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Full Paper

Bile acid is a responsible host factor for high-fat diet-induced gut microbiota alterations in rats: proof of the “bile acid hypothesis”

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High-fat diet (HFD)-induced alterations in gut microbiota may be associated with host pathophysiology, prompting increased interest in elucidating their causal relationships. However, the mechanisms by which HFDs induce these alterations require further clarification. Our previous study using cholic acid (CA)-fed rats suggested that bile acid drives the HFD-induced microbiota alterations as a host factor, a concept termed the “bile acid hypothesis”. We analyzed the alterations in the cecal microbiota and bile acid composition in HFD-fed rats and compared the results with those of rats on a CA-supplemented diet. In both cases, the concentrations of total bile acids, including highly bactericidal deoxycholic acid (DCA), increased, concomitant with the increases in the Firmicutes (Bacillota)/Bacteroidetes (Bacteroidota) ratio. Operational taxonomic units (OTUs), accounting for 63.39% of the cecal microbiota of control rats, showed a significant correlation with the total bile acid concentration in HFD-fed rats. A DCA sensitivity test conducted in Firmicutes isolates, corresponding to the predominant OTUs from the HFD-fed rats, exhibited significantly higher DCA resistance compared with Bacteroidetes. The top 12 most abundant OTUs of Firmicutes and Bacteroidetes showing positive or negative correlations with the total bile acid concentration were selected from the HFD-fed rats, and their dynamics were compared with those in the CA-fed rats. Of the 24 OTUs, 18, which constituted 48.28% of the cecal population in the control rats, were altered in the same direction (increase or decrease) in the HFD- and CA-supplemented diet groups. Therefore, approximately half of the cecal populations in the control rats were affected by bile acids, substantiating the bile acid hypothesis microbiologically and quantitatively.

Key words: gut microbiota, bile acid, high-fat diet, cholic acid, deoxycholic acid

INTRODUCTION

The composition of the gut microbiota affects the host’s health. Accumulating evidence suggests that gut microbiota dysbiosis is associated with diseases such as obesity, inflammatory bowel disease, Crohn’s disease, type 2 diabetes, and cancer [1–3]. In particular, high-fat diet (HFD)-induced dysbiosis is associated with various diseases, including obesity, obesity-related metabolic phenotypes, and liver cancer [4, 5]. Given these implications, understanding the mechanism underlying HFD-induced alterations in the gut microbiota is pivotal for elucidating the causal relationship between dysbiosis and disease development.

In many rodent models [6, 7] and probably in humans [8], an HFD disrupts the balance of two major phyla of the gut microbiota, increasing Firmicutes (currently reassigned as Bacillota) and decreasing Bacteroidetes (Bacteroidota). Although the mechanism behind this phenomenon remains somewhat elusive, our previous research demonstrated that bile acid is a host

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factor that regulates cecal microbiota in rats [9]. Feeding cholic acid (CA), a common primary bile acid in rodents and humans, increases total bile acid concentrations in the rat cecum, with a preferential increase in deoxycholic acid (DCA) levels, a potent secondary bile acid with strong bactericidal properties [10]. Under these conditions, the composition of the cecal microbiota was drastically altered at the phylum level, resembling the changes typically observed in response to the consumption of an HFD; Firmicutes became more predominant, leading to a decrease in the abundance of Bacteroidetes [9]. Bile acids, synthesized from cholesterol in the liver in a conjugated form with taurine or glycine, are secreted into the duodenum to aid in the digestion of dietary lipids in the small intestine [11]. Another important feature of bile acids is their bactericidal activity. During intestinal transit, the majority of bile acids are deconjugated and further modified by the indigenous gut microbes to produce secondary bile acids [12]. Our previous studies showed that DCA, a typical secondary bile acid formed from CA, exhibits approximately 10 times higher bactericidal activity than CA [10, 13]. As intestinal bile acid flow increases in response to the consumption of dietary fat, a higher concentration of DCA may exert a strong selective pressure in the cecum to yield Firmicutes-enriched gut microbiota on an HFD (termed the "bile acid hypothesis") [14, 15]. In this context, Zheng *et al.* [16] demonstrated that bile acid was the primary factor associated with cecal microbiota alterations on an HFD, using metabolome data and 16S rRNA gene sequence data of the microbiota in mice on an HFD or a bile acid-supplemented diet. Interestingly, HFD-induced secretion of bile acids in the intestine occurred before observable alterations in the microbiota. However, further research is needed to quantitatively analyze the bile acid effects at the operational taxonomic unit (OTU) level and to understand microbiologically how bile acids influence gut microbiota composition.

Besides working as a detergent, bile acids function as signaling molecules that maintain host homeostasis [17, 18]. Bile acids serve as ligands that activate the farnesoid X receptor, a nuclear receptor that regulates bile acid metabolism in hepatocytes, as well as host glucose and lipid metabolism. Furthermore, bile acids activate Takeda G protein-coupled receptor 5 (TGR5), the first known G-protein-coupled receptor specifically responsive to bile acids. This activation leads to enhanced energy expenditure in adipose tissue [19] and the suppression of proinflammatory cytokine release [19, 20]. As the structure of bile acids significantly influences their affinity for these receptors, gut microbes are profoundly involved in the maintenance of host homeostasis through bile acid biotransformation [21].

Further studies have identified a link between bile acid levels and disease development. Increased DCA levels, produced by gut microbes during HFD consumption, facilitate obesity-associated hepatocellular carcinoma development in mice pretreated with chemical carcinogens [5]. Conversely, primary bile acids, such as taurocholic acid (TCA) and chenodeoxycholic acid (CDCA), were found to enhance the chemokine-dependent accumulation of hepatic natural killer T cells, thus leading to the inhibition of tumor growth in the liver. However, DCA did not promote such accumulation [22]. Therefore, inhibiting the formation of secondary bile acids in the gut may reduce the risk of liver cancer development. Furthermore, feeding a diet high in milk-derived saturated fat promoted colitis in *Il10*$^{-/-}$ genetically susceptible mice [23]. This exacerbation was triggered by the increased excretion of TCA resulting from milk fat consumption; the delivery of taurine-conjugated bile acid to the intestine promoted the outgrowth of *Bilophila wadsworthia* by providing the bacterium with taurine as an electron acceptor for anaerobic respiration to produce H_2S, a causative agent of colitis.

Disease development often affects the bile acid profile, which in turn can impact the composition of the gut microbiota, potentially affecting the course of the disease. In patients with advanced cirrhosis, the total fecal bile acid concentrations were significantly reduced, with significantly lower DCA/CA ratios. These changes are accompanied by dysbiosis, characterized by a decreased ratio of Firmicutes to Bacteroidetes and significant overgrowth of the potentially pathogenic family *Enterobacteriaceae* [24]. Our previous findings [9] suggest that reducing bile acid input could lead to a decrease in the Firmicutes to Bacteroidetes ratio, potentially resulting in a reduction in the abundance of DCA-producing bacteria belonging to the order Clostridiales. In patients with inflammatory bowel disease, bile acid dysmetabolism, linked to dysbiosis, leads to a significantly decreased proportion of secondary bile acids [25]. This reduction may result in the loss of the anti-inflammatory effects of secondary bile acids mediated by TGR5 activation, thereby exacerbating chronic inflammation [16]. Interestingly, in pediatric patients with Crohn's disease, sustained remission by exclusive enteral nutrition was achieved in patients with a secondary bile acid-dominant profile but not in those with a primary bile acid-dominant profile [26], aligning with the consequence of the TGR5 activation [17, 25]. Therefore, there is growing interest in validating the bile acid hypothesis.

In this study, we aimed to obtain quantitative and microbiological evidence supporting the bile acid hypothesis. Alterations in cecal microbiota composition and bile acid profiles in rats fed an HFD or a normal diet supplemented with CA for 8 weeks were analyzed. HFD-induced microbiota alterations were then compared with CA-induced alterations at the OTU level. Active cultures were obtained from the cecal contents, and their sensitivity to DCA was evaluated to elucidate the observed alterations.

