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Identification of short chain fatty acid esters of hydroxy fatty acid (SFAHFA) in murine model by nontargeted analysis using UHPLC/ LTQ Orbitrap MS

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Abstract:**Rationale**

Fatty acid esters of hydroxy fatty acids (FAHFAs) are the recently discovered endogenous lipids with outstanding health benefits. FAHFAs are known to exhibit antioxidant, antidiabetic and anti-inflammatory properties. The number of known long chain FAHFAs in mammalian tissues and dietary resources increased recently because of the latest developments in high-resolution tandem mass spectrometry techniques. However, there are no reports on identification of short chain fatty acid esterified hydroxy fatty acids (SFAHFAs).

Methods

Intestinal contents, tissues, and plasma of rats fed with high-fat diet (HFD) and normal diet (ND) was analysed for fatty acids, hydroxy fatty acids, and FAHFAs using ultra - high - performance liquid chromatography (UHPLC) and linear trap quadrupole - Orbitrap mass spectrometry (LTQ Orbitrap MS) with negative heated electrospray ionization.

Results

Untargeted analysis of total lipid extracts from murine samples (male 13-week old WKAH/HKMSlc rats) lead to the identification of several new SFAHFAs of acetic acid or propanoic acid esterified long chain (>C20)-hydroxy fatty acids. Further, MS³ analysis revealed the position of hydroxyl group in long chain fatty acid as C-2. The relative amount of SFAHFAs were relatively quantified in intestinal contents and their tissues (caecum, small intestine, and large intestine), liver, and plasma of rats fed with HFD and ND. Large intestinal contents showed the highest abundance of SFAHFAs with a concentration range from 0.84 to 57 pmoles/mg of contents followed by cecum contents with a range of 0.66 to 28.6 pmoles/mg of contents. SFAHFAs were significantly altered between HFD and ND groups, with a strong decreasing tendency under HFD conditions.

Conclusions

Identification of these novel SFAHFAs can contribute to better understanding the chemical and biological properties of individual SFAHFAs and their possible sources in the gut. Which in turn help us tackle the role of these lipids in various metabolic diseases.

1. Introduction

Fatty acid esters of hydroxy fatty acids (FAHFAs) are a novel class of endogenous lipids, identified recently in mammalian adipose tissues and blood plasma^{1,2}. Multiple combinations of fatty acids (FAs) and hydroxy fatty acids (HFAs) forms the various isomers with the ester linkage at position C5, C7, C8, C10, C11, C12, and C13 respectively^{1,3}. To date FAHFAs biosynthesis and degradation pathways are not well characterized. Their biosynthesis may occur *denovo* in tissues by the action of fatty acyltransferases and transported to circulating system¹. Many studies in mice and humans suggest that FAHFAs can act as endocrine mediators of insulin sensitivity and exert anti-diabetic properties^{1,4-6}. Oral administration of 5- and 9-palmitic acid hydroxy stearic acid esters (PAHSA) to mice, attenuated the ulcerative colitis (an intestinal chronic inflammation) by reducing the proliferation of pro-inflammatory T lymphocytes⁷. Dietary supplementation of ω -3 fatty acid derived FAHFAs to diabetic patients and obese mice increased the circulating levels of polyunsaturated fatty acid derived FAHFAs and are also known to inhibit the inflammation by stimulating immune cells and limit macrophage activation⁶. Besides, PAHSA levels are drastically decreased in serum of obese mice¹, milk of obese women compared to lean mothers⁸, and increased in high-fat diet fed aP2-WisP2 transgenic mice⁹. All these studies suggest the biological significance and greater therapeutic potential of FAHFAs for the treatment of various metabolic diseases such as obesity, cancer, and diabetes.

