Synthesis and quantification of short-chain fatty acid esters of hydroxy fatty acids in rat intestinal contents and fecal samples by LC-MS/MS

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Abstract

Short-chain fatty acid esters of hydroxy fatty acids (SFAHFAs) are a new class of endogenous lipids belonging to the fatty acid esters of the hydroxy fatty acid family. We previously uncovered their chemical structure and discussed their potential biological significance. We anticipate an increased need for SFAHFA measurements as markers of metabolic and inflammatory health. In this study, we synthesized sixty isomeric SFAHFAs by combining 12 hydroxy fatty acids (C16-C24) and five short-chain fatty acids (C2-C6) including a labelled internal standard. SFAHFA enrichment was achieved by solid-phase extraction and established a sensitive method for their quantitation by targeted LC-MS/MS. The method was applied to profile SFAHFAs in intestinal contents and fecal samples collected from rats fed a high-fat diet (HFD). The results demonstrated a significant decrease in SFAHFAs in the intestinal contents of the HFD group compared with the control group. The fecal time course (0-8 weeks) profile of SFAHFAs showed significant downregulation of acetic and propanoic acid esters in just 2 weeks after HFD administration. This study offers the first synthesis and quantitation method for SFAHFAs, demonstrating their potential use in elucidating SFAHFA sources, their role in various diseases, and potential biochemical signalling pathways.

Key words: SFAHFAs, chemical synthesis, gut microbial lipids, intestinal contents, liquid chromatography, mass spectrometry.

1. Introduction

Lipids are the most important biomolecules with unique structures and functions. They are structurally diverse, and the search for new lipid metabolites is of great scope to understand the key biological mechanisms. In 2014, a lipidomic analysis of white adipose tissue revealed the existence of a new class of lipids named fatty acid esters of hydroxy fatty acids (FAHFAs) which have anti-inflammatory and anti-diabetic effects[1]. FAHFAs are structurally composed of a fatty acid esterified with a hydroxy fatty acid having more than 300 regioisomers[2]. Since their initial discovery, many FAHFAs have been identified in daily foods, animals, plants, and human samples[3]. The most studied FAHFAs have ester linkages at 5, 7, 8, 9, 10, 11, 12, and 13 respectively[1,4]. Different regioisomers are often shown to have different potencies for biological function. For example, 5 and 9-palmitic acid esters of hydroxy stearic acids (PAHSA) are known to improve glucose tolerance in mice fed a high-fat diet (HFD)[5]. 9-PAHSA exhibits anti-inflammatory effects via free fatty acid acceptor 4 (FFA4, GPR 120) and modulates inflammatory responses in a mouse colitis model[6]. Furthermore, our previous reports demonstrated the plausible antioxidant effects of 12-eicosapentaenoic acid hydroxystearic acid (12-EPAHSA) and 12-docosahexanoic acid ester of hydroxystearic acid (12-DHAHSA) via Nrf2 activation[7,8]. The links between the gut and long-chain FAHFAs have also been explored, including stimulation of GLP-1 secretion from the enteroendocrine cell line STC-1 and metabolism of FAHFAs derived from colon carcinoma cells to escape the apoptotic pathway[9,10].

Many studies have demonstrated the numerous biological activities of FAHFAs; hence, hunting begins to find their new structural isomers. In 2019, Tan et al., identified and characterized FAHFA containing triacylglycerols[11]. We also recently uncovered a new class of lipids in the FAHFA family, named short-chain fatty acid esters of hydroxy fatty acids (SFAHFA)[12]. These lipids are found to be abundant in the gut, especially in the colon and cecum contents of a rat[13]. Because the gut-microbiota is identified as an important source of short-chain fatty acids (SFA) and hydroxy fatty acids (HFA), we assume that SFAHFAs could be formed by the involvement of the gut-microbiota. To date, we have identified and characterized >20 SFAHFA structural isomers by the combination of acetic, propanoic, butyric, and pentanoic acid with various 2-HFAs. These lipids have attracted considerable attention because in our previous studies, we found a sharp decrease of SFAHFA levels in the colon contents of rat fed HFD and a significant increase in colon contents of influenza virus[12,14]. Downregulation of propanoic- and butyric acid derived SFAHFAs (3:0/26:0, 3:0/24:0, 3:0/22:0, 4:0/24:0, 4:0/24:1, and 4:0/26:0) are observed in fecal samples of mice

fed with antibiotics, suggesting that these lipids are gut-microbiota-specific[15]. Analysis of cecum contents collected from infection of a laying hen with *Campylobacter jejuni*, which causes gastrointestinal illness, showed an increase in SFAHFAs (5:0/26:0 and 4:0/25:0) at 24 weeks of post infection compared with 8 weeks[16]. Increased levels of (2:0/20:0), (4:0/24:1), and (4:0/24:2) SFAHFAs were observed in the colonic contents of weaned piglets fed a low-protein diet compared with a normal protein diet[17]. Furthermore, SFAHFAs (2:0/22:0, 2:0/23:0, 2:0/24:0, 2:0/24:1 and 4:0/24:0) were decreased in the stool sample of *Pla2g2a* deficient mice compared with the control, indicating that the regulation of these lipids by group IIA secreted phospholipase A2[18]. All these research studies suggest the importance of these novel lipids in the various causes of metabolic diseases.

Despite their significance, SFAHFAs, their sources, absolute levels, biological pathways, and functions remain enigmatic. Several research studies on highly sensitive liquid chromatography/mass spectrometry (LC/MS) quantitative methods for long-chain FAHFAs determination in biological matrices have been established[19,20]. However, there are no reports aiming at the absolute quantification of SFAHFAs. This is because of the low abundance and unavailability of SFAHFA commercial standards, causing significant delays in SFAHFAs research. Although we tried to increase the detection sensitivity of SFAHFAs by the DMED derivatization strategy in intestinal contents[13], it was not an absolute quantitative and results need to be verified with authentic standards. To overcome these limitations, we designed and chemically synthesized 55 homologous series of SFAHFAs. They are derived from eleven saturated fatty acids (C16 to C26) and five internal standards of each short-chain fatty acid (C2 to C6) esterified with deuterated 2-hydroxy palmitic acid. The abbreviations of the homolog series of SFAHFAs are described in **Figure 1**. Due to its high sensitivity, specificity, accuracy, and reproducibility, a targeted LC/MS with single reaction monitoring channels (SRM) in negative ionization mode was established for all sixty isomeric SFAHFAs and their precursors, such as free fatty acids (FFA) and 2-hydroxy fatty acids (2-HFA). To minimize the matrix effects and enrich SFAHFAs, a solid-phase extraction strategy was applied. Further, after the analytical validation, the developed method was applied for the quantification of SFAHFAs in intestine contents or feces collected from rats fed a high-fat diet and a control diet. This is the first absolute quantitation method for SFAHFAs, and this established method is a promising analytical tool for the precise identification and quantification of SFAHFAs in biological samples.

