has been included in this published article under Supplementary Dataset files. Supplementary materials can be found via https://doi.org/10.15430/JCP.2021.26.4.277.

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