Bacteria are identified as an important source of the hydroxy fatty acid (HFA) in the natural environment¹⁰. For instance, 2-HFA are known to occur in sulfate-reducing bacteria¹¹ whereas, 3-HFA are constituents of endotoxins of gram-negative bacteria¹². 2-HFA are also important components of a subset of mammalian sphingolipids¹³. Although 2-HFAs and 3-HFAs are widely spread in nature, yet there are limited reports on identification of FAHFAs with ester linkage at C2 or C3¹⁴. In mammals, gut microbiota could be a noble source of HFAs, and it is also reported that intestinal bacteria's produce several short chain fatty acids (SFAs) such as acetic acid, propanoic acid, and butyric acid, with diverse biological functions¹⁵. It is of our interest that, due to the large abundance of SFAs in gut, there is a possible existence SFA derived FAHFAs. So far, there are many saturated or unsaturated long chain fatty acid derived FAHFAs were identified^{1,3}, but no reports on SFA derived fatty acid esters.

Electron ionization-mass spectrometry is a well-established technique for fatty acids (FA)¹⁶ and FAHFAs analysis^{1,2,6,14,17}, however, it is limited by the exact structure determination due to complex rearrangements occurring during fragmentation process¹⁶. In other words, collision-induced dissociation gives rather specific fragment ions, which often useful in elucidating the structural information. More recently, chemical derivatization of FAHFAs followed by shotgun lipidomic analysis approach was employed¹⁸. In this study, we aim to identify and characterize the SFA derived FAHFAs in plasma, liver, intestinal tissues, and their contents in a rodent rat obesity

model using ultra-high-performance liquid-chromatography with linear ion trap quadrupole-Orbitrap mass spectrometry (UHPLC-LTQ Orbitrap MS) by collision induced dissociation. Also, we relatively quantified and compared their levels with the effect of diet, between high-fat diet and normal diet fed rats.

2. Materials and Methods

2.1 Chemicals

The LC/MS grade solvents such as methanol, isopropanol, chloroform, and ammonium acetate solution (1M) were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Oleic acid-d₉, a deuterated internal standard was obtained from Avanti Polar Lipids (Alabaster, AL). All other reagents of analytical grade were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2 Animal experiments

The animal experiments were carried out by the method reported earlier by our group¹⁹. In brief, male 3-week-old WKAH/HkmSlc rats were acclimatized for 2 weeks by feeding a normal diet (ND), then divided into two groups (n=6 each) and fed ND or high fat diet (HFD) based on the AIN-93G formulation for 8 weeks. The lard was used in place of dextrin to prepare HFD and the detailed diet composition is provided in our earlier report¹⁹. At 8 weeks, portal blood was collected from rats with heparin as an anticoagulant agent for preparation of plasma. Then, the rats were killed by exsanguination without food deprivation and required intestinal contents, tissues were collected for LC/MS analysis. All experimental procedures were carried out with a prior approval (No. 17-0050) from Hokkaido University Ethics Review Committee.

2.3 Sample preparation

The total lipids were extracted from the rat plasma by the previously established method in our laboratory with minor modifications¹⁹. Concisely, 50 µL of each plasma samples were extracted with 200 µL of methanol having internal standard (FA 18:1 (d₉), 17 µM) and were vortexed (30 sec, 3500 rpm) at room temperature. Then, 200 µL of chloroform (vortex, 5 min, 3500 rpm) and 50 µL of Milli-Q (vortex, 30 sec, 3500 rpm) were added. The extracts were centrifuged (15000 rpm, 10 min) at 4°C and the residue was re-extracted with additional 200 µL of chloroform. The combined chloroform extracts were dried under vacuum and redissolved in 100 µL of methanol, centrifuged (15000 rpm, 10 min), and were transferred to the LC/MS vials. In the case of tissues, about 50-100 mg of the snap-frozen tissue was weighed into a 1.5 mL eppendorf tube and approximately 5-6 ceramic beads (1.4 mm, catalog no. 15-340-159, Fisherbrand) were added and homogenized for a period of 1-2 repeated 30-s cycles using Bead Mill 4 (Fisherbrand) homogenizer. Then, cold methanol was added into the homogenate to normalize the weight (10 µL/mg of tissue) and repeated the homogenization for a period of 30s. About 100 µL of the methanol homogenate is used for the extraction of total lipids by the protocol described above. The cecum, small

intestine, and large intestine contents were taken from the rats soon after sacrifice and stored at -80 °C before the analysis. To the appropriately weighed contents cold methanol was added (10 µL/mg of content) and vortexed for a min. About 100 µL of the vortexed sample mixture is directly used for extraction of total lipids. The limitation of this extraction method is that, there is a possibility of loss of volatile fatty acids during the drying procedures for redissolution. The injection volume of all samples in each run was set to 10 µL.