2. Experimental Section

2.1. Materials

2-hydroxypalmitic acid (2-HPA), propionyl chloride, phosphorus red, hexanoyl chloride, acetic anhydride, diethyl ether, bromine, LC/MS grade chloroform, and isopropanol were purchased from Wako Pure Chemical Industry (Tokyo, Japan). Heptadecanoic acid, stearic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid, lignoceric acid, pentacosanic acid, cerotic acid, $CDCl_3$, butyryl chloride, and valeryl chloride were obtained from Tokyo Chemical Industry (Tokyo, Japan). Palmitic acid-d31 was purchased from Cayman Chemical (Arbor, Michigan, USA). LC/MS grade acetic acid was purchased from Merck (Darmstadt, Germany). Pyridine, methanol (for LC/MS), hexane, ethyl acetate, and all other solvent-grade reagents were obtained from Kanto Chemical (Tokyo, Japan). Solid-phase extraction cartridges (MonoSpin C18-AX) were supplied by GL Science (Tokyo, Japan). Silica Gel N60 (spherical type, particle size 40–50 μm; Kanto Chemical Industry) was used for column chromatographic purification. Thin-layer chromatography was performed on Merck pre-coated plates (20 cm × 20 cm; layer thickness, 0.25 mm; Silica Gel 60F254); the spots were visualized by spraying ceric ammonium molybdate solution or 5% H_2SO_4 in methanol. ^{1}H and ^{13}C NMR spectra were obtained using 400 MHz JNM-ECX400P (JEOL, Tokyo, Japan; 1 H: 400 MHz, 13C: 100 MHz). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded using a Linear Trap Quadrupole (LTQ) Orbitrap XL (Thermo Fisher Scientific).

2.2. Chemical synthesis of the SFAHFAs

The synthesis of a homologous series of isomeric SFAHFAs was accomplished in three steps, starting with commercially available FFAs. The synthetic scheme for the SFAHFAs is shown in **Figure 2**. The saturated fatty acids from C16 to C24 were converted into their respective 2-HFAs using the method reported earlier with minor modifications[21]. Furthermore, 2-HFAs were esterified with short-chain fatty acids from C2 to C6 by direct acylation using respective acid chlorides or anhydrides. In brief, to a known amount of fatty acid in a round bottom flask, red phosphorous (1.3eq) was added and heated to 95 °C under a reflux condenser. Then, bromine (3eq) was added dropwise, and the reaction was stirred for 6 h at 95 °C. After cooling the reaction mixture to room temperature, 20 mL of water was added and allowed to be stirred for an additional 20 min. The reaction mixture was extracted with diethyl ether, and the residue was subjected to column chromatography using 95:5 (v/v) to 90:10 (v/v) hexane: ethyl acetate to yield crude 2-bromo fatty acid. To a 2-bromo fatty acid, an excess of 2M NaOH was added and refluxed at 85 °C for 30 h. The progress of the reaction was monitored by thin-layer chromatography (TLC). After completion, the flask was cooled down and the reaction mixture was neutralized with 2M HCl and extracted with

diethyl ether. The extracted residue was purified by column chromatography using chloroform: methanol 95:5 (v/v) to 85:15 (v/v) to yield 2-HFA.

Furthermore, the respective SFAHFAs from 2-HFAs were prepared by dissolving the known amount of 2-HFA in dichloromethane,100 µL of pyridine (for every 50 mg 2HFA), and acid chlorides (2eq) were added dropwise at 0 °C and stirred at room temperature for overnight. The reaction mixture was extracted with dichloromethane and purified by silica gel column chromatography using chloroform: methanol 90:10 (v/v) to yield SFAHFAs. The detailed experimental procedures, characterization by HR-ESI-MS, ^{1}H and ^{13}C NMR spectra are provided in the supporting information (page S9-S56).

2.3. Animal Samples

All animal experimental procedures were performed in accordance with the regulations of the Hokkaido University Ethics Review Committee (approval No. 17-0050) using method reported earlier by our group[22]. Male 3-week-old WKAH/HkmSlc rats were maintained under constant temperature with free access to water and a standard chow diet (control) for 2 weeks. Then, they were divided into two groups (n=6 each) and fed a control diet or a high-fat diet (HFD) based on the AIN-93G formulation for 8 weeks. Lard was used in place of dextrin to prepare the HFD, and the detailed diet composition is provided in supporting information in **Table S1**. Rat feces from each group were collected at an interval of 0, 2, 4, 6, and 8 weeks to obtain the time course lipid profile with HFD. At 8 weeks, the rats were killed by exsanguination without food deprivation, and required intestinal contents from the colon, cecum, ileum, duodenum, and jejunum were collected. All samples were stored at −80 °C until further analysis.

2.4. Solid-phase extraction

The samples (feces/intestinal contents) were weighed into a 1.5 mL Eppendorf preloaded with 10 volumes of methanol and 5-6 ceramic beads (1.4 mm, catalog no. 15-340-159, Fisherbrand) and homogenized for a period of 30 s in two cycles using a Bead Mill 4 (Fisherbrand) homogenizer. Then, 50 µL (i.e. 5 mg) of homogenate was transferred to a new Eppendorf and 100 µL internal standard mixture in methanol (5 µM deuterated SFAHFA standard mixture (PAHPA-d30, BAHPA-d30, VAHPA-d30, and HAHPA-d30), 20 µM 2-HPA-d30 and 500 µM palmitic acid-d31) and 450 µL Milli-Q were added. The mixture was vortexed (3500 rpm, 1 min) and centrifuged (15000 rpm, 1 min, 4 °C) to collect the centrifugate as a sample for the loading step. Solid-phase extraction was performed using

MonoSpin C18-AX cartridges. First, the columns were pre-conditioned with 300 µL methanol and 300 µL Milli-Q by spinning at 15000 rpm for 1 min at room temperature. Then, the samples were loaded into the column and washed with 300 µL of Milli-Q followed by 300 µl of 40% MeOH (by spinning at 15000 rpm, 1 min). Subsequently, the lipids of our interest were eluted at two stages by 100 µL of methanol/Milli-Q/acetic acid (90:8:2, v/v/v) and 300 µL of chloroform -acetic acid (98:2, v/v). The combined lipid elutes (400 µL) were dried under vacuum at 4 °C. The extracts were redissolved in 100 µL of methanol followed by gentle vortexing and transferred to LC/MS vials for analysis.

2.5. LC/MS analysis

The selected reaction monitoring (SRM) channels for all the SFAHFA standards were established using a TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) under electrospray ionization (ESI) in negative mode. The optimized SRM parameters are summarized in **Table 1**. The optimized ion source parameters were set as follows: Spray Voltage is 3 kV, Vaporizer Temperature is 300 ℃, Capillary Temperature is 270 °C, Skimmer Offset is 5, Collision Pressure is 1.5, the nitrogen-sheath gas and auxiliary gas flow was set to 50 and 5 units, respectively. Chromatographic separation was performed using an ultra-fast liquid chromatograph (UFLC) system (Shimadzu Corporation, Kyoto, Japan) at a flow rate of 300 µL/min using a reversed-phase Atlantis T3 C18 column (50 mm × 2.1 mm, 3 µm, Waters, Milford, USA) maintained at 40 °C. The mobile phases consist of A: 10 mM ammonium acetate, B: isopropanol, and C: methanol. The gradient was set as follows: 0–7 min, 40% B and 20% C; 7–14 min, 80% B and 10% C; 14–18 min, 30% B and 35% C; 18–22 min, 30% B and 35% C to the initial condition. The injection volume was set to 10 µL.