MATERIALS AND METHODS

Animal trial

Male WKAH/HkmSlc rats (3 weeks old; Japan SLC, Inc., Hamamatsu, Japan) were individually housed in a controlled environment with a 12-hr light and 12-hr dark cycle at 22 ± 2°C and 55 ± 5% humidity following a two-week acclimatization period on a control diet. All experimental rats had *ad libitum* access to food and water throughout the study period. Details of the feed ingredients for the control, HFD, and CA diets are provided in Supplementary Table 1. For the HFD feeding experiment, 27 rats were divided into two groups: one group received a control diet (n=13), while the other group received an HFD (n=14). At weeks 2, 4, and 8, four to six rats from each group were euthanized using sodium pentobarbital anesthesia (50 mg/kg body weight) to examine the time-dependent profiles (Supplementary Table 2). Cecal contents were immediately collected for analysis of pH levels, bile acid concentrations, and microbiota populations. In the CA feeding experiment, similar experimental conditions were maintained, including the rat strain, housing, lighting, and feeding period. To prepare the CA diet, 0.5 g CA/kg diet was added to the control diet. Ten to 12 rats were

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divided into two groups: one group was fed the control diet (n=5), while the other group was fed the CA diet (n=5–7). A total of 32 rats were used in three rounds of feeding experiments for 2, 4, and 8 weeks. Rats from each group were euthanized at the end of each feeding period (Supplementary Table 2) as described above, and the cecal contents were collected. This study was approved by the Institutional Animal Care and Use Committee of the National University Corporation, Hokkaido University (approval numbers 14-0026 and 17-0050). All animals were managed in accordance with the Hokkaido University Manual for Implementing Animal Experimentation.

Bile acid analysis

Bile acids in cecal contents were extracted and quantified using an Accela UHPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) or an ACQUITY UPLC system (Nihon Waters K.K., Tokyo, Japan) with an ACQUITY UPLC® BEH C18 column as the main column (100 × 2.0 mm i.d., 1.7 µm thickness, Nihon Waters K.K.), as previously described [27, 28]. Mass analysis of bile acids was performed using a TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific Inc.) or a Quattro Premier XE Quadrupole Tandem Mass Spectrometer (Nihon Waters K.K.) equipped with an electrospray ionization probe in the negative ion mode. The bile acids analyzed are listed in Supplementary Table 3.

Community DNA extraction

The microbial community DNA was extracted from the cecal contents using a previously described method [29]. After homogenization in 5 mL of washing buffer (200 mM Tris-hydrochloride [HCl] and 80 mM ethylenediaminetetraacetic acid (EDTA); pH 9.0), the bacterial pellet was washed three times by centrifugation at 7,000 × g for 10 min and resuspended in 1 mL of extraction buffer (500 mM sodium chloride, 50 mM Tris-HCl, 50 mM EDTA, and 4% sodium dodecyl sulfate; pH 8.0), along with 300 mg of glass beads (diameter, 0.1 mm). Subsequently, microbial cells were disrupted using a Multi-Beads Shocker (Yasui Kikai Corporation, Osaka, Japan) three times at 2,500 rpm for 20 sec, with 10-sec breaks between cycles, for a total of 80 sec. The samples were incubated at 70°C for 15 min, and the bead-beating and incubation steps were repeated once. After centrifugation at 18,700 × g for 10 min at 4°C, 260 µL of 10 mM ammonium acetate was added to the collected supernatant (~700 µL) and mixed. After centrifugation, the collected supernatant was treated with 1 µL of 100 mg/mL RNase A (Nippon Gene Co., Ltd., Tokyo, Japan) for 1 hr at 37°C, and then nucleic acids were purified with 400 µL of a phenol-chloroform-isoamyl alcohol mixture (25:24:1, v/v). DNA was precipitated from 600 µL of the supernatant by adding the same volume of isopropanol. The precipitated DNA was collected by centrifugation (18,700 × g for 15 min at 4°C), washed with 70% ethanol, dried, and resuspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA; pH 8.0).

Cecal microbiota analysis

To determine the cecal microbiota composition in HFD-fed and CA-fed rats, pyrosequencing and Illumina MiSeq analyses were performed, respectively. For pyrosequencing analysis, the V3–V4 variable regions of the 16S rRNA genes were amplified using 100 ng of extracted community DNA as a template. A primer pair, 347F (5′-*CCATCTCATCCCTGCGTGTCTCCGAC TCAG*NNNNNNNNNNGGAGGCAGCAGTRRGGAAT-3′) and 803R (5′-*CCTATCCCCTGTGTGCCTTGGCAGTCC* TACCRGGGTATCTAATCC-3′) [30], was used for amplification. Adaptor sequences A and B (italicized sequences) were added to the 5′ end of the forward and reverse primers, respectively. Unique multiplex identifier sequences, indicated as Ns, were added between adaptor A and forward primer 347F to distinguish the samples. The target region of the 16S rRNA gene was amplified and purified as previously described [28]. The amplicons purified from multiple samples were mixed at equal ratios and sequenced using a 454 GS Junior (F. Hoffmann-La Roche Ltd., Basel, Switzerland), resulting in a total of 267,426 reads (9,905 ± 1,343 [average ± standard deviation] reads per sample).

For Illumina MiSeq analysis, the same regions of the 16S rRNA genes were amplified using the primer sets, 347F_MiSeqF (5′-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*-GGAGGCAGCAGTRRGGAAT-3′) and 803R_MiSeqR (5′-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* CTACCRGGGTATCTAATCC-3′), as the first polymerase chain reaction (PCR). Adapters C and D (italicized sequences) were incorporated into the 5′ end of the forward and reverse primers, respectively. The target region of the16S rRNA gene was amplified and purified as previously described [28]. A second PCR was conducted to incorporate the index sequences using Nextera XT Index Kit v2 Set D for 96 indices and 384 samples (FC-131-2004; Illumina, Inc., San Diego, CA, USA). The amplification was conducted in a total reaction volume of 50 µL containing 4.5 µL of purified first PCR products, 25 µL of 2× PCR buffer, 0.4 mM each of the four types of deoxynucleotide triphosphate, 0.3 µM of each primer, and 0.5U of KOD Fx Neo (Toyobo Co., Ltd., Osaka, Japan) using the following 3-step protocol: preheating at 94°C for 2 min; 8 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 68°C for 30 sec; and a final terminal extension at 68°C for 7 min. The amplified DNA was subjected to agarose gel electrophoresis to verify the product size and purified using a MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified products were quantified using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific Inc.). Subsequently, amplicons from multiple samples were mixed in equal ratios, and primer dimers were removed using a GeneRead Size Selection Kit (Qiagen). Mixed libraries were sequenced on an Illumina MiSeq instrument using MiSeq Reagent Kit V3 (600 cycles; Illumina, Inc.). After the sequences from PhiX were removed from the raw Illumina reads, paired-end reads that overlapped at least 20 bp in length were assembled using the Paired-End reAd mergeR software (version 0.9.10) [31]. In total, 3,137,949 high-quality paired reads (98,061 ± 36,117 average reads per sample) were obtained. The raw sequences obtained by pyrosequencing and Illumina MiSeq analyses were deposited in the Sequence Read Archive of the DNA Data Bank of Japan (DRA) under accession numbers DRA017244 and DRA017254, respectively.

Taxonomic analysis

The sequences on the 454 GS Junior or Illumina MiSeq were analyzed using the open-source software package Quantitative Insights into Microbial Ecology (QIIME, version 1.8.0) [32]. For the pyrosequencing data, the raw sequence data were denoised and assigned to individual rats using sequence-quality filtering commands. High-quality sequences were then extracted, assigned

to OTUs using de novo OTU picking with a 97% threshold of identity, and then classified taxonomically using the Greengenes reference database (http://greengenes.secondgenome.com/downloads/database/13_5) [33]. The microbial diversity within each control and HFD group was assessed using α-diversity metrics, including Chao1, the number of observed species (the number of OTUs), phylogenetic distance (PD) whole tree index, and Shannon diversity index. These analyses were conducted according to a previously published protocol [34].