2.4 Lipidomic analysis by UHPLC-LTQ Orbitrap MS

Prominence UHPLC system (Shimadzu Corp., Kyoto, Japan) connected to an LTQ Orbitrap MS (Thermo-Fisher Scientific Inc., San Jose, CA) was used to perform the lipidomic analysis. Chromatographic separation was achieved at a flow rate of 200 µL/min using reverse phase-Atlantis T3 C18 column (2.1 × 150 mm, 3 µm, Waters, Milford, MA) and maintaining the oven temperature at 40 °C. The mobile phase consists of A: Milli-Q (with 10 mM CH₃COONH₄), B: Isopropanol and C: Methanol with a linear gradient flow of: 0-1 min (30% B and 35% C), 1-14 min (80% B and 10% C), 14-27 min (85% B and 10% C), followed by reequilibration to initial conditions for 3 min. The MS analysis was carried out in ESI-negative ionization mode with the following parameters. The MS capillary voltage was set at 3 kV, the nitrogen-sheath gas flow was set to 50 units, and the nitrogen-auxiliary gas to 5 units. The MS¹ data were acquired in Fourier Transform mode with a resolving power of 60,000 and a 2 Hz scan speed, with a scan range *m/z* 150–1900.

Data-dependent acquisition with MS² and MS³ scans were performed in the ion-trap mode at an isolation width of *m/z* 3, and a collision energy of 40 V and 45 V, respectively. The minimum signal threshold of each scan was set to 500 and 100 counts respectively. The obtained raw data were processed by MS DIAL (ver 3.9) software to identify the lipid molecular species²⁰. Then, peak processing and integration were drawn within the mass tolerance of 5.0 ppm by Xcalibur 2.2 (Thermo-Fisher Scientific Inc.). The fatty acids, hydroxy fatty acids, and SFAHFAs in the samples were relatively quantified by using Oleic acid-d9 as an internal standard.

2.5 Statistical analysis

Data were plotted in Excel 2016 and GraphPad Prism 6, as the mean ± standard error (n=6, for each ND and HFD groups) with applied student's t test (p<0.05 is considered to be statistically significant).

3. Results and Discussion

3.1 Identification of fatty acids and derivatives

The comprehensive lipidomic analysis of murine samples leads to the identification of fatty acids (FA), hydroxy fatty acids (HFA), and their derivatives (FAHFA) by the aid of MS DIAL and Xcalibur data processing computational tools. All the detected FAs and HFAs are listed in the **Table 1** with their retention time and exact masses. The

extracted ion chromatograms (EIC) and their CID mass spectra of representative species from each class were shown in **Figure 1**. In general, the saturated and less unsaturated FAs (FA 24:0, m/z 367) lose a molecule of water to produce a most significant ion (m/z 349) (**Figure 1a**) whereas, HFAs (FAOH 24:0, m/z 383) (**Figure 1b**) commonly, loses m/z 46 to produce its characteristic ion (m/z 337) in their mass spectra^{21,22}. However, the polyunsaturated fatty acids predominately lose a molecule of CO₂ rather than H₂O to give the most significant ion in their spectra²³. The fatty acid derivative (**Figure 1c**) elutes between FA and HFA, shows the loss of ions m/z 42 (CH₂CO) and m/z 56 (CH₃CHCO) from the molecular ion (m/z 425) but their MS spectrum did not match any of reported FAHFAs suggest, it could be a new type of FAHFAs. Then, we extracted these novel FAHFAs by the similarity of their MS spectrum and named them as unknowns U1 to U10 (**Figure 2**). Most of the FAHFAs reported so far are hydroxy fatty acid ester at C5, C7, C8, C10, C11, C12, and C13 respectively^{1,3} with specific fragment ions of each long chain fatty acids.