2.6. Data processing and quantification

The obtained raw data were processed by Xcalibur 2.2 (Thermo-Fisher Scientific Inc.), and the integrated peak area was exported. To calculate the SFAHFAs HFA, and FFA concentrations in subsequent experiment samples, calibration curves were constructed under neat conditions for representative SFAHFAs of concentration range 0.005 to 25 µM (2-PAHPA, 2-BAHPA, 2-VAHPA, and 2-CAHPA), 0.01 to 500 µM HFAs (HPA) (internal standard 20 µM HPA-d30) and 0.5 to 5000 µM FFAs (PA) (internal standard 500 µM PA-d31) by plotting area ratios (analyte to internal standard) versus concentration. The limit of quantification (LOQ) and limit of detection (LOD) were evaluated as the signal-to-noise (S/N) ratios 10 and 3, respectively. The recovery, intra-day, and inter-day assays were performed

for SFAHFAs at low (0.5 μM), medium (2.5 μM), and high concentrations (5 μM) respectively and results are summarized in supporting information **Table S2**.

2.7 Statistical analysis

The data were plotted in Excel 2019 and GraphPad Prism 8 software as the mean ± standard error. Student's *t*-test ($p < 0.05$) or one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant) were used to study statistically significant differences between the groups. Cluster correlation analysis was performed using MetaboAnalyst (ver 5.0). Network analysis was performed using a cystoscape (ver 3.1).

3. Results and Discussion

3.1 Chemical synthesis and optimization of the LC/MS method

A two-step synthetic strategy was developed for the gram-scale preparation of a homologous series of SFAHFAs, as depicted in **Figure 2**. Commercially available saturated fatty acids of the C16 to C26 carbon chains were used as starting materials. In the first step, an α-hydroxylation reaction was carried out by bromination and successive alkali hydrolysis at high temperature to yield 2-HFAs by the method established earlier with minor modifications[21]. All 11 synthesized 2-HFAs were then subjected to O-acylation using respective short-chain fatty acid chlorides (C3-C6) or anhydride (C2) to yield a homologous series of fifty-five SFAHFAs. The next challenge was to prepare a suitable internal standard for the quantification of these new classes of lipids. To achieve this, palmitic acid-d31 was used as the starting material and synthesized the respective deuterated SFAHFAs (2-AAHPA-d30, 2-PAHPA-d30, 2-BAHPA-d30, 2-VAHPA-d30, and 2-CAHPA-d30) by the α-hydroxylation followed by an O-acylation reaction similar to that described for analytes. Although many methods have been established for the facile synthesis of FAHFAs, these methods were recently reviewed by Balas et al[23]. To our knowledge, this is the first synthetic method for the preparation of SFAHFAs in high yield in just two-steps, with detailed structural characterization by MS and NMR techniques.

Our next aim was to establish an analytical method for the quantification of SFAHFAs using synthetically prepared authentic standards and internal standards. To achieve this, highly sensitive and selective liquid chromatography coupled with targeted tandem mass spectrometry (LC/MS) was applied because targeted LC/MS techniques have been demonstrated to have robust sensitivity and selectivity compared with untargeted methods[24,25]. First, 1 μM SFAHFAs standard solutions were directly injected into the triple quadrupole mass spectrometer by T-infusion, and mass parameters, including multiple

reaction monitoring (MRM) channels for each analyte and internal standard, were established in negative ionization mode. The exact mass, MS/MS spectra, and MRM channels of representative standards and internal standards of short-chain fatty acid derivatives of 2-HPA and 2-HPA-d30 are shown in **Figure 3A**. For example, the compound 2-AAHPA ionizes in the negative mode to give a deprotonated molecule, $[M-H]^-$ at *m/z* 313.2384 (obtained by high-resolution mass spectra (HRMS)). Which further ionizes to give fragment ions *m/z* 271.2 $[M-H-CH_2O]^-$, *m/z* 253.2 $[M-H-CH_3COOH]^-$, and *m/z* 225.2 (by the loss of CH_2O_2 (formic acid, 46 Da) from *m/z* 271.2) respectively. The ionization pattern of the internal standard 2-AAHPA-d30 was similar to that of 2-AAHPA, as demonstrated by their similar MS/MS spectra shown in **Figure 3A**. The most abundant fragment ion, i.e. $[M-H-CH_2CO]^-$ was used for both 2-AAHPA and 2-AAHPA-d30 as a product ion. The MS/MS behaviour of SFAHFAs was identical to that of our previous reports, which described the first detection and characterization of SFAHFAs in intestinal contents[14,12]. The detailed HRMS and MS/MS results for each analyte acquired using LTQ-Orbitrap MS are shown in the supporting information, **Figure S1.**

Furthermore, the chromatographic separation of all the homologous series of SFAHFAs was performed on a Shimadzu UFLC system using an Atlantis C18 column maintained at 40 °C by reversed-phase column chromatography. The extracted ion chromatograms of the representative SFAHFAs and their internal standards are shown in **Figure 3B**. The elution of polar lipids follows that of non-polar lipids. SFAHFAs with acetate (C2) esters elute first followed by propionate (C3), butyrate (C4), valerate (C5), and caproate (C6) esters in both authentic standard and internal standards. Separation of all 84 standards, including SFAHFAs, FFAs, and hydroxy fatty acids (HFAs). was achieved within 22 min. The analysis results of linearity, LOD, and LOQ of authentic FFAs, HFAs, and SFAHFAs are summarized in **Table 2**. A value of R^2 >0.99 was observed for most of the lipid species measured. The LOD and LOQ for SFAHFAs range from 0.1 to 5 and 1 to 50 femtomoles, respectively. The chemical derivatization of the carboxylic acid group could improve the sensitivity by enhanced ionization[13,19], however, without having any additional derivatization, we could achieve the detection of SFAHFAs at low femtomolar levels.

3.2 High-diet rat model and optimization of the lipid extraction method

The high-fat diet-fed rat model was established by the protocols as described in our earlier studies [22]. The changes in the body weight and epididymal adipose tissue weight between tge control and high-fat diet (HFD) groups were shown in **Figure 4A**. Body weight and epididymal adipose tissue were slightly increased in the HFD group compared with controls. A schematic representation of the established solid-phase extraction (SPE) strategy for

FFAs, HFAs, and SFAHFAs is depicted in **Figure 4B**. Further, the results of extraction recovery, accuracy, intraday, and interday method validations of representative SAFHFAs, HFAs, and FFAs are shown in supporting information **Table S2.** In most of the case's extraction, recoveries were>70% for SFAHFAs, and intraday, interday relative standard deviations are <15%. The matrix effects were also validated in the colon contents, and the results showed significant ion suppression (<100%) for most of the analytes (**Table S2**). The extracted ion chromatograms of representative standards *vs* homologous series of SFAHFAs detected in samples are shown in supporting information **Figure S2**. Data C16-C19 hydroxy fatty acids esterified SFAHFAs eluted before 12 min, followed by C20-C26 series between 12-18 min. SPE techniques are widely applied in FFA and FAHFA analytical research studies to eliminate the matrix effects[19,27]. However, these methods require extensive modifications for SFAHFA extraction because they have both polar and non-polar molecular species. Overall, this is the first effort to establish an efficient extraction method for SFAHFAs with a broad coverage of its molecular species.