Illumina MiSeq data were analyzed using a previously published protocol [34]. Paired-end sequences were assigned to individual rats and were filtered based on the sequence quality using the script "split_libraries_fastq.py". Potential chimeric sequences were removed using Usearch 61. Non-chimeric, high-quality sequences were assigned to OTUs using open-reference OTU picking with a 97% threshold of pairwise identity and then classified taxonomically using the Greengenes reference database (http://greengenes.secondgenome.com/downloads/database/13_8) [33]. The α-diversity was analyzed according to the protocol described above.

To compare the altered microbes between the HFD and CA interventions, representative sequences of OTUs from the Illumina MiSeq analysis of the CA intervention were subjected to a local nucleotide BLAST analysis. The analysis identified the OTUs that corresponded to the top 12 abundant OTUs from Firmicutes and Bacteroidetes, which showed a significant positive or negative correlation with the total bile acid concentration in the HFD feeding experiment. The local BLAST analysis was conducted using the Genetyx 11 software (GENETYX Corp., Tokyo, Japan), with a minimum 97% nucleotide sequence identity threshold.

Isolation of bacteria from cecal contents and DCA sensitivity test

The cecal contents (50 mg) of rats were serially diluted in 1× phosphate-buffered saline. The diluted suspension was inoculated onto a solid medium supplemented with 15 g/L agar. EG [35] and Brucella agar media (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), containing 2 μg/mL of colistin sodium methanesulfonate (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and 1 mM DCA, were mainly used for the isolation of bacteria from the HFD-fed rat cecal contents. Colistin was used to inhibit the growth of *Escherichia coli* [36]. EG agar medium and Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 2 μg/mL of colistin were used for the isolation of bacteria from the control rat cecal contents. All cultures were conducted at 37°C under anaerobic conditions (N_2:CO_2:H_2=80:10:10) in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA). Agar plates were incubated for 96 hr. The isolated colonies were cultured overnight in 200 μL of GAM in a 96-well microtiter plate. A 50 μL aliquot of 80% (w/v) glycerol was added to each well and mixed, and then the plates were stored at −80°C. A suspension of stored culture in saline was used as a PCR template, and the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGMTACCTTGTTACGACTT-3′) were used to amplify the 16S rDNA region. Partial nucleotide sequences containing V3–V4 regions in the amplified 16S rDNA fragments were determined using the primer 926r (5′-CCGTCAATTYYTTTRAGTTT-3′). The determined nucleotide sequences were then subjected to similarity analysis with the sequences from pyrosequencing to determine isolates that belonged to the identified OTUs in the microbiota analysis using the Genetyx 11 software with a minimum nucleotide sequence identity threshold of 97%. Isolates corresponding to the Firmicutes and Bacteroidetes OTUs were used for the DCA sensitivity test. Nearly full-length nucleotide sequences of the 16S rDNA region, amplified using primers 27F and 1492R, were determined in the isolates used for the DCA sensitivity test. Species names of the isolates were assigned from the known bacterial species showing the highest sequence similarity (≥97% identity) to the determined 16S rDNA sequence. OTUs corresponding to the isolates were also identified by sequence similarity analysis based on the same criteria. The selected isolates were precultured in Gifu anaerobic medium medium containing 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS; pH 7.0) overnight. For the DCA sensitivity test, the precultures were inoculated to 5 mL of GAM containing 0.1 M MOPS (pH 7.0) and DCA (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM; 0, 0.3, 0.4, 0.5, 0.6, and 0.7 mM; or 0, 0.5, 0.75, 1, and 1.5 mM) and cultured for 24 hr. All cultures were conducted at 37°C under the same anaerobic conditions. MOPS was added to prevent the precipitation of DCA along with a decrease in pH due to the production of organic acids by bacteria. At least three independent strains from each OTU were used for the DCA sensitivity testing. In cases where only one (OTU 1781 and OTU 214) or two (OTU 1027 and OTU 1353) isolates were available for the same OTU, the culture experiment was repeated more than twice using the same isolate. Bacterial growth was monitored by measuring OD_{660}. The DCA concentration resulting in 50% growth inhibition (IC_{50}) was calculated through a nonlinear regression analysis of the OD_{660} data across various DCA concentrations.

Statistical analysis

Statistical analysis of the data between the control group and HFD- or CA-fed groups was performed using Student's t-test. For the DCA sensitivity test among the isolated bacteria, the IC_{50} values were analyzed by analysis of variance, followed by the Tukey–Kramer honestly significant difference (HSD) test. A p-value of <0.05 was considered significant. The data are expressed as the mean ± standard error of the mean (SEM). A single regression analysis was performed to clarify the correlation between the relative abundance of OTUs and total bile acid concentration in individual rat ceca using R package version 4.2.2. The statistical significance of the coefficients in the regression analysis was evaluated using an *F*-test.

RESULTS

Rat growth and tissue weights during HFD feeding

To verify the bile acid hypothesis, HFD feeding experiments on rats were conducted. Five-week-old rats were fed either a control diet or HFD for 2, 4, or 8 weeks. During the study period, no significant differences were observed in final body weight between the groups (Table 1). The food intake of the HFD group was significantly lower than that of the control group after 2 weeks of treatment (Table 1). This difference persisted until the end of the study. The liver and cecal tissue weights did not differ between the two groups during the experimental period. The epididymal adipose tissue weight tended to increase in the HFD group and exhibited a significant increase at 4 weeks (Table 1). These results indicated that although no apparent phenotypic

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differences were observed between the HFD-fed rats and the control diet-fed rats, body weight and epididymal adipose tissue tended to increase in the HFD-fed rats at 4 and 8 weeks.

Profiles of bile acids in cecal contents

Bile acid analysis was conducted to verify the effect of the HFD on the cecal bile acid profiles. As shown in Fig. 1A, the concentration of total bile acids increased in response to the HFD at 4 weeks and significantly increased at 8 weeks. At week 4, the levels of DCA; DCA-related bile acids, such as 12-oxo-lithocholic acid (12-oxo-LCA) and 3-oxo-12α-5β-cholanoic acid (3-oxo-12α); TCA; ω-muricholic acid (MCA); and ursocholic acid increased significantly in the cecum of HFD-fed rats (Fig. 1B). At week 8, the levels of CA, 7-oxo-DCA, and ursocholic acid increased significantly in the HFD groups, with a notable trend of increase observed for β-MCA, ω-MCA, and DCA (Fig. 1B). Our previous study showed that free bile acids (FBAs), particularly β-MCA, HDCA, ω-MCA, and DCA, exhibit bactericidal activity at physiological concentrations [10]. These results showed that total bile acids, including bactericidal FBAs such as DCA, β-MCA, and ω-MCA, increased following HFD feeding for 4 and/or 8 weeks, thereby potentially exerting strong selective pressure in determining the gut microbiota composition.

Alterations in the composition of the cecal microbiota in response to HFD feeding

As the concentrations of total bile acids in the cecal contents significantly increased in response to the HFD at 8 weeks, our subsequent experiments focused on the 8-week samples. To elucidate the effects of the HFD on gut microbiota composition, we performed 454 pyrosequencing of the 16S rRNA gene sequences in the community DNA. Composition analysis revealed the profound impact of the 8 weeks of HFD feeding on the composition of the cecal microbiota. The α-diversity (community diversity within a sample) analysis revealed that the α-diversity metrics other than the Shannon index were significantly lower in the HFD group compared with the control (Fig. 2A). In the HFD group, the relative abundance of Firmicutes increased, while those of Bacteroidetes and Proteobacteria decreased (Fig. 2B). In the phylum Bacteroidetes, the abundances of the genera

Table 1. Effects of HFD feeding on rat growth, tissue weights, and cecal pH

	2 w		4 w		8 w	
	Control	HFD	Control	HFD	Control	HFD
Rat growth						
Final body weight (g)	189.93 ± 6.06	189.28 ± 4.73	258.00 ± 3.29	267.45 ± 10.12	348.00 ± 10.22	355.28 ± 13.88
Total food intake (g)	204.87 ± 3.86	153.54 ± 4.30**	485.67 ± 5.95	368.28 ± 5.55**	1014.29 ± 51.30	750.22 ± 23.07**
Tissue (g/100 g body weight)						
Liver	4.54 ± 0.04	4.47 ± 0.11	3.85 ± 0.03	3.81 ± 0.04	3.43 ± 0.04	3.51 ± 0.09
Epididymal adipose tissue	1.63 ± 0.10	1.65 ± 0.20	2.35 ± 0.08	2.68 ± 0.10*	2.68 ± 0.16	2.87 ± 0.10
Cecal tissue	0.24 ± 0.01	0.22 ± 0.01	0.28 ± 0.11	0.19 ± 0.02	0.19 ± 0.02	0.16 ± 0.01
Cecal contents	0.75 ± 0.13	0.46 ± 0.02	0.42 ± 0.07	0.37 ± 0.05	0.43 ± 0.02	0.28 ± 0.02**
Cecal pH	7.00 ± 0.08	6.15 ± 0.13**	6.91 ± 0.16	6.60 ± 0.15	6.90 ± 0.03	6.60 ± 0.11*

Data are represented as the mean ± SEM (n=4 except for 8w Control group, n=5 and 8w HFD group, n=6). Asterisks indicate significant differences between two diet groups (*p<0.05; **p<0.01; Student's t-test). HFD: high-fat diet; SEM: standard error of the mean; w: weeks.

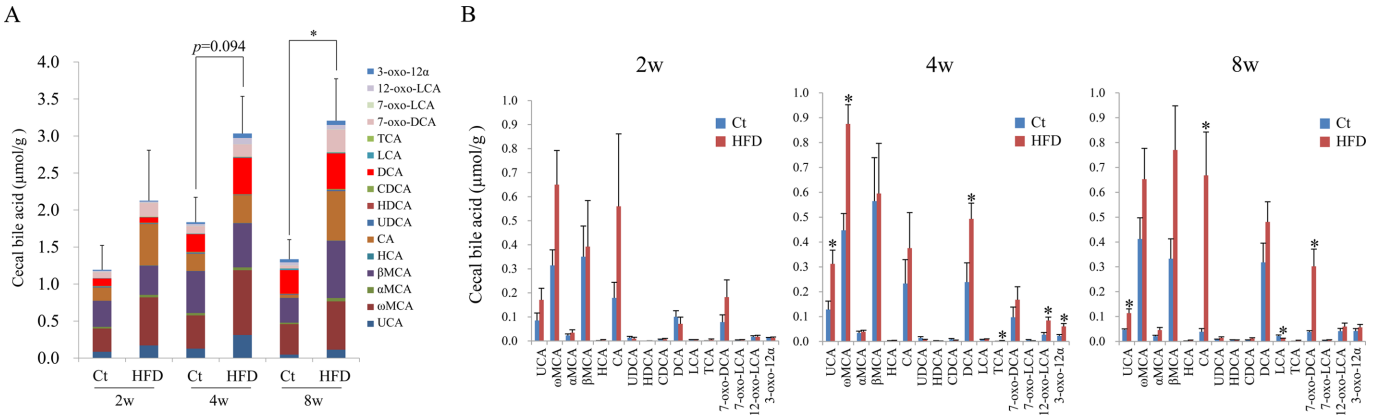

Fig. 1. Cecal bile acid profiles of WKAH rats fed the HFD for 8 weeks. (A) The bile acid composition in cecal contents in control rats (Ct group) and HFD-fed rats (HFD group) after 2, 4, and 8 weeks (2 w, 4 w, and 8 w). Each bile acid species is distinguished by the colors indicated at the right side of the panel. (B) Concentration of each bile acid in the cecal contents of the Ct (blue bars) and HFD (red bars) groups. The values are expressed as the mean ± SEM of 4–6 rats in each group. Asterisks indicate a significant difference compared with the control in the same week evaluated using Student's t-test ($p<0.05$). The abbreviated names of the bile acids are provided in Supplementary Table 3.

Ct: control; HFD: high-fat diet; SEM: standard error of the mean; 3-oxo-12α: 3-oxo-12α-hydroxy-5β-cholan-24-oic acid; LCA: lithocholic acid; DCA: deoxycholic acid; TCA: taurocholic acid; CDCA: chenodeoxycholic acid; HDCA: hyodeoxycholic acid; UDCA: ursodeoxycholic acid; CA: cholic acid; HCA: hyocholic acid; MCA: muricholic acid; UCA: ursocholic acid.

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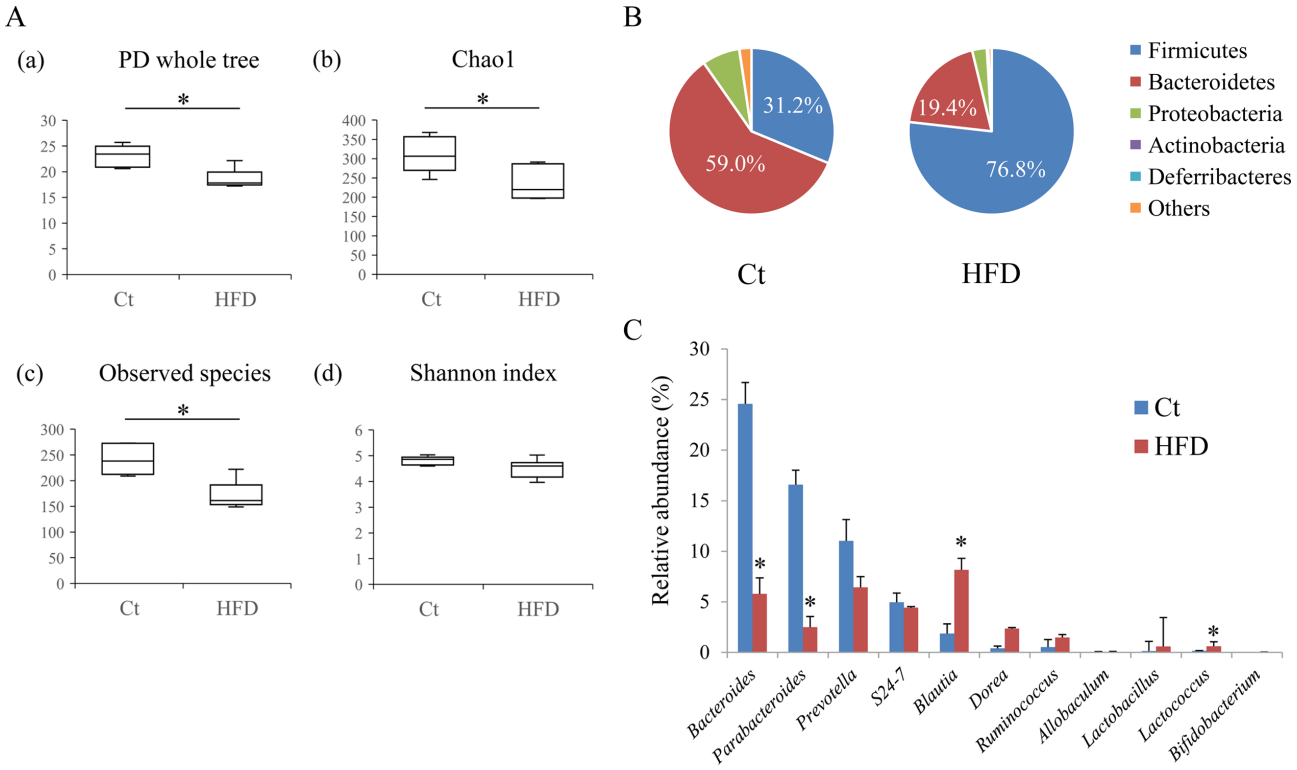

Fig. 2. Modulation of the cecal microbiota composition in WKAH rats fed the HFD for 8 weeks. (A) α-Diversity metrics of cecal microbiota at the OTU level. Each α-diversity metric between the control diet (Ct) group and HFD group are indicated as boxplots: (a) PD whole tree for phylogenetic diversity; (b) Chao1 index; (c) observed species for species richness; and (d) Shannon index for species diversity. (B) Relative abundances of bacterial phyla in the cecal contents. Each phylum is distinguished by the colors indicated at the right side of the panel. (C) Relative abundances of the representative bacterial families or genera from Firmicutes and Bacteroidetes in the Ct (blue bars) and HFD (red bars) groups. The values are expressed as the mean ± SEM of 5–6 rats in each group. Asterisks indicate a significant difference compared with the control in the same week evaluated by Student's t-test ($p<0.05$).

Ct, control; HFD, high-fat diet; SEM, standard error of the mean.

Bacteroides and *Parabacteroides* decreased significantly in the HFD-fed rats (Fig. 2C). In the phylum Firmicutes, the relative abundances of the genera *Blautia* and *Lactococcus* significantly increased with HFD feeding (Fig. 2C). Importantly, an increased tendency in the relative abundance of OTU 1096, which showed the highest similarity (99%) with the 16s RNA sequence of the 7α-dehydroxylating bacterium *Clostridium scindens,* was observed in the HFD-fed rats (Supplementary Table 4). The 7α-dehydroxylation is required to produce DCA from CA, and the result was consistent with the increase in DCA levels observed in the HFD-fed rats. These results indicate that 8 weeks of HFD feeding induced dysbiosis in the gut microbiota, probably due to the elevated levels of bactericidal bile acids, including DCA, in the cecum.

Effects of the HFD intervention on the relative abundances of OTUs in rats

To demonstrate the effect of bile acids on each microbe in detail, we performed an OTU analysis of the cecal microbiota (Supplementary Table 5). OTUs that showed the highest similarity (≥97% identity) to the near-full-length 16S rRNA gene sequences of the isolates used in the following DCA sensitivity test were described using bacterial species names. Other OTUs were described using the classification in the Greengenes database. Initially, we assessed the alterations in the relative abundance following HFD feeding. The results showed that the 8 weeks of HFD feeding significantly altered the relative abundances of 69 OTUs, which accounted for 62.34% of the relative abundance of the cecal microbiota in the control diet-fed group (hereafter abbreviated as Cont, xx%; Fig. 3, OTUs marked with *, Supplementary Table 6). However, these alterations could potentially stem from the differences in diet composition between the control diet and HFD. To eliminate this possibility, we conducted a correlation analysis between the relative abundance of a given OTU and the bile acid concentration in the cecum. We selected the appropriate FBAs for correlation analysis. Of all the FBAs previously evaluated, DCA showed the highest toxicities, while β-MCA displayed toxicities closely resembling those of DCA [10]. In addition, CA and ω-MCA, which are the abundant bile acids in rodent intestines, displayed moderate toxicities. Therefore, we investigated the correlation of OTUs with the concentrations of two FBAs (DCA and β-MCA), four FBAs (DCA, β-MCA, CA, and ω-MCA), or total bile acids. Contrary to our expectations, in the single regression analysis, the total bile acid concentrations showed significant correlations with the highest number of OTUs (66 OTUs; Cont, 63.39%) among the tested concentrations of bile acid species (Fig. 3, OTUs marked with φ, Supplementary Table 6). The two FBAs and four FBAs

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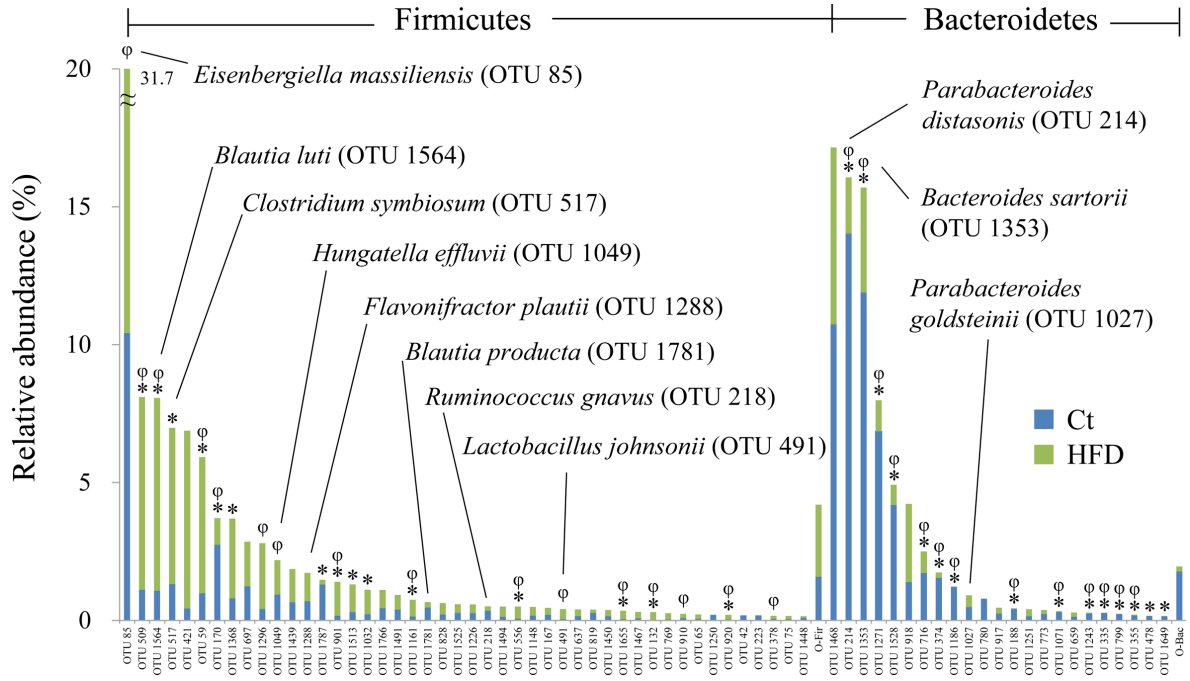

Fig. 3. OTU analysis of rat cecal microbiota populations in WKAH rats fed the HFD for 8 weeks. OTUs are indicated with numbers identified by de novo OTU picking. The sum of the mean relative abundances of Firmicutes and Bacteroidetes OTUs in rats of the control diet (Ct) group (blue bars) and HFD group (green bars) is indicated. OTUs representing at least 0.15% of the sum of the mean relative abundance in Ct and HFD groups are indicated along with each OTU number. OTUs comprising less than 0.15% of the sum of the mean relative abundance of the Ct and HFD groups are combined into other Firmicutes (O-Fir) and other Bacteroidetes (O-Bac). Asterisks indicate a significant difference compared with the control in the same week, evaluated using Student's t-test ($p<0.05$). Phi (φ) indicates a significant correlation with total bile acid concentrations ($p<0.05$). The species names of bacterial isolates obtained from the cecal contents of WKAH rats fed the control diet and/or HFD are also displayed.

HFD: high-fat diet; Ct: control; OTUs: operational taxonomic units; O-Fir: other Firmicutes; O-Bac: other Bacteroidetes.

showed significant correlations with 55 OTUs (Cont, 45.96%) and 63 OTUs (Cont, 63.19%), respectively. These results suggest that the total bile acid concentrations are the most appropriate parameters for correlation analysis.

Figure 4 shows the results of the correlation analysis of the top three abundant OTUs in Fig. 3 from the phyla Firmicutes and Bacteroidetes. Among them, OTU 85 (*Eisenbergiella massiliensis*; $p<0.01$, $R^2=0.556$), OTU 509 (f_*Lachnospiraceae*; $p<0.05$, $R^2=0.423$), and OTU 1564 (*Blautia luti*; $p<0.05$, $R^2=0.388$) in Firmicutes were positively correlated with the total bile acid concentrations, while OTU 214 (*Parabacteroides distasonis*; $p<0.05$, $R^2=0.487$) and OTU 1353 (*Bacteroides sartorii*; $p<0.05$, $R^2=0.515$) in Bacteroidetes were negatively correlated (Fig. 4). These results suggest that the 66 OTUs (Cont, 63.39%) including the five abundant OTUs from Firmicutes (OTUs 85, 509, and 1,564) and Bacteroidetes (OTUs 214 and 1,353) are affected by the HFD-induced increase in bile acids. Moreover, among the 66 OTUs, 38 OTUs (Cont, 51.23%) showed significant alterations following HFD feeding (Fig. 3, OTUs with * and φ, Supplementary Table 6). These OTUs represent the core members of the cecal microbiota in response to increased bile acid levels. However, in this study, the correlation (φ) is more important than the difference in relative abundance (*) because the latter may not always achieve statistical significance due to the limited number of cecal samples analyzed in the experiments.

Isolation of intestinal bacteria from rat cecal contents and determination of DCA IC_{50} values

To validate whether the increased bile acid levels caused by HFD feeding altered the cecal microbiota, bacteria from cecal contents were isolated, and their susceptibility to DCA was compared in *in vitro* growth experiments. A total of 279 colonies were isolated for DCA sensitivity testing and subjected to sequencing of the V3–V4 regions of the 16S rRNA gene. As a result of clustering analysis, 271 sequences were revealed to belong to 32 OTUs (≥97% identity). Among them, bacteria corresponding to 13 OTUs in the phylum Firmicutes or Bacteroidetes were successfully isolated, including 6 OTUs (OTU 1564 [*B. luti*], OTU 1049 [*Hungatella effluvii*], OTU 491 [*Lactobacillus johnsonii*], OTU 85 [*E. massiliensis*], OTU 1353 [*B. sartorii*], and OTU 214 [*P. distasonis*]) that showed significant correlation with the total bile acid concentrations (Fig. 3). These bacteria were taxonomically assigned by sequencing the nearly full-length 16S rRNA gene and were subjected to DCA sensitivity testing. DCA was used as a representative bile acid species showing the highest antibacterial activity among the bile acids analyzed in this study [10]. As indicated in Fig. 5, the IC_{50} values (DCA concentrations giving 50% growth inhibition) of the seven Firmicutes species (*Clostridium symbiosum* [OTU 517], *L. johnsonii* [OTU 491], *Ruminococcus gnavus* [OTU 218], *H. effluvii* [OTU 1049], *B. luti* [OTU 1564], *Limosilactobacillus reuteri* [OTU 1047], and *Blautia producta* [OTU 1781]; 0.9–1.5 mM) were significantly higher than those of the four

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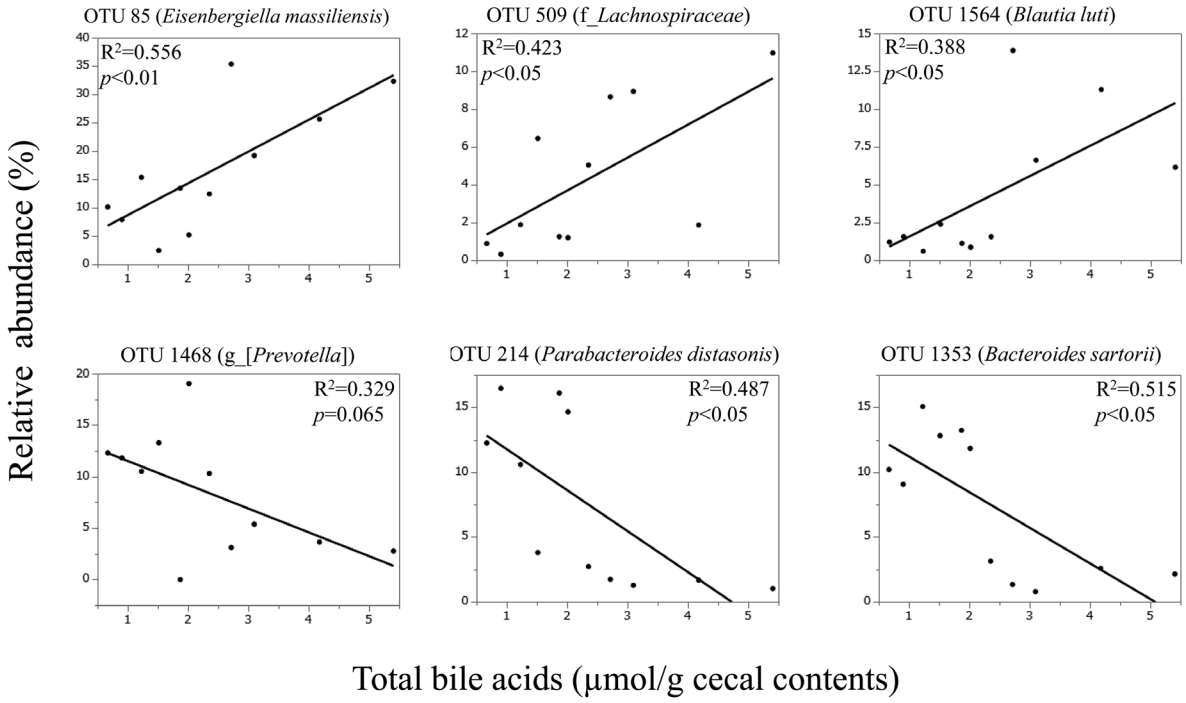

Fig. 4. Scatterplots of the relative abundances of predominant OTUs from the phyla Firmicutes and Bacteroidetes (vertical axis) versus the concentrations of total bile acids in the cecal contents of WKAH rats fed the control diet or HFD for 8 weeks (horizontal axis). Their correlations were evaluated using single regression analysis. R-squared and p-values are indicated within each plot. A p-value of <0.05 indicates a significant correlation.

OTUs: operational taxonomic units.

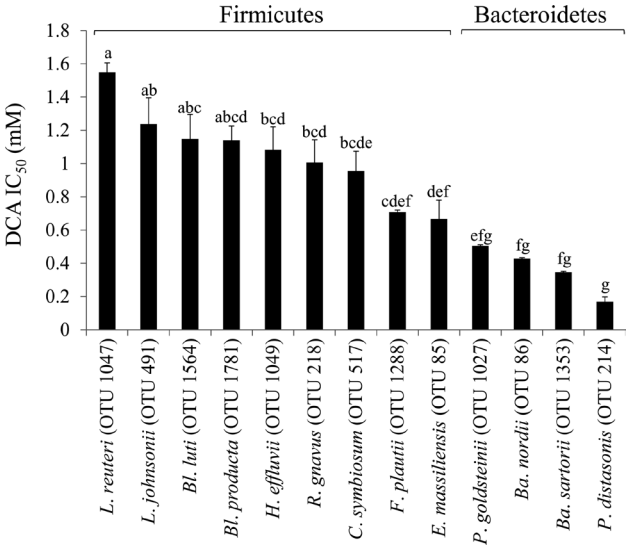

Fig. 5. The IC_{50} values for DCA in bacteria isolated from the rat cecal contents. The bacteria were anaerobically cultured in GAM-0.1 M MOPS (pH 7.0) supplemented with different concentrations of DCA. The IC_{50} values are expressed as the mean ± SEM of at least three independent culture experiments. Data are indicated in descending order of the averaged DCA IC_{50} values. The Tukey–Kramer HSD test was used for clarification of the significant differences in IC_{50} values among the isolates. Values not sharing the same letter are considered significantly different ($p<0.05$).

IC_{50}: 50% inhibition concentration; GAM: Gifu anaerobic medium; SEM: standard error of the mean.

Bacteroidetes species (*B. sartorii* [OTU 1353], *P. distasonis* [OTU 214], *Parabacteroides goldsteinii* [OTU 1027], and *Bacteroides nordii* [OTU 86]; 0.2–0.5 mM). Among the top three abundant OTUs from the phyla Firmicutes and Bacteroidetes (Fig. 4), four abundant OTUs, *E. massiliensis* (OTU 85) and *B. luti* (OTU 1564) in Firmicutes and *P. distasonis* (OTU 214) and *B. sartorii* (OTU 1353) in Bacteroidetes, also showed the same trends in IC_{50} values for each phylum. These results confirmed our previous observations using a limited number of isolates from CA-fed rat cecal contents, wherein Firmicutes showed higher IC_{50} values (1.2–1.3 mM) compared with Bacteroidetes (0.7–0.9 mM) [9]. The IC_{50} values of *E. massiliensis* (OTU 85) and *B. luti* (OTU 1564) in Firmicutes and *P. distasonis* (OTU 214) and *B. sartorii* (OTU 1353) in Bacteroidetes also explained their positive and negative correlations, respectively, with the total bile acid concentrations in the cecum (Fig. 4). The inclusion of ~1 mM DCA in a growth medium severely inhibits the growth of many intestinal bacteria, including *Clostridium perfringens*, *Bacteroides fragilis*, *Lactobacillus*, and bifidobacteria [13, 15]. Thus, our results demonstrate that Firmicutes are generally more resistant to DCA than Bacteroidetes, with significantly higher IC_{50} values for Firmicutes than for Bacteroidetes.

Cecal microbiota alterations in response to CA feeding and a comparison with those observed in HFD feeding

To verify the direct impact of bile acids on the gut microbiota structure, rats were fed a normal diet or a CA-supplemented diet, and their cecal bile acids and microbiota were evaluated. As shown in Fig. 6A and Supplementary Table 7, the CA feeding

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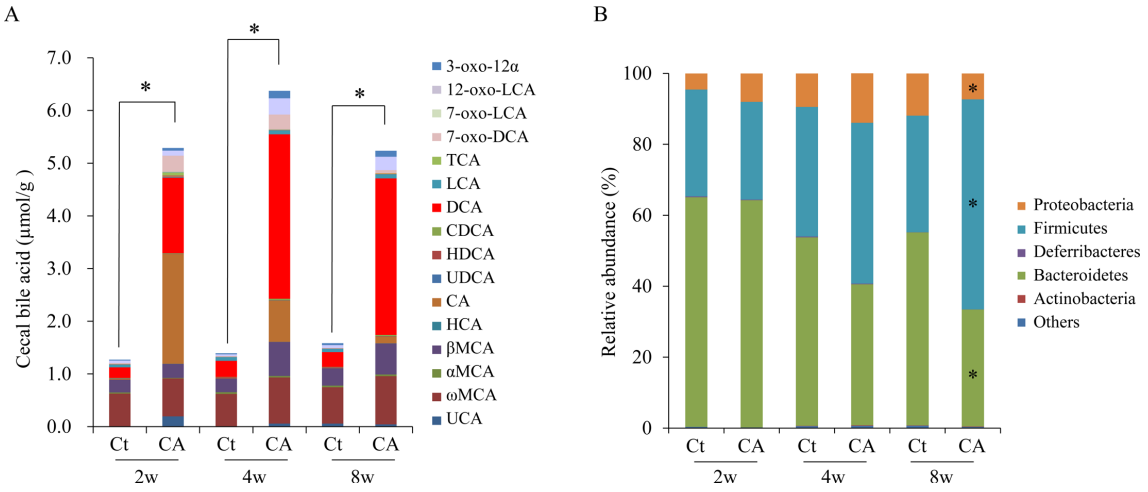

Fig. 6. Cecal bile acid profiles and microbiota composition in WKAH rats fed the CA-supplemented diet for 8 weeks. The bile acid profiles (A) and microbiota composition at the phylum level (B) in the cecal contents of control rats (Ct group) and CA-supplemented-diet-fed rats (CA group) after 2, 4, and 8 weeks (2 w, 4 w, and 8 w). Each bile acid species (A) and each phylum (B) are distinguished by the colors indicated at the right side of each panel. The values are expressed as the mean ± SEM of 5–7 rats in each group. Asterisks indicate a significant difference compared with the control in the same week, evaluated using Student's t-test ($p<0.05$). The abbreviated names of the bile acids are provided in Supplementary Table 3.

Ct: control; CA: cholic acid; SEM:standard error of the mean; 3-oxo-12α: 3-oxo-12α-hydroxy-5β-cholan-24-oic acid; LCA: lithocholic acid; DCA: deoxycholic acid; TCA: taurocholic acid; CDCA: chenodeoxycholic acid; HDCA: hyodeoxycholic acid; UDCA: ursodeoxycholic acid; CA: cholic acid; HCA: hyocholic acid; MCA: muricholic acid; UCA: ursocholic acid.

significantly increased the concentrations of total bile acids, including CDCA, β-MCA, DCA, 12-oxo-LCA, and 3-oxo-12α after week 8, mirroring the changes observed in HFD feeding. In the CA-fed group, the relative abundance of Firmicutes increased, while that of Bacteroidetes significantly decreased after week 8 (Fig. 6B).

To elucidate the impact of bile acids on gut microbiota alterations during HFD feeding, we compared the alterations at the OTU level between the HFD and CA feeding experiments. For this analysis, we selected the top 12 abundant OTUs that showed positive or negative correlations with the total bile acid concentrations in the HFD feeding experiment from Firmicutes and Bacteroidetes, respectively (total of 24 OTUs; Cont, 61.03%; Supplementary Table 8). These were then matched with their corresponding OTUs in the CA feeding experiments for 8 weeks. A comparison of the alterations in the relative abundance of each OTU between the two feeding experiments revealed that 18 out of the 24 OTUs (Cont, 48.28%) exhibited alterations in the same direction (increase or decrease) in both diet groups (Fig. 7). These results indicate that increased bile acid levels predominantly regulate microbiota alterations in the cecum of HFD-fed rats. OTUs corresponding to OTU 1655 (g_*Oscillospira*) and OTU 170 (f_*Lachnospiraceae*) in the HFD feeding experiment were not detected during the CA feeding experiment due to their low populations in the cecum.

DISCUSSION

In this study, we provided microbiological and quantitative evidence supporting the bile acid hypothesis, which proposes that bile acids play a crucial role in the alterations of gut microbiota induced by an HFD. This hypothesis had not been fully elucidated in a previous study [16]. In the HFD feeding experiment, OTUs comprising 63.39% of the cecal population in control rats exhibited changes in their relative abundances, showing significant correlations with the total bile acid concentration (Figs. 3 and 4). A comparison of the alterations in the relative abundances of the top 12 abundant OTUs between the HFD- and CA-supplemented diet groups revealed that 18 out of the 24 OTUs (Cont, 48.28%) exhibited alterations in the same direction (increase or decrease) in both diet groups (Fig. 7). Although not all OTUs showed alterations in the same direction, likely due to other factors, our findings unequivocally demonstrate that approximately half of the cecal populations in control rats were affected by bile acids under HFD-fed conditions.

We have clearly shown that CA diet intake increased the concentration of total bile acids and DCA in the cecum and that the accompanying changes in the cecal microbiota were similar to those observed in the case of HFD intake (Figs. 2, 6, and 7). Therefore, we believe that the causality of the changes in the microbiota being due to bile acids has been demonstrated in the case of HFD intake, which strongly supports the bile acid hypothesis. This hypothesis might be reinforced by further experiments inhibiting endogenous bile acid synthesis using obeticholic acid, a synthetic bile-acid derivative, under HFD-intake conditions [37].

Here, we have to mention that as seen from Figs. 1A and 1B, at 8 weeks, the total bile acid concentrations in the case of HFD intake differed significantly between the two diet groups, while the DCA concentration was not significantly different between the diet groups. These results imply that some synergistic interactions exist between FBA molecules to enhance their toxicities rather than their simple additive effects under physiological conditions. In our previous study, rodent-specific FBAs, including β-MCA, did not show positive correlations between the bactericidal activity of the FBA molecules and their hydrophobicity, while the FBAs common to humans and rodents showed such correlations [10]. Therefore, some interactions in bactericidal activities

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between the rodent-specific FBAs and those common to humans and rodents that await further clarification might exist under physiological conditions. It is also possible that other bile acid molecules not analyzed in this study may contribute to changes in the gut microbiota. Recently, several novel bile acid species have been identified. For example, isoalloLCA (3β-monohydroxy-5α-cholan-24-oic acid), a derivative of LCA that is abundant in the intestines of centenarians, was shown to exhibit strong bactericidal activity against Gram-positive bacteria but not against Gram-negative bacteria at the micromolar level [38]. In addition, 3-isoLCA (3β-monohydroxy-5β-cholan-24-oic acid), another derivative of LCA, has been reported to show high bactericidal activity against *Clostridioides difficile* and major DCA-producing bacterial species, but not against other gut microbiota species [39]. These bile acids showed antibacterial activity opposite to the alterations in Firmicutes and Bacteroidetes observed in this study. Thus, their concentrations in the intestine should be quantified to evaluate their contribution to the alterations of the gut microbiota.

In addition to the bactericidal activity, as other functions of bile acids, the induction of endospore germination of Firmicutes by conjugated bile acids may contribute to Firmicutes-dominant cecal microbiota compositions. Bacterial isolation studies using human feces have shown that various intestinal Firmicutes species can form endospores and that they germinate and grow by recognizing conjugated bile acids such as TCA or tauro-CDCA [40, 41]. Although we did not observe an increase in the cecal concentrations of conjugated bile acids, most bile acids return to the liver via the enterohepatic circulation, are re-conjugated, and are secreted back into the small intestine [12]. Therefore, a transient increase in conjugated bile acids may occur in the cecum and colon, contributing to the expansion of Firmicutes.

Generally, the gut microbiota composition is controlled by nutritional availability in the cecum and competition for nutritional substances among populations. Therefore, minor differences in the IC_{50} values for DCA may synergistically affect the survival of intestinal bacteria under nutritionally competitive conditions, which determines the population shift at the phylum level following HFD feeding and increases the relative abundance of Firmicutes over Bacteroidetes (Fig. 2B). However, it is unclear why Firmicutes show higher DCA IC_{50} values than Bacteroidetes (Fig. 5). The difference in DCA IC_{50} values among the tested bacteria (Fig. 5) was thought to be due to the differences in the resistance mechanism against bile acids in each bacterium [42]. The bactericidal mechanism of hydrophobic FBAs such as DCA and CA is cell membrane damage [10, 13]. Historically, Gram-negative bacteria harboring dual membranes were thought to be intrinsically more resistant to bile acids than Gram-positive bacteria [11]. However, examinations on *Blautia coccoides* (Firmicutes) and *Bacteroides thetaiotaomicron* (Bacteroidetes) revealed that *B. thetaiotaomicron* lost membrane integrity and viability at lower DCA concentrations (0.2 mM) than *B. coccoides* (0.6 mM), suggesting that the above concept is not necessarily adaptable in all Firmicutes and Bacteroidetes [10]. The major mechanisms of bacterial bile acid resistance have been reported to include 1) bile acid efflux pumps, 2) changes in cell membrane lipids, and 3) exopolysaccharide production [42–44]. In fact, we have previously reported a cholate efflux pump in a Firmicutes species, *Lactococcus lactis* [45], and the bile acid adaptation mechanism in *Lactobacillus gasseri* due to changes in cell membrane lipid composition [46]. However, the distribution

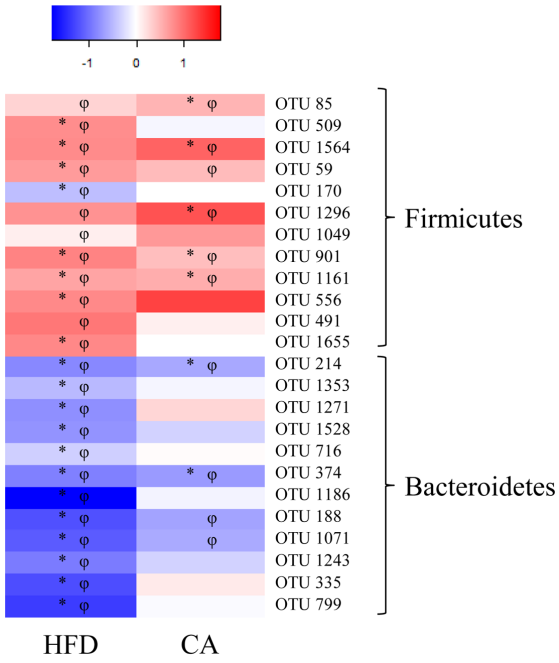

Fig. 7. Comparison of alterations in the representative cecal microbiota population between the HFD and CA interventions. The top 12 abundant OTUs from Firmicutes and Bacteroidetes, which showed a significant positive or negative correlation with the total bile acid concentrations in the HFD feeding experiment, were compared with the OTUs in the CA-supplemented diet feeding experiment. The heatmap showed the $\log_{10}$ values of the ratio of the relative abundance of each OTU in the HFD or CA-supplemented diet to that in the control. Red cells represent the increased OTUs in HFD feeding or CA-supplemented diet feeding, while blue cells represent the decreased OTUs in those interventions. The difference in the ratio among the cells is depicted by the shading of each color, as indicated in the scale bar for the ratio at the top of the figure. Asterisks indicate a significant difference in the relative abundance of each OTU compared with the control in each feeding experiment, evaluated using Student's t-test ($p<0.05$). Phi (φ) indicates a significant correlation with total bile acid concentrations, evaluated using single regression analysis ($p<0.05$).

OTUs: operational taxonomic units; CA: cholic acid; SEM: standard error of the mean.

of each of the above mechanisms (1 to 3) among individual intestinal bacteria is unclear. Therefore, at present, it is difficult to explain the differences in bile acid resistance between Firmicutes and Bacteroidetes from the bile acid resistance mechanism.

Here, we need to discuss some discrepancies observed in the direction of alterations in several OTUs between the two diet groups (Fig. 7). The direction of alterations in OTUs 509, 1271, 716, and 335 were inconsistent, implying that the alterations in these four OTUs in the case of the HFD might be due to other factors (Fig. 7). Among them, OTU 509 (f_*Lachnospiraceae*) (Cont, 1.11%) and OTU 716 (f_*S24-7*; Cont, 1.72%) were classified by family level, which may result in the OTUs comprising diverse bacterial species. Both OTU 1271 and OTU 335 were assigned to *Bacteroides acidifaciens*, which can grow in the presence of 20% bile [47]. Conversely, the administration of *B. acidifaciens* to HFD-fed C57BL/6 mice resulted in the rapid disappearance of this species in the intestine after 4 days [48], which is consistent with the decrease in this species in the cecal

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microbiota of HFD-fed rats observed in our study. Therefore, although this species exhibits bile tolerance, its survival under HFD-fed conditions may be compromised.

We observed a significant expansion of OTU 1564 (*B. luti*; Cont, 1.08%; HFD, 6.99%), which was among the top 3 abundant OTUs in Firmicutes, in the gut microbiota of HFD-fed rats (Fig. 3). The relative abundance of OTU 1564 (*B. luti*) increased for 2–8 weeks (9th, 3rd, and 3rd in terms of relative abundance at weeks 2, 4, and 8, respectively; Supplementary Table 9). We have previously reported that a CA-supplemented diet increased the relative abundance of the genus *Blautia* [28]. Therefore, this study identified the specific species of *Blautia* that experienced an increase under selective pressure from bile acids during HFD or CA feeding. In our previous study, we observed that alongside the increase in *Blautia* abundance, CA feeding induced liver lipid accumulation in non-obese rat models [28]. An increase in the abundance of the genus *Blautia* was also observed in patients with non-alcoholic fatty liver disease (NAFLD), characterized by excess fat accumulation in the liver [49]. Therefore, an elevation in the abundance of *B. luti* may be associated with lipid accumulation in the liver and NAFLD development. However, further investigation is warranted to elucidate the causal relationship between *B. luti* and liver disease development.

The bile acid hypothesis can be adapted to humans. A comparison of fecal microbiota between children in Europe (consuming a Western diet) and rural Africa (consuming a traditional African diet) showed a high proportion of Firmicutes in the former and a high proportion of Bacteroidetes, including the fiber-degrading *Prevotella* and *Xylanibacter*, in the latter [50]. Although fecal bile acid data were not available in that study, higher bile acid levels were expected in the former population compared with the latter population. Similarly, a high-fat, low-fiber diet intervention increased the fecal levels of DCA and LCA in rural Africans, which was associated with fecal microbiota alterations such as increases in the abundance of *Clostridium orbiscindens* and copy numbers of the *baiCD* gene, which is responsible for DCA and LCA production [51]. David *et al.* reported increased levels of fecal DCA, total bile acids, and the abundance of bile-tolerant microorganisms, including *Bilophila*, in subjects consuming animal-based diets compared with those in subjects taking baseline or plant-based diets [52]. Therefore, the observed gut microbiota alterations induced by consuming a Western diet can be attributed to the increased bile acid levels, supporting the bile acid hypothesis.

In conclusion, the findings from this study have substantiated the validity of the bile acid hypothesis. These findings not only enhance our understanding of the mechanisms underlying gut microbiota alterations induced by consumption of a Western diet but also contribute to the development of novel bile acid-targeted therapies for various diseases, including obesity, obesity-related metabolic phenotypes, type 2 diabetes, and liver cancer.

CONFLICT OF INTEREST

We declare no conflicts of interest associated with this manuscript.

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