3.2 Characterization of unknown FAHFAs by MSⁿ analysis

The **Figure 3** and **Figure 4** shows the detailed MS spectrum of all the unknown FAHFAs. The MS¹ spectrum is acquired in negative FT mode and MS², MS³ were obtained by CID in ion trap mode. The ionization of compound U1 ([M-H]⁻, m/z 369.3010; C₂₂H₄₁O₄, error: -1.62 ppm) shows the MS² fragment ions m/z 327 [M-H-CH₂CO]⁻ and m/z 309 [M-H-CH₃COOH]⁻ respectively, indicating the possible esterification of acetic acid with the hydroxy fatty acid. Further the MS³ spectra of fragment ion m/z 327 gives m/z 281 by the loss of m/z 46, indicates the position of hydroxyl group is at C-2 (2-HFA)²², hence U1 is identified as short chain fatty acid ester of hydroxy fatty acid (SFAHFA) 2:0/20:0 (**Figure 3a**), or alternatively named as, 2-acetic acid ester of hydroxy arachidic acid.

The unknown FAHFA U2 ([M-H]⁻, m/z 397.3323; C₂₄H₄₅O₄, error: -0.25 ppm), shows the MS² fragment ions m/z 355 [M-H-CH₂CO]⁻ and m/z 337 [M-H-CH₃COOH]⁻, and loss of m/z 46 in MS³ spectra of fragment ion m/z 355, indicating the identical fragmentation pattern to U1 and hence, named U2 as SFAHFA 2:0/22:0 or 2-acetic acid ester of hydroxy docosanoic acid. However, the existence of another low abundant MS² ion at m/z 341 [M-H-CH₃CHCO]⁻ shows the presence of structural isomer as SFAHFA 3:0/21:0 or 2-propanoic acid ester of hydroxy heneicosanoic acid (**Figure 3b**). The limitation of our analysis method is that unable to separate the structural isomer, which may be achieved by isotope labelling experiments³. The unknown, U3 (**Figure 3c**) shows the spectra identical to U1 spectrum by the loss of 42 Da and 60 Da from the molecular ion ([M-H]⁻, m/z 395.3166; C₂₄H₄₃O₄, error: -1.01 ppm) and a loss of 46 Da from the fragment ion m/z 353. Hence, the compound U3 is identified as SFAHFA 2:0/22:1. Whereas the compound U4 (**Figure 3c**) shows the spectra partially matches with U2 by the loss of 56 Da and 74 Da from the molecular ion ([M-H]⁻, m/z 411.3480; C₂₅H₄₇O₄, error: 0 ppm) and loss of 46 Da from the fragment ion m/z 355 to give m/z 309. Thus, compound U4 is identified as SFAHFA 3:0/22:0 or 2-propanoic acid ester of hydroxy docosanoic acid. The U5 shows the fragmentation

pattern identical to U2 ($[M-H]^-$, m/z 425.3636; $C_{26}H_{49}O_4$, error: 0 ppm), indicating the presence of isomeric mixtures of acetate and propionate esters of 2-hydroxy fatty acids, which is further confirmed by their MS^3 fragment ions m/z 337 and m/z 323 from m/z 383 and 369 by the loss 46 Da in each case. Hence U5 is identified as isomeric mixture of SFAHFA 3:0/23:0 or 2-propanoic acid ester of tricosanoic acid and SFAHFA 2:0/24:0 or 2-propanoic acid ester of hydroxy tetracosanoic acid (**Figure 3e**).