3.3 Application of the method for the quantification of SFAHFAs in rat intestinal contents

The analysis results of significantly altered SFAHFAs in the cecum (detected 54 SFAHFAs), colon (22 SFAHFAs), duodenum (54 SFAHFAs), ileum (25 SFAHFAs), and jejunum (53 SFAHFAs) contents of HFD-fed-rats are represented in volcanic plots, as shown in **Figure 5A**. In the cecum, colon, and ileum contents, propanoic acid esters hydroxy fatty acids such as 2-PAHTA, 2-PAHLA, 2-PAHAA, 2-PAHBA, 2-PAHHA, and 2-PAHPSA were significantly decreased in the HFD group compared with the control. Contradictorily, caproic acid esters of hydroxy fatty acids such as 2-CAHMA, 2-CAHSA, and 2-CAHPA were significantly increased in the cecum contents of the HFD group. In the duodenum and jejunum, mainly acetic acid esters of hydroxy fatty acids such as 2-AAHSA, and 2-AAHAA are decreased in the HFD group. Acetic acid and caproic acid esters such as 2-AAHHA, and 2-CAHSA were significantly increased in the jejunum contents of rats fed with HFD. Furthermore, the distribution of changes in total SFAHFA (calculated by the sum of individual concentrations) is depicted in the pi-charts shown in **Figure 5B.** The data clearly indicate that SFAHFAs are downregulated specifically in the colon and cecum contents in the HFD group compared with controls. Interestingly, the SFAHFA concentration was higher in the colon contents (range (min-max): 1076-73632 nmol/100g) followed by the cecum (range: 3239-56940 nmol/100g) contents. The detailed concentrations of individual molecular species of SFAHFAs, FAs, and HFAs are provided in the supporting information **Table S3** and **Table S4** respectively.

The cluster correlation analysis and distribution of the molecular species of SFAHFAs are represented by the heatmap as shown in **Figure 5C**. The intense red color indicates a higher concentration of SFAHFAs. The data clearly show diminished levels of several SFAHFAs in the colon and cecum contents of the HFD group compared with controls. These results are consistent with our previous studies using the same model, but SFAHFAs but were determined by Orbitrap MS[13,26]. Diminished SFAHFAs (also known as acyl α-hydroxyl fatty acids) were also observed in fecal samples of mice fed with antibiotics[15] and stool samples of *Pla2g2a*-deficient mice compared with the control[18]. However, increased SFAHFAs in cecal samples of laying hen infected with *campylobacter jejuni* [16], and the colon contents of weaned piglets fed with low protein diet were also reported [17]. A recent study also identified SFAHFAs in human stool samples and found that their levels varied among individuals and are decreased with antibiotic treatment [27]. All these facts could support that these novel lipids are gut-microbiota dependent, and their metabolism is dysregulated during high-fat diet. The mechanism behind these decreases requires further investigation.

3.4 Time-course profile and network analysis of SFAHFAs in rat feces

In addition to the intestinal contents, we analyzed the fecal samples collected over different time periods. The time-course profiles of total SFAHFAs, acetic acid esters, and propanoic acid esters in feces are shown in **Figure 6A**. The data distinctly showed a sharp decrease in their levels in just 2 weeks after HFD, and the trend remained the same until 8 weeks. The significantly altered SFAHFAs in rat feces at 2 weeks (2w), 4 weeks (4w), 6 weeks (6w), and 8 weeks (8w) of HFD, are shown by network analysis in **Figure 6B.** The size of the bubble indicates the p-values, larger the size, the data are more significant, and the intense blue color indicates a sharp decrease of SFAHFAs in the HFD group. The majority of SFAHFAs decreased in the feces of the HFD group were acetic acid and propanoic acid esters of hydroxy fatty acids, suggesting these particular lipids as specific targets.

The plausible reason behind this decrease would be (i) the changes in gut-microbiota composition because a previous study demonstrated that antibiotics could reduce the SFAHFA levels in feces suggesting these lipids are microbiome dependent[15]. (ii) Decrease of their precursors, such as SFAs, because many studies have demonstrated that high-fat diet can affect the SFAs and decrease their levels and (iii) Decrease in the expression of their biosynthesis-related enzymes. Although SFAHFA biosynthesis is unknown, a recent study suggested that phospholipase A2 Group IIA (*Pla2g2a*) deficient mice as reduced levels of acetic acid-derived SFAHFAs in their feces samples[18]. With all these possibilities, there remains a scientific ambiguity behind the sharp decrease in SFAHFAs, specifically in the colon and cecum contents. Further mechanistic investigations are essential to reveal this

unexplored area of research. The study has some limitations, including specific internal standards for each lipid species, which were not used for quantitation due to the handle in synthesizing labelled standards and other potential biological matrices need to be evaluated.

Conclusions

We have chemically synthesized and established an absolute LC-MS/MS-based quantitation method for the determination of SFAHFAs using a solid-phase extraction strategy. The method shows high sensitivity at low femtomolar levels and robustness to cover 84 lipid molecular species analysis in a period of 22 min. Further, application of the established method to profile SFAHFAs and their precursors in the intestinal contents and feces of rats fed HFD showed a significant decrease in acetic acid and propanoic acid esters of hydroxy fatty acids in the HFD group compared with controls. These results demonstrate the biological significance of these novel lipids in obesity associated with HFD and their links to gut microbiota. The mechanistic investigations behind the decreased SFAHFAs in HFD and the application of the method to elucidate the role of SFAHFAs in various disease models are our future interests.

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CRediT authorship contribution statement

Siddabasave Gowda B. Gowda: Conceptualization, Methodology, Supervision, Writing - original draft. Fengjue Hou: Formal analysis, Visualization, Writing - original draft. Divyavani Gowda: Data curation, visualization, Writing - review & editing. Hitoshi Chiba: Supervision, Writing - review & editing. Kentaro Kawakami: Data curation, Investigation. Satoru Fukiya: Investigation, Writing - review & editing. Atsushi Yokota: Supervision, Resources, Writing - review & editing. Shu-Ping Hui: Supervision, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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482 **Tables: Table 1**: Optimized mass parameters for SFAHFAs detection. (Q1: precursor ion,

483 Q3: product ion, CE: collision energy)

SFAHFA	Q1 (*m/z*)	Q3 (*m/z*)	Tube lens (V)	CE (V)	SFAHFA	Q1 (*m/z*)	Q3 (*m/z*)	Tube lens (V)	CE (V)
16:0/2:0	313.2		49	17	22:0/2:0	397.3		74	22
16:0/3:0	327.2		59	17	22:0/3:0	411.3		76	23
16:0/4:0	341.2	271.2	63	18	22:0/4:0	425.3	355.3	77	23
16:0/5:0	355.2		59	19	22:0/5:0	439.3		79	24
16:0/6:0	369.3		71	20	22:0/6:0	453.3		81	24
17:0/2:0	327.2		50	20	23:0/2:0	411.3		76	22
17:0/3:0	341.2		67	20	23:0/3:0	425.3		77	23
17:0/4:0	355.2	285.2	66	20	23:0/4:0	439.3	369.3	79	23
17:0/5:0	369.3		64	21	23:0/5:0	453.3		81	24
17:0/6:0	383.3		72	21	23:0/6:0	467.4		138	24
18:0/2:0	341.2		60	20	24:0/2:0	425.3		77	22
18:0/3:0	355.2		57	20	24:0/3:0	439.3		79	22
18:0/4:0	369.3	299.2	59	20	24:0/4:0	453.3	383.3	99	22
18:0/5:0	383.3		84	21	24:0/5:0	467.4		82	24
18:0/6:0	397.3		72	21	24:0/6:0	481.4		84	24
19:0/2:0	355.2		69	21	25:0/2:0	439.3		79	24
19:0/3:0	369.3		84	21	25:0/3:0	453.3		81	24
19:0/4:0	383.3	313.2	72	21	25:0/4:0	467.4	397.3	149	25
19:0/5:0	397.3		75	22	25:0/5:0	481.4		76	24
19:0/6:0	411.3		84	22	25:0/6:0	495.4		97	26
20:0/2:0	369.3		71	21	26:0/2:0	453.3		81	24
20:0/3:0	383.3		93	21	26:0/3:0	467.4		156	25
20:0/4:0	397.3	327.3	84	22	26:0/4:0	481.4	411.3	32	25
20:0/5:0	411.3		75	22	26:0/5:0	495.4		93	25
20:0/6:0	425.3		77	22	26:0/6:0	509.4		87	25
21:0/2:0	383.3		68	21	16:0/2:0(IS)	343.4		79	20
21:0/3:0	397.3		70	22	16:0/3:0(IS)	357.4		84	21
21:0/4:0	411.3	341.3	65	23	16:0/4:0(IS)	371.4	301.4	112	20
21:0/5:0	425.3		74	22	16:0/5:0(IS)	385.4		94	21
21:0/6:0	439.4		84	23	16:0/6:0(IS)	399.4		94	21

484

485 **Table 2.** The analysis results of linearity, LOD, and LOQ of authentic FFAs, HFAs, and
486 SFAHFAs (* In both SFAHFA and HFA, the hydroxyl group is at C-2 carbon).

487

Lipids*	Linearity	R^2	LOD (fmol)	LOQ (fmol)
AAHPA	y = 0.2614x	0.9981	5	50
PAHPA	y = 0.2224x	0.9992	5	50
BAHPA	y = 0.3206x	0.9987	5	50
VAHPA	y = 0.3807x	0.9997	5	50
CAHPA	y = 0.4132x	0.9989	5	50
AAHMA	y = 0.117x	0.9976	5	50
PAHMA	y = 0.0978x	0.9967	5	50
BAHMA	y = 0.1001x	0.9983	5	50
VAHMA	y = 0.2098x	0.9976	5	50
CAHMA	y = 0.3148x	0.9969	5	50
AAHSA	y = 0.0933x	0.9959	1	10
PAHSA	y = 0.1523x	0.9981	1	10
BAHSA	y = 0.1847x	0.9965	1	10
VAHSA	y = 0.3204x	0.9989	1	10
CAHSA	y = 0.3034x	0.9986	1	10
AAHNA	y = 0.1757x	0.9995	5	50
PAHNA	y = 0.082x	0.9983	5	50
BAHNA	y = 0.2259x	0.9972	5	50
VAHNA	y = 0.3418x	0.9976	5	50
CAHNA	y = 0.1731x	0.9961	5	50
AAHAA	y = 0.265x	0.9982	0.5	5
PAHAA	y = 0.1145x	0.9935	0.5	5
BAHAA	y = 0.1968x	0.9984	0.5	5
VAHAA	y = 0.3843x	0.9994	0.5	5
CAHAA	y = 0.1967x	0.9971	0.5	5
AAHHA	y = 0.3077x	0.9991	0.5	5

PAHHA	y = 0.2383x	0.9928	0.5	5
BAHHA	y = 0.1748x	0.9939	0.5	5
VAHHA	y = 0.399x	0.9932	0.5	5
CAHHA	y = 0.5159x	0.9985	0.5	5
AAHBA	y = 0.2363x	0.9988	5	50
PAHBA	y = 0.1743x	0.9931	5	50
BAHBA	y = 0.2097x	0.9985	0.5	5
VAHBA	y = 0.1865x	0.9830	0.5	5
CAHBA	y = 0.2334x	0.9859	0.5	5
AAHTA	y = 0.3224x	0.9992	5	50
PAHTA	y = 0.1609x	0.9679	5	50
BAHTA	y = 0.3141x	0.9822	0.5	5
VAHTA	y = 0.3515x	0.9903	0.5	5
CAHTA	y = 0.5459x	0.9966	0.5	5
AAHLA	y = 0.3607x	0.9920	0.5	5
PAHLA	y = 0.33x	0.9959	0.5	5
BAHLA	y = 0.3156x	0.9936	0.5	5
VAHLA	y = 0.2509x	0.9885	0.5	5
CAHLA	y = 0.2772x	0.9970	0.5	5
AAHPSA	y = 0.1326x	0.9800	0.5	5
PAHPSA	y = 0.0752x	0.9569	0.5	5
BAHPSA	y = 0.1119x	0.9891	0.5	5
VAHPSA	y = 0.1121x	0.9823	0.5	5
CAHPSA	y = 0.2235x	0.9962	0.5	5
AAHCA	y = 0.3412x	0.9707	0.1	1
PAHCA	y = 0.2562x	0.9945	0.1	1
BAHCA	y = 0.2473x	0.9862	0.1	1
VAHCA	y = 1.0535x	0.9883	0.1	1
CAHCA	y = 0.7736x	0.9983	0.1	1
HPA	y = 0.0753x	0.9997	10	100
HMA	y = 0.0727x	0.9995	10	100

HSA	y = 0.0967x	0.9995	10	100
HNA	y = 0.074x	0.9999	10	100
HAA	y = 0.0538x	0.9992	1	10
HHA	y = 0.0785x	0.9974	1	10
HBA	y = 0.0682x	0.9977	1	10
HTA	y = 0.0735x	0.9974	1	10
HLA	y = 0.0219x	0.9880	1	10
HPSA	y = 0.0237x	0.9876	1	10
HCA	y = 0.0535x	0.9709	1	10
PA	y = 0.0002x	0.9997	5000	50000
MA	y = 0.0003x	0.9956	5000	50000
SA	y = 0.0004x	0.99122	5000	50000
NA	y = 0.0004x	0.98799	5000	50000
AA	y = 0.0005x	0.99085	5000	50000
HA	y = 0.0008x	0.99057	5000	50000
BA	y = 0.0006x	0.98393	5000	50000
TA	y = 0.0009x	0.97901	5000	50000
LA	y = 0.001x	0.97521	500	5000
PSA	y = 0.0015x	0.97117	500	5000
CA	y = 0.0015x	0.98462	500	5000

Figures

Figure 1: Abbreviations of homologues series of short chain fatty acid ester of hydroxy fatty acids (SFAHFA).

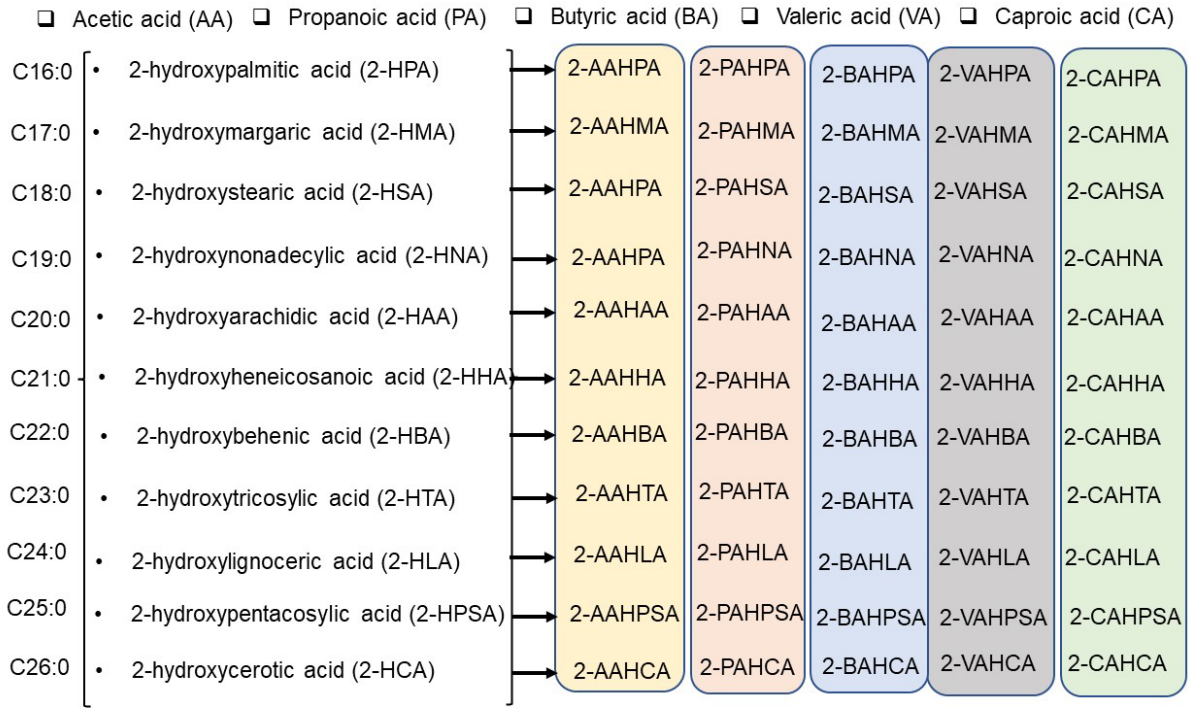


Figure 2: Schematic representation of synthesis of SFAHFAs derivatives of palmitic acid and its deuterated internal standard.

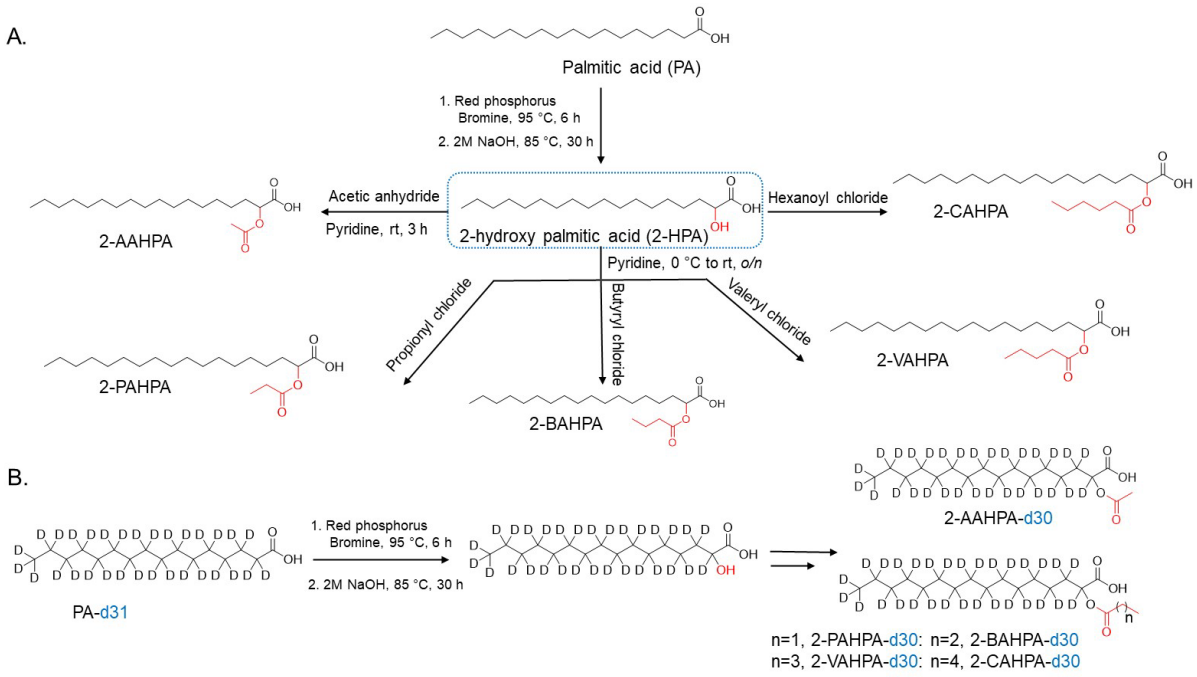


Figure 3: MS spectra and extracted ion chromatogram of representative SFAHFAs. (**A**) High resolution MS spectra obtained using LTQ-orbitrap MS and MS/MS spectra are obtained from TSQ Quantum Access MAX, and energy-resolved dissociation curves of C2-C6 fatty acid esters of 2-hydroxypalmitic acid and 2-hydroxypalmitic acid-d30. (**B**) Extracted ion chromatograms of C2-C6 fatty acid esters of 2-hydroxypalmitic acid and its deuterated forms**.** (RA: relative abundance, RT: retention time).

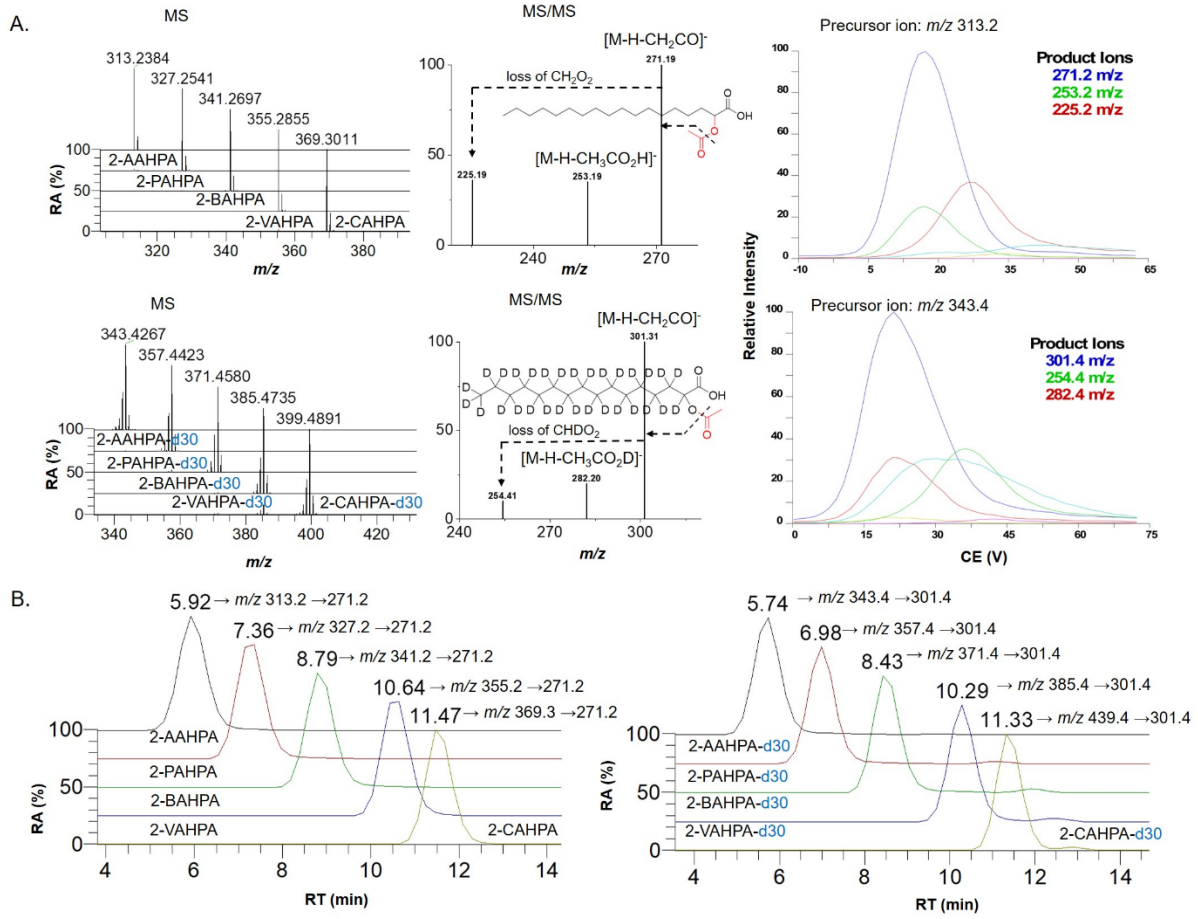


Figure 4: (A) Characteristics of high-fat diet: changes in body weight and adipose tissue weight (mean±SEM, t-test with $p<0.05$ is considered to be significant). (B) Sample preparation and solid-phase extraction workflow for SFAHFAs and their precursors.

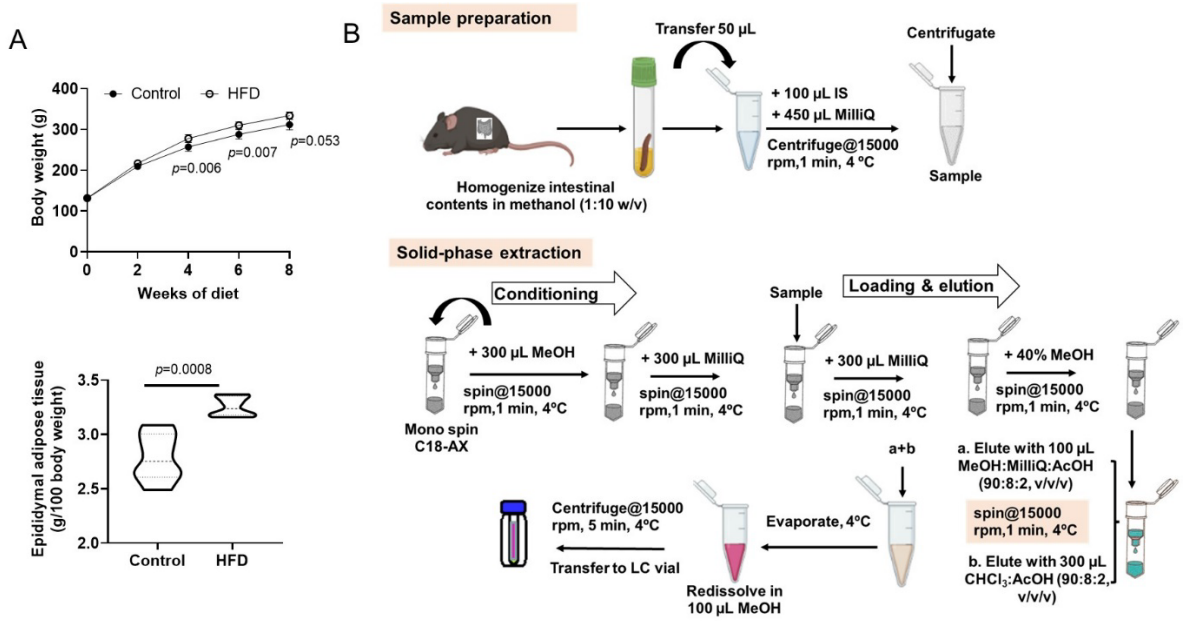


Figure 5: Change in SFAHFA level by the high-fat diet (**A**) Volcano plots showing significantly altered SFAHFAs (**B**) Gut-distribution of SFAHFAs (**C**) Cluster correlation analysis results of SFAHFA.

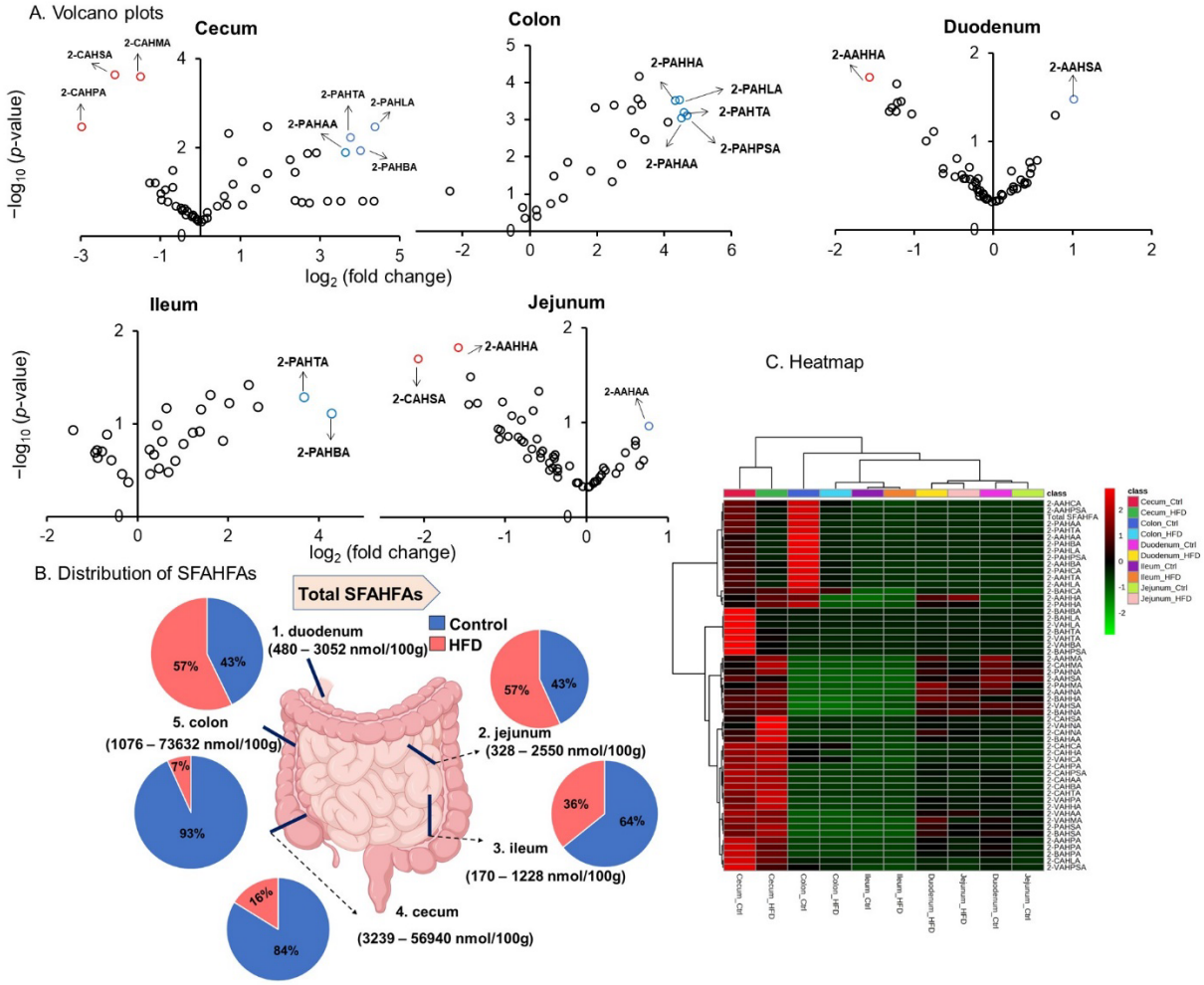
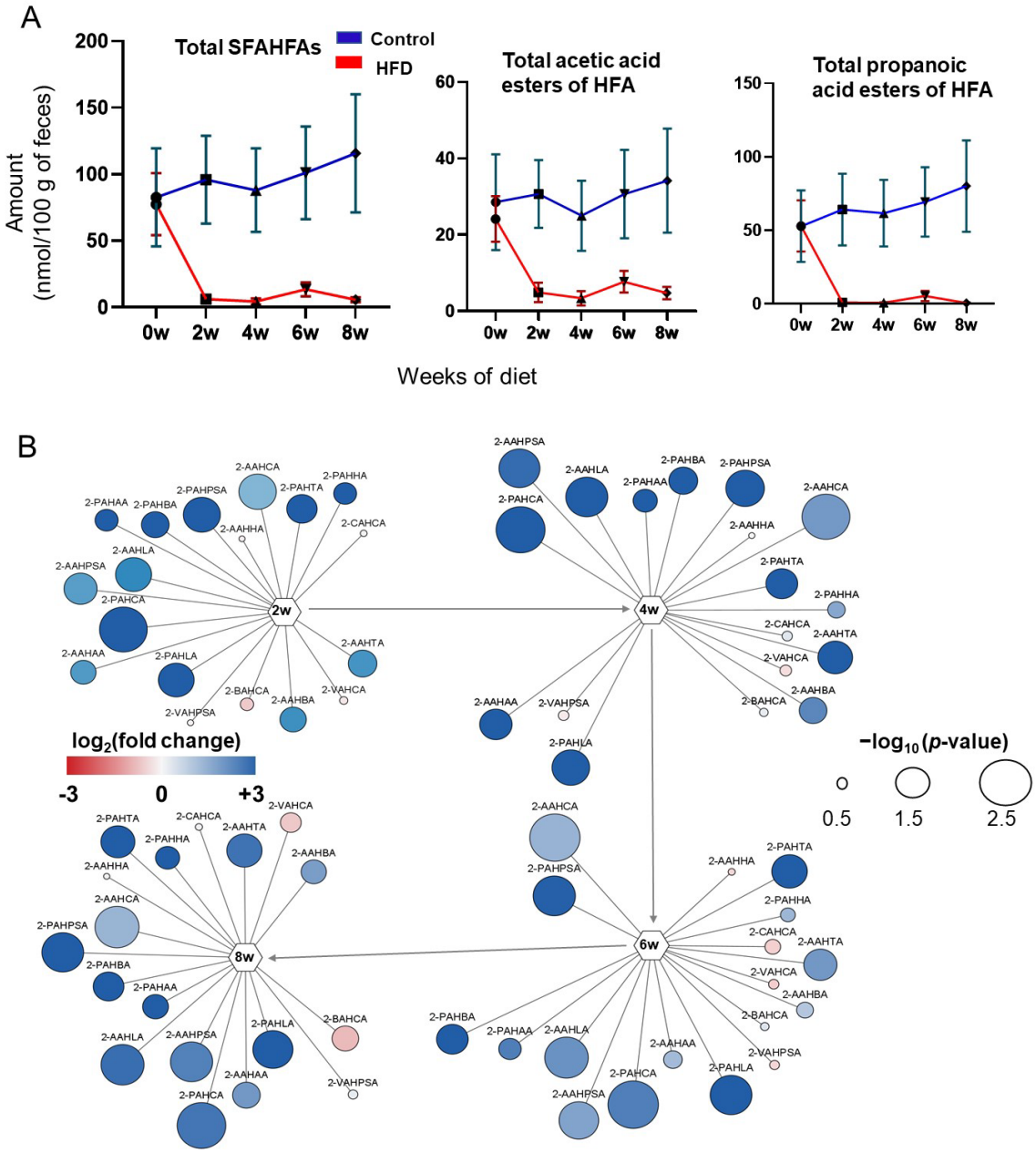


Figure 6: Time-course kinetics of SFAHFAs in feces during high-fat diet feeding in rats (A) Change in SFAHFAs in feces every 2 week after the diet. (B) Network analysis of significantly altered SFAHFAs in feces.



Graphical abstract: