Research Paper

Comprehensive analysis of fatty acid metabolites produced by gut microbiota using LC-MS/MS-based lipidomics

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Abstract The saturation metabolism of polyunsaturated fatty acids represents a mode of detoxification metabolism by the gut microbiota. It generates a series of functional fatty acids, such as hydroxy fatty acids (e.g., 10-hydroxy-*cis*-12-octadecenoic acid (HYA) from linoleic acid), oxo fatty acids, conjugated fatty acids, and trans fatty acids. In this study, we prepared 45 synthetic standards, including HYA and related fatty acid metabolites, and developed liquid chromatography-tandem mass spectrometry-based targeted lipidomics. This method was successfully applied for the comprehensive analysis of the gut microbiota-dependent production of fatty acid metabolites in mouse feces, plasma, and tissues under different dietary conditions.

Key words: mass spectrometry, bacteria, hydroxy fatty acids, lipidomics

Introduction

Dietary fats are metabolized by host digestive enzymes and gut microbiota. The saturation metabolism of polyunsaturated fatty acids (PUFAs), which is the detoxification metabolism of anaerobic bacteria, produces unique fatty acid metabolites that are not produced by host enzymes[1]. C18 fatty acids, such as linoleic acid (C18:2 n-6) (LA), α-linolenic acid (C18:3 n-3) (ALA), and γ-linolenic acid (C18:3 n-6) (GLA), are metabolized by enzymes, such as CLA-HY, CLA-DH, CLA-DC, and CLA-ER, to generate a series of functional fatty acids, including hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and *trans* fatty acids. For example, 10-hydroxy-*cis*-12-octadecenoic acid (HYA) and 10-oxo-*cis*-12-octadecenoic acid (KetoA) are produced as intermediates when linoleic acid is metabolized to oleic acid. In addition to this metabolic pathway, some lactic acid bacteria hydrate the double bond at the Δ12-position of 18-carbon fatty acids to produce 13-hydroxy fatty acids[2]. A combination of different fatty acid substrates and metabolic enzymes contributes to the production of a series of functional metabolites (Fig. 1). HYA, a LA-derived metabolite, exerts an anti-obesity effect and enhances intestinal barrier function via G-protein-coupled receptor (GPR)40 and GPR120[3,4]. 10-Oxo-*cis*-6,*trans*-11-octadecadienoic acid (γKetoC), a GLA-derived metabolite, suppresses LPS-induced inflammatory cytokine production in monocytes[5]. 13-Hydroxy-*cis*-9,*cis*-15-octadecadienoic acid (αHYD), an ALA-derived metabolite, induces M2 macrophage differentiation from bone marrow-derived cells via GPR40[6]. Therefore, it is important to monitor these metabolites comprehensively in biological systems to explore their significance and therapeutic potential. Liquid chromatography tandem mass spectrometry (LC-MS/MS), particu-

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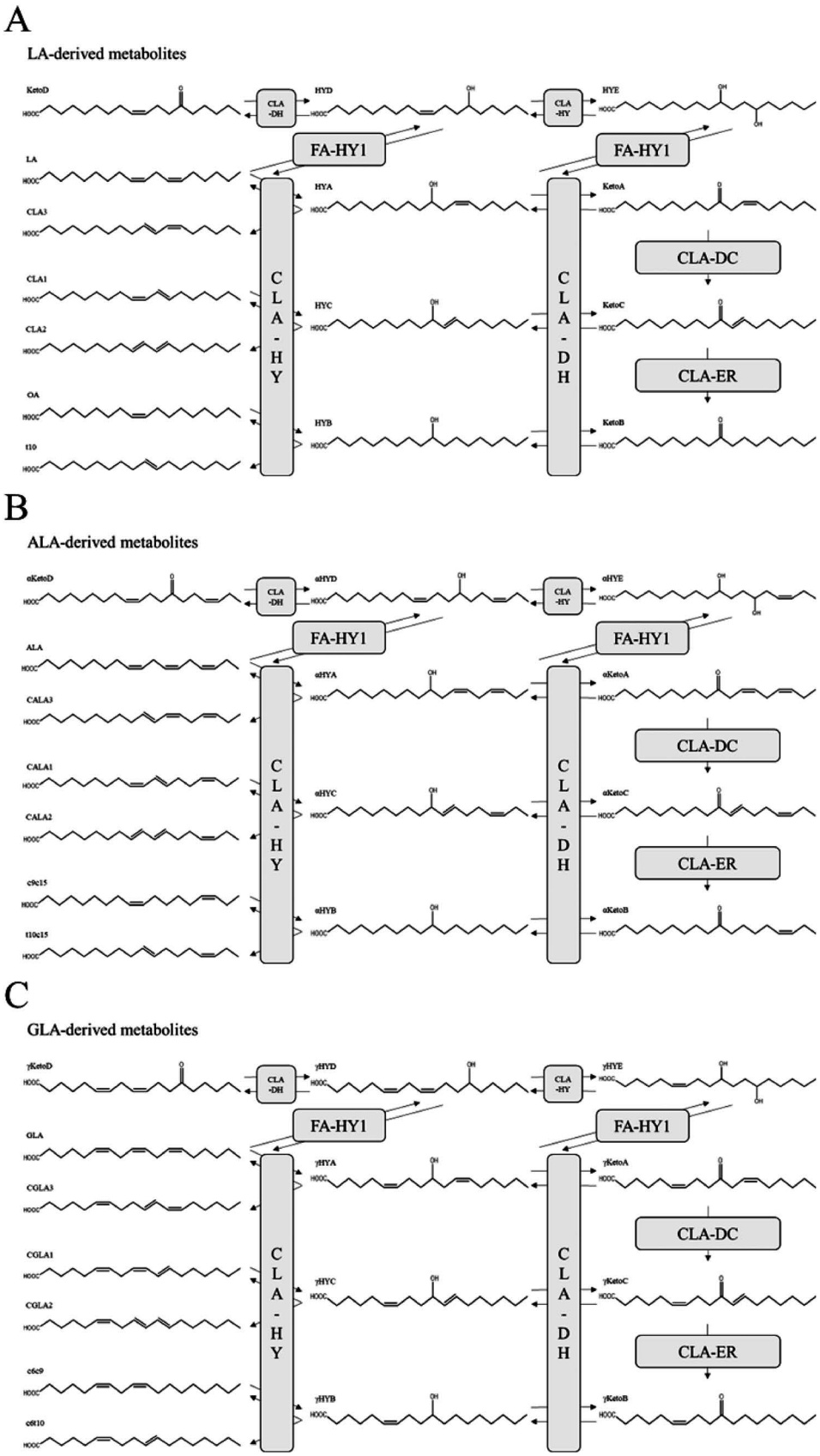

Fig. 1. Saturation metabolism pathway of C18 fatty acid. A. Linoleic acid (LA)-derived metabolites B. α-linolenic acid (ALA)-derived metabolites C. γ-linolenic acid (GLA)-derived metabolites.

Table 1. Classification and formal name of the C18 fatty acid metabolites quantified using LC-MS/MS

Group	Compound	Formal name
LA	LA	*cis*-9,*cis*-12-octadecadienoic acid
	HYA	10-hydroxy-*cis*-12-octadecenoic acid
	HYB	10-hydroxy-octadecanoic acid
	HYC	10-hydroxy-*trans*-11-octadecenoic acid
	HYD	13-hydroxy-*cis*-9-octadecenoic acid
	HYE	10,13-dihydroxy-octadecanoic acid
	KetoA	10-oxo-*cis*-12-octadecenoic acid
	KetoB	10-oxo-octadecanoic acid
	KetoC	10-oxo-*trans*-11-octadecenoic acid
	KetoD	13-oxo-*cis*-9-octadecenoic acid
	CLA1	*cis*-9,*trans*-11-octadecadienoic acid
	CLA2	*trans*-9,*trans*-11-octadecadienoic acid
	CLA3	*trans*-10,*cis*-12-octadecadienoic acid
	OA	*cis*-9-octadecenoic acid
	t10	*trans*-10-octadecenoic acid
ALA	ALA	*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid
	α HYA	10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid
	α HYB	10-hydroxy-*cis*-15-octadecenoic acid
	α HYC	10-hydroxy-*trans*-11,*cis*-15-octadecadienoic acid
	α HYD	13-hydroxy-*cis*-9,*cis*-15-octadecadienoic acid
	α HYE	10,13-dihydroxy-*cis*-15-octadecenoic acid
	α KetoA	10-oxo-*cis*-12,*cis*-15-octadecadienoic acid
	α KetoB	10-oxo-*cis*-15-octadecenoic acid
	α KetoC	10-oxo-*trans*-11,*cis*-15-octadecadienoic acid
	α KetoD	13-oxo-*cis*-9,*cis*-15-octadecadienoic acid
	CALA1	*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid
	CALA2	*trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid
	CALA3	*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid
	c9c15	*cis*-9,*cis*-15-octadecadienoic acid
	t10c15	*trans*-10,*cis*-15-octadecadienoic acid
GLA	GLA	*cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid
	γ HYA	10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid
	γ HYB	10-hydroxy-*cis*-6-octadecenoic acid
	γ HYC	10-hydroxy-*cis*-6,*trans*-11-octadecadienoic acid
	γ HYD	13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid
	γ HYE	10,13-dihydroxy-*cis*-6-octadecenoic acid
	γ KetoA	10-oxo-*cis*-6,*cis*-12-octadecadienoic acid
	γ KetoB	10-oxo-*cis*-6-octadecenoic acid
	γ KetoC	10-oxo-*cis*-6,*trans*-11-octadecadienoic acid
	γ KetoD	13-oxo-*cis*-6,*cis*-9-octadecadienoic acid
	CGLA1	*cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid
	CGLA2	*cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid
	CGLA3	*cis*-6,*trans*-10,*cis*-12-octadecatrienoic acid
	c6c9	*cis*-6,*cis*-9-octadecadienoic acid
	c6t10	*cis*-6,*trans*-10-octadecadienoic acid
IS	EPA-d5	*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic-19,19,20,20,20-d5 acid
	15(*S*)-HETE-d8	15*S*-hydroxy-*cis*-5,*cis*-8,*cis*-11,*trans*-13-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid
	LTB_4-d4	5*S*,12*R*-dihydroxy-*cis*-6,*trans*-8,*trans*-10,*cis*-14-eicosatetraenoic-6,7,14,15-d4 acid

larly triple quadrupole LC-MS/MS, is suitable for targeted analysis to quantitatively measure the levels of lipid metabolites, such as eicosanoids and bile acids[7–9]. We previously demonstrated the targeted analysis of LA- and ALA-derived metabolites including HYA, KetoA and 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid (αKetoA)[3]; however in the present study, we expanded the number of metabolites up to 45 by systemically preparing synthetic standards. Furthermore, we developed a comprehensive analytical method including GLA-derived metabolites and metabolites with different positional isomers (Table 1), and applied it to biological samples, including mouse feces, plasma and tissues, under different dietary conditions.

Material & Method

Chemicals

Synthetic standards, including HYA, 10-hydroxy-octadecanoic acid (HYB), 10-hydroxy-*trans*-11-octadecenoic acid (HYC), KetoA, 10-oxo-octadecanoic acid (KetoB), 10-oxo-*trans*-11-octadecenoic acid (KetoC), 13-hydroxy-*cis*-9-octadecenoic acid (HYD), 13-oxo-*cis*-9-octadecenoic acid (KetoD), 10,13-dihydroxy-octadecanoic acid (HYE), *cis*-9,*trans*-11-octadecadienoic acid (CLA1), *trans*-9,*trans*-11-octadecadienoic acid (CLA2), *trans*-10,*cis*-12-octadecadienoic acid (CLA3), *trans*-10-octadecenoic acid (t10), 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid (αHYA), 10-hydroxy-*cis*-15-octadecenoic acid (αHYB), 10-hydroxy-*trans*-11,*cis*-15-octadecadienoic acid (αHYC), αKetoA, 10-oxo-*cis*-15-octadecenoic acid (αKetoB), 10-oxo-*trans*-11,*cis*-15-octadecadienoic acid (αKetoC), αHYD, 13-oxo-*cis*-9,*cis*-15-octadecadienoic acid (αKetoD), 10,13-dihydroxy-*cis*-15-octadecenoic acid (αHYE), *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA1), *trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA2), *trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid (CALA3), *cis*-9,*cis*-15-octadecadienoic acid (c9c15), *trans*-10,*cis*-15-octadecadienoic acid (t10c15), 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid (γHYA), 10-hydroxy-*cis*-6-octadecenoic acid (γHYB), 10-hydroxy-*cis*-6,*trans*-11-octadecadienoic acid (γHYC), 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid (γKetoA), 10-oxo-*cis*-6-octadecenoic acid (γKetoB), γKetoC, 13-hydroxy-*cis*-6,*cis*-9-octadecadienoic acid (γHYD), 13-oxo-*cis*-6,*cis*-9-octadecadienoic acid (γKetoD), 10,13-dihydroxy-*cis*-6-octadecenoic acid (γHYE), *cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid (CGLA1), *cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid (CGLA2), *cis*-6,*trans*-10,*cis*-12-octadecatrienoic acid (CGLA3), *cis*-6,*cis*-9-octadecadienoic acid (c6c9), and *cis*-6,*trans*-10-octadecadienoic acid (c6t10), were synthesized from OA, LA, ALA, and GLA by lactic acid bacteria-derived enzymes (CLA-HY, CLA-DH, CLA-DC, CLA-ER and FA-HY1) and chemical reactions and purified, as described previously[1,2]. Organic solutions of fatty acid standards and internal standards (IS) used in this study (LA, OA, ALA, GLA, leukotriene B_4 (LTB_4)-d4, 15*S*-hydroxyeicosatetraenoic acid (15(*S*)-HETE)-d8 and eicosapentaenoic acid (EPA)-d5) were obtained from Cayman Chemical (Michigan, USA). LC-MS grade acetonitrile, acetic acid and methanol (all from Fujifilm Wako Pure Chemical, Osaka, Japan) were used for sample preparation and LC-MS/MS measurements. Ultrapure water was obtained using a Milli-Q water purification system (Merck, Darmstadt, Germany). Ampicillin, vancomycin, neomycin, and metronidazole purchased from Fujifilm Wako Pure Chemical, were used to prepare antibiotic-cocktail (AbX) solutions.

LC-MS/MS

C18 fatty acid metabolites were quantified using a Nexera X2 HPLC system (Shimadzu, Kyoto, Japan) coupled with an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu). The best resolutions of chromatographic peaks and separation of analytes were achieved using an Acquity LC BEH C18 (1.0×150 mm, 1.7 μm, Waters, Milford, Massachusetts, USA) analytical column. The mobile phases consisted of 0.1% acetic acid in water (A) and acetonitrile/methanol (4:1, v/v) (B) and were delivered using the following gradient elution program: 27% B for 5.0 min, 27−70% B from 5.0 to 15.0 min, 70−80% B from 15.0 to 25.0 min, hold 80% B from 25.0 to 33.0 min, 80−100% B from 33.0 to 35.0 min, hold 100% B from 35.0 to 45.0 min, and hold 27% B for 5.0 min for column equilibration with flow rates of 50 μL/min (0−30 min), 80 μL/min (30−33 min),100 μL/min (33−45 min), and 50 μL/min (45−50 min). Data acquisition was carried out in multiple reaction monitoring (MRM) mode with negative electrospray ionization for all C18 fatty acid metabolites and deuterated internal standards. The ion transitions (Q1/Q3) selected for quantification and the collision energy (CE) are listed in Table 2. The desolvation, interface, heatblock, desolvation line and source temperatures were set at 526, 300, 400, 250 and 150°C, respectively. MS detection was performed in negative ionization mode, and the source capillary voltage was set to 2 kV.

Table 2. Mass spectrometry parameters of C18 fatty acid metabolites

Group	Name	RT	Precursor ion	Product ion	CE	Q1 pre bias	Q3 pre bias
LA	LA	32.3	279.2	279.2	25	11	35
	HYA	23.1	297.2	185.2	23	15	21
	HYB	25.7	299.2	141.1	26	19	12
	HYC	23.4	297.2	185.2	23	19	19
	HYD	22.7	297.2	197.2	22	11	11
	HYE	18.2	315.3	185.2	27	16	18
	HYE (Reference)	18.2	315.3	197.2	26	13	11
	KetoA	23.8	295.2	139.1	23	11	12
	KetoB	26.6	297.2	139.1	23	11	12
	KetoC	24.3	295.2	139.1	22	12	12
	KetoD	23.6	295.2	179.2	23	19	22
	CLA1	32.8	279.2	261.2	25	11	17
	CLA2	33.5	279.2	279.2	25	18	18
	CLA3	32.7	279.2	261.2	25	19	11
	OA	35.4	281.2	281.2	30	11	11
	t10	35.9	281.2	281.2	30	19	17
ALA	ALA	29.1	277.2	277.2	11	20	17
	αHYA	21.0	295.2	185.2	19	11	10
	αHYB	22.6	297.2	141.1	25	19	13
	αHYC	21.0	295.2	185.2	21	12	18
	αHYD	21.1	295.2	225.2	21	12	20
	αHYE	17.1	313.2	243.2	22	12	15
	αHYE (Reference)	17.1	313.2	185.2	24	12	17
	αKetoA	21.7	293.2	139.1	22	12	13
	αKetoB	23.4	295.2	155.1	23	12	14
	αKetoC	21.8	293.2	139.1	22	12	12
	αKetoD	21.8	293.2	205.2	20	11	12
	CALA1	29.3	277.2	277.2	12	18	18
	CALA2	30.1	277.2	277.2	12	11	11
	CALA3	29.5	277.2	277.2	12	11	17
	c9c15	32.2	279.2	279.2	25	18	11
	t10c15	32.9	279.2	279.2	25	19	12
GLA	GLA	29.6	277.2	277.2	12	11	17
	γHYA	21.9	295.2	183.2	21	20	19
	γHYB	24.2	297.1	155.1	23	11	13
	γHYC	22.1	295.2	183.2	30	20	17
	γHYD	21.3	295.2	195.2	19	11	19
	γHYE	17.7	313.2	155.2	25	12	14
	γHYE (Reference)	17.7	313.2	195.2	25	12	20
	γKetoA	22.6	293.2	137.1	23	19	13
	γKetoB	24.8	295.2	155.1	22	11	14
	γKetoC	22.8	293.2	137.1	22	11	12
	γKetoD	21.9	293.2	195.1	22	11	11
	CGLA1	30.1	277.2	277.2	12	11	11
	CGLA2	31.0	277.2	277.2	12	11	18
	CGLA3	29.8	277.2	277.2	11	11	17
	c6c9	33.0	279.2	261.2	25	11	18
	c6t10	33.4	279.2	279.2	25	11	18
IS	15-HETE-d8	21.6	306.2	262.2	16	24	12
	LTB_4-d4	18.5	327.2	226.2	11	17	28
	EPA-d5	28.5	339.2	197.1	14	22	14

RT: retention time, CE: collision energy, Reference: parameters set as qualitative ions to aid in compound identification.

Lipid extraction from biological samples

Frozen biological samples (10−100 mg) excluding plasma were weighted and placed in a 3 mL reinforced homogenization tube. A metal corn was placed in the tube and placed it in liquid nitrogen for 1 min. The samples were homogenized (2,500 rpm, 15 s ×2, pause time, 5 s) by a multiple bead shocker (Yasuikikai, Osaka, Japan). Homogenized samples were added with methanol to 50 or 100 mg/mL, and 20 μL of plasma was added with methanol to 400 μL before vortex. The metal cone was removed and stored at −30°C for at o/n. After centrifuging (2,500 rpm, 10 min, 4°C) the tube, sample were transfered 100 μL of the supernatant (300 μL for plasma) to a 1.5 mL tube and scaled-up with methanol to 300 μL. Then, 10 μL of IS and an equal volume of water as in total methanol were added. The solid phase extraction was performed using Monospin AX-18 (GLScience, Tokyo, Japan). Methanol was added 300 μL to the set column and centrifuged (2,500 rpm, 1 min, RT). The same process was carried out with water, and the prepared sample solution was added and centrifuged (2,500 rpm, 2 min, RT); washed with 300 μL volume of water and 50% methanol, and 75 or 100 μL volume eluted with eluent (acetic acid : methanol : water=2 : 90 : 8).

Method validation

Quantification curves were prepared from the standard mixture at ten different concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1,000 ng/mL) using methanol as the diluent. Accuracy and precision were investigated using quality control (QC) samples prepared from the standard mixture at three concentrations of 2, 20, and 200 ng/mL: namely, low QC (LQC), medium QC (MQC), and high QC (HQC). All QC samples were pretreated and analyzed, with three replicates for each concentration. Accuracy was calculated as the average percentage difference between the measured and real C18 fatty acid metabolite concentrations. Precision was calculated as the relative standard deviation (RSD) of the measured C18 fatty acid metabolite concentrations in three replicates of the QC samples.

Mice

Male wild-type C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan) were maintained in a specific pathogen-free environment. Ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin (1 g/L), and metronidazole (1 g/L) were administrated in drinking water were administrated for 3 weeks from 7 weeks of age. Mice were fed normal chow (CE-2; CLEA, Tokyo, Japan)[10]. Diets composed of chemically defined materials with 4% each dietary oil (safflower oil [Saff], linseed oil [Lin], evening primrose oil [Eve]) (Oriental Yeast, Tokyo, Japan) were supplied for 1 month from 8 weeks of age[11]. Five mice were used in each experimental group. All animal experiments were approved by the Animal Care and Use Committee of Keio University.

Data analysis

Data were analyzed using LabSolutions INSIGHT software (Shimadzu, Kyoto, Japan). The chromatogram peaks were manually curated. Data are shown as mean±standard error of the mean (SEM).

Result

Optimization of LC-MS/MS condition

For each fatty acid metabolite, the fragment ion with sufficient intensity and specificity was optimized from the multiple fragment ions generated by collision-induced dissociation. The fragmentation patterns are shown in Fig. S1. The fragment ion at *m/z* 185.2, generated by cleavage near the hydroxy group at position 10 in HYA, was selected as a diagnostic ion. Similarly, a fragment ion at *m/z* 139.1 was selected for KetoA, and those at *m/z* 185.2 and 197.2 were selected as diagnostic ions for HYE. LA does not cause specific fragmentation, hence, the product ion was set to the same parameters at *m/z* 279.2 as the precursor ion. The collision energy, Q1 pre bias, and Q3 pre bias for each compound in the measurement were optimized (Table 2).

LC conditions were implemented based on previous studies. Previous LC conditions used acetonitrile and 2-propanol as organic solvents in the mobile phase and were optimized only for LA and ALA metabolites[3]; when GLA metabolites and metabolites with a modified group at position 13 were also measured, some compounds could not be separated by the previous method. Therefore, we developed an optimized method by changing the mobile phase from 2-propanol to methanol to reduce the elution power and by improving the separation performance by further examining the gradient conditions.

The MRM chromatograms of the LA-, ALA- and GLA-derived metabolites are shown in Fig. 2A, S2A, and B. In total, 31 of the 45 compounds displayed well-separated peaks. Most LA-derived metabolites showed a good separation, but some metabolites with the same MRM