The detailed MS spectrum of unknown FAHFAs U6 to U10 were provided in **Figure 4**. The fragmentation pattern of compounds U6 ($[M-H]^-$, m/z 423.3480; $C_{26}H_{47}O_4$, error: -1.8 ppm), and U7 ($[M-H]^-$, m/z 439.3793; $C_{27}H_{51}O_4$, error: 0 ppm), were similar to that of U2 and U5 with the loss of 42 Da and 56 Da respectively, indicating them as structural isomers of acetic acid and propanoic esterified FAHFAs (**Figures 4a, 4b**). Thus, U6 is identified as isomeric mixture of SFAHFA 2:0/24:1 and SFAHFA 3:0/23:1, whereas U7 as SFAHFA 3:0/24:0 or 2-propanoic acid ester of hydroxy tetracosanoic acid and SFAHFA 2:0/25:0 or 2-acetic acid ester of hydroxy pentacosanoic acid, with esterification at C2-hydroxy position of long chain fatty acid moiety, confirmed by their MS^3 spectra showing common loss of 46 Da. The compound U8 ($[M-H]^-$, m/z 437.3636; $C_{27}H_{49}O_4$, error: 0 ppm), U9 ($[M-H]^-$, m/z 435.3480; $C_{27}H_{47}O_4$, error: 0 ppm), and U10 ($[M-H]^-$, m/z 453.3949; $C_{28}H_{53}O_4$, error: -0.22 ppm) shows the fragmentation pattern identical to U4, by the loss of m/z 56 from their molecular ions and m/z 46 from MS^2 fragment ions, hence they were identified as propanoic acid esters of 2-hydroxy long chain fatty acids. U8 is identified as SFAHFA 3:0/24:1, U9 as SFAHFA 3:0/24:2 and U10 as SFAHFA 3:0/25:0 or 2-propanoic acid ester of hydroxy pentacosanoic acid (**Figures 4c, 4d, 4e**) respectively. The compounds U7 and U8 as odd chain hydroxy fatty acids, which are rarely exist in mammals, but have been known to be possibly biosynthesised in gut and incorporated to complex lipids²⁴.

3.3 Relative quantification of SFAHFAs in murine samples

The relative concentration of these novel SFAHFAs was semi-quantified by an internal standard method in tissues, intestinal contents, and plasma of high-fat diet induced rodent-rat obesity model. The results are given in **Figure 5**. The data shows the highest abundance of SFAHFAs in the large intestine and cecum contents, but their levels relatively low in small intestine contents and plasma samples (**Figure 5A**). The concentrations of SFAHFA in tissues such as the cecum, small intestine, large intestine and liver were significantly low (**Figure 5B**) compared to their contents. It has been known that gut bacteria in the large intestine and cecum produce the short chain fatty acids and hydroxy fatty acids²⁵. And gut microbiota is largely colonized in large intestine and cecum indicating possible role of gut microbiota in the synthesis of SFAHFAs. In cecum contents (SFAHFA 2:0/22:1 and SFAHFA 3:0/24:2), small intestine contents (SFAHFA 24:0, and SFAHFA 26:0), large intestine contents (SFAHFA 2:0/22:1) and plasma (SFAHFA 2:0/22:1, SFAHFA 3:0/24:2) respective SFAHFAs were significantly decreased in high fat diet (HFD) group compared to

normal diet (ND) group. Previous studies, also showed the decreased levels of SFAs under rats fed with HFD²⁶, could account for possible reduction of SFAHFAs. However, some of the SFAHFAs such as SFAHFA 3:0/22:0 and SFAHFA 3:0/24:1, shows the increased under high-fat diet condition.

In the tissues, SFAHFAs concentrations were relatively low and not significantly altered between HFD and ND groups except decreased levels of SFAHFA 26:0, SFAHFA 27:0 and increased levels of SFAHFA 24:0 over the large intestine. Overall, many SFAHFAs were decreased under HFD condition compared to ND, with some exceptions for few molecular species. In previous reports, FAHFAs of long chain fatty acid esters are reduced in milk of obese women⁸, serum of obese mice¹ and in our experimental results SFAHFAs are also showed the same trend in plasma of obese rats. Whereas, PAHSAs are positively associated with the weight gain during pregnancy²⁷ suggesting the structure specific variations of FAHFAs. Metabolization of FAHFAs may be regulated at both local and systemic levels and could be differently distributed among type of tissues⁴. The irregular variations among SFAHFAs between HFD and ND groups in intestinal contents could be due to the distribution of differential microbiota over the gastrointestinal tract. Moreover, the analysis of samples of meconium from healthy new-borns, showed the presence of complex regio-isomeric FAHFAs and possible involvement of gut microbiota in their metabolism⁸. The dysregulation of fatty acid metabolism is commonly observed in obesity associated disorders such as cardiac failure in aged mice^{28,29} and hence, it could be also possible in FAHFAs metabolism as fatty acids are their precursors. All these results suggest that, there is a need for more research on identification of novel FAHFAs and their functions over the intestine in connection with metabolic disorders such as obesity.

4. Summary and conclusions

The detailed chromatographic and mass spectrometric analysis of fatty acids and their derivatives in murine samples lead to the identification of several new FAHFAs of acetic acid or propanoic acid esters of long-chain 2-hydroxy fatty acids. All the identified compounds were further relatively quantified, indicating that SFAHFAs are abundant in contents of large intestine followed by cecum contents. This is the first report of the identification and quantification of a series of SFAHFAs, which are mostly decreased in obese rats. Further investigation is required to better understand the sources, biochemical pathways, and importance of these novel lipid's in metabolic syndromes.

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References:

1. Yore MM, Syed I, Moraes-Vieira PM, et al. Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell*. 2014;159(2):318-332. doi:10.1016/j.cell.2014.09.035
2. Kolar MJ, Nelson AT, Chang T, et al. Faster Protocol for Endogenous Fatty Acid Esters of Hydroxy Fatty Acid (FAHFA) Measurements. *Anal Chem*. 2018;90(8):5358-5365. doi:10.1021/acs.analchem.8b00503
3. Zhu QF, Yan JW, Gao Y, Zhang JW, Yuan BF, Feng YQ. Highly sensitive determination of fatty acid esters of hydroxyl fatty acids by liquid chromatography-mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci*. 2017;1061-1062:34-40. doi:10.1016/j.jchromb.2017.06.045
4. Balas L, Feillet-Coudray C, Durand T. Branched Fatty Acyl Esters of Hydroxyl Fatty Acids (FAHFAs), Appealing Beneficial Endogenous Fat against Obesity and Type-2 Diabetes. *Chem - A Eur J*. 2018;24(38):9463-9476. doi:10.1002/chem.201800853
5. Moraes-Vieira PM, Saghatelian A, Kahn BB. GLUT4 expression in adipocytes regulates de novo lipogenesis and levels of a novel class of lipids with antidiabetic and anti-inflammatory effects. *Diabetes*. 2016;65(7):1808-1815. doi:10.2337/db16-0221
6. Kuda O, Brezinova M, Rombaldova M, et al. Docosahexaenoic acid-derived fatty acid esters of hydroxy fatty acids (FAHFAS) with anti-inflammatory properties. *Diabetes*. 2016;65(9):2580-2590. doi:10.2337/db16-0385
7. Lee J, Moraes-Vieira PM, Castoldi A, et al. Branched fatty acid esters of hydroxy fatty acids (FAHFAs) protect against colitis by regulating gut innate and adaptive immune responses. *J Biol Chem*. 2016;291(42):22207-22217. doi:10.1074/jbc.M115.703835
8. Brezinova M, Kuda O, Hansikova J, et al. Levels of palmitic acid ester of hydroxystearic acid (PAHSA) are reduced in the breast milk of obese mothers. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2018;1863(2):126-131. doi:10.1016/j.bbalip.2017.11.004
9. Grünberg JR, Hoffmann JM, Hedjazifar S, et al. Overexpressing the novel autocrine/endocrine adipokine WISP2 induces hyperplasia of the heart, white and brown adipose tissues and prevents insulin resistance. *Sci Rep*. 2017;7:43515. doi:10.1038/srep43515
10. Fautz E, Rosenfelder G, Grotjahn L. Iso-branched 2- and 3-hydroxy fatty acids as characteristic lipid constituents of some gliding bacteria. *J Bacteriol*. 1979;140(3):852-858. doi:10.1128/jb.140.3.852-858.1979
11. Řezanka T, Sokolov MY, Viden I. Unusual and very-long-chain fatty acids in *Desulfotomaculum*, a sulfate-reducing bacterium. *FEMS Microbiol Lett*. 1990;73(3):231-237. doi:10.1016/0378-1097(90)90735-9
12. Lee AKY, Chan CK, Fang M, Lau APS. The 3-hydroxy fatty acids as

- biomarkers for quantification and characterization of endotoxins and Gram-negative bacteria in atmospheric aerosols in Hong Kong. *Atmos Environ*. 2004;38(37):6307-6317. doi:10.1016/j.atmosenv.2004.08.013
13. Gowda SGB, Nakahashi A, Yamane K, et al. Facile Chemoselective Strategy toward Capturing Sphingoid Bases by a Unique Glutaraldehyde-Functionalized Resin. *ACS Omega*. 2018;3(1):753-759. doi:10.1021/acsomega.7b01440
 14. Zhu QF, Yan JW, Zhang TY, Xiao HM, Feng YQ. Comprehensive Screening and Identification of Fatty Acid Esters of Hydroxy Fatty Acids in Plant Tissues by Chemical Isotope Labeling-Assisted Liquid Chromatography-Mass Spectrometry. *Anal Chem*. 2018;90(16):10056-10063. doi:10.1021/acs.analchem.8b02839
 15. Ohira H, Tsutsui W, Fujioka Y. Are short chain fatty acids in gut microbiota defensive players for inflammation and atherosclerosis? *J Atheroscler Thromb*. 2017;24(7):660-672. doi:10.5551/jat.RV17006
 16. Oursel D, Loutelier-Bourhis C, Orange N, Chevalier S, Norris V, Lange CM. Identification and relative quantification of fatty acids in *Escherichia coli* membranes by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom*. 2007;21(20):3229-3333. doi:10.1002/rcm.3177
 17. Zhang T, Chen S, Syed I, et al. A LC-MS-based workflow for measurement of branched fatty acid esters of hydroxy fatty acids. *Nat Protoc*. 2016;11(4):747-763. doi:10.1038/nprot.2016.040
 18. Hu C, Wang M, Duan Q, Han X. Sensitive analysis of fatty acid esters of hydroxy fatty acids in biological lipid extracts by shotgun lipidomics after one-step derivatization. *Anal Chim Acta*. 2020;1105:105-111. doi:10.1016/j.aca.2020.01.026
 19. Gowda SGB, Gao Z-J, Chen Z, et al. Untargeted lipidomic analysis of plasma from high fat diet-induced obese rats using UHPLC- linear trap quadrupole - Orbitrap MS. *Anal Sci*. 2020;Article ID 19P442. doi:10.2116/analsci.19p442
 20. Tsugawa H, Cajka T, Kind T, et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nat Methods*. 2015;12(6):523-526. doi:10.1038/nmeth.3393
 21. Li J, Xu J, Zhang R, et al. Strategy for Global Profiling and Identification of 2- and 3-Hydroxy Fatty Acids in Plasma by UPLC-MS/MS. *Anal Chem*. 2020;92(7):5143-5151. doi:10.1021/acs.analchem.9b05627
 22. Murphy RC, Fatty acids, in *Tandem Mass Spectrometry of Lipids: Molecular Analysis of Complex Lipids*. Royal Society of Chemistry, eISBN:978-1-78262-635-0. 2014:1-39. doi:10.1039/9781782626350-00001
 23. Yang K, Zhao Z, Gross RW, Han X. Identification and quantitation of unsaturated fatty acid isomers by electrospray ionization tandem mass spectrometry: A shotgun lipidomics approach. *Anal Chem*. 2011;83(11):4243-4250. doi:10.1021/ac2006119
 24. Pfeuffer M, Jaudszus A. Pentadecanoic and Heptadecanoic Acids: Multifaceted Odd-Chain Fatty Acids. *Adv Nutr An Int Rev J*. 2016;7(4):730-734. doi:10.3945/an.115.011387
 25. Hirata S ichiro, Kunisawa J. Gut microbiome, metabolome, and allergic

- diseases. *Allergol Int.* 2017;66(4):523-528. doi:10.1016/j.alit.2017.06.008
26. Jakobsdottir G, Xu J, Molin G, Ahrné S, Nyman M. High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects. *PLoS One.* 2013;8(11):e80476. doi:10.1371/journal.pone.0080476
 27. The American College of Obstetricians and Gynecologists. ACOG Committee Opinion. Weight Gain During Pregnancy. *Obstet Gynecol.* 2013;121(1):210-212.
 28. Lopez EF, Kabarowski JH, Ingle KA, et al. Obesity superimposed on aging magnifies inflammation and delays the resolving response after myocardial infarction. *Am J Physiol - Hear Circ Physiol.* 2015;308(4):H269-H280. doi:10.1152/ajpheart.00604.2014
 29. Halade G V., Kain V, Black LM, Prabhu SD, Ingle KA. Aging dysregulates D- and E-series resolvins to modulate cardiosplenic and cardiorenal network following myocardial infarction. *Aging (Albany NY).* 2016;8(11):2611-2634. doi:10.18632/aging.101077

Tables:

Table 1: List of characterized FAs and HFAs (as [M-H]⁻ ions) in murine samples (rat intestinal contents, tissues, and plasma).

Name	RT, min	Theoretical, <i>m/z</i>	Experimental, <i>m/z</i>	Mass error, ppm
FA 14:0	7.62	227.2017	227.2016	-0.44
FA 16:0	9.94	255.2330	255.2327	-1.18
FA 17:0	10.89	269.2486	269.2485	-0.37
FA 18:0	11.90	283.2643	283.2639	-1.41
FA 18:1	10.78	281.2486	281.2484	-0.71
FA 18:2	9.29	279.2330	279.2329	-0.36
FA 18:3	8.13	277.2173	277.2172	-0.36
FA 19:1	11.45	295.2643	295.2641	-0.68
FA 20:0	13.49	311.2956	311.2955	-0.32
FA 20:1	12.06	309.2799	309.2799	0.00
FA 20:4	9.29	303.2330	303.2328	-0.66
FA 20:5	8.13	301.2173	301.2168	-1.66
FA 21:0	13.98	325.3112	325.3111	-0.28
FA 22:0	14.99	339.3269	339.3268	-0.29
FA 22:1	13.73	337.3112	337.3111	-0.30
FA 22:4	10.88	331.2643	331.2640	-0.91
FA 22:6	9.01	327.2330	327.2327	-0.92
FA 23:0	15.64	353.3425	353.3424	-0.28
FA 24:0	16.27	367.3582	367.3580	-0.54
FA 24:1	15.14	365.3425	365.3424	-0.27
FA 24:2	14.23	363.3269	363.3267	-0.55
FA 25:0	16.65	381.3738	381.3738	0.00
HFA 16:0	7.00	271.2279	271.2279	0.00
HFA 17:0	7.17	285.2435	285.2435	0.00
HFA 18:1	8.31	297.2435	297.2434	-0.34
HFA 20:0	11.36	327.2905	327.2902	-0.92
HFA 22:0	12.04	355.3218	355.3217	-0.28
HFA 22:1	11.35	353.3061	353.3060	-0.28
HFA 24:0	13.07	383.3531	383.3531	0.00
HFA 24:1	12.04	381.3374	381.3374	0.00
HFA 24:2	11.48	379.3218	379.3217	-0.26

Figures:

Figure 1: Extracted ion chromatograms (EICs) of representative molecular species from each class and their acquired MS¹ and MS² spectra

Figure 2: Extracted ion chromatograms (EICs) of unknown FAHFAs

Figure 3: Characterization of SFAHFAs U1 to U5, and their MSⁿ analysis (M* represents the most abundant MS² ion).

Figure 4: Characterization of SFAHFAs U6 to U10, and their MSⁿ analysis (M* represents the most abundant MS² ion).

Figure 5: Amount of SFAHFAs in intestinal contents and plasma (**A**), and Intestinal tissues and liver (**B**) of rats fed normal diet and high fat diet. (n=6 or 5 each group***p<0.05, ** p<0.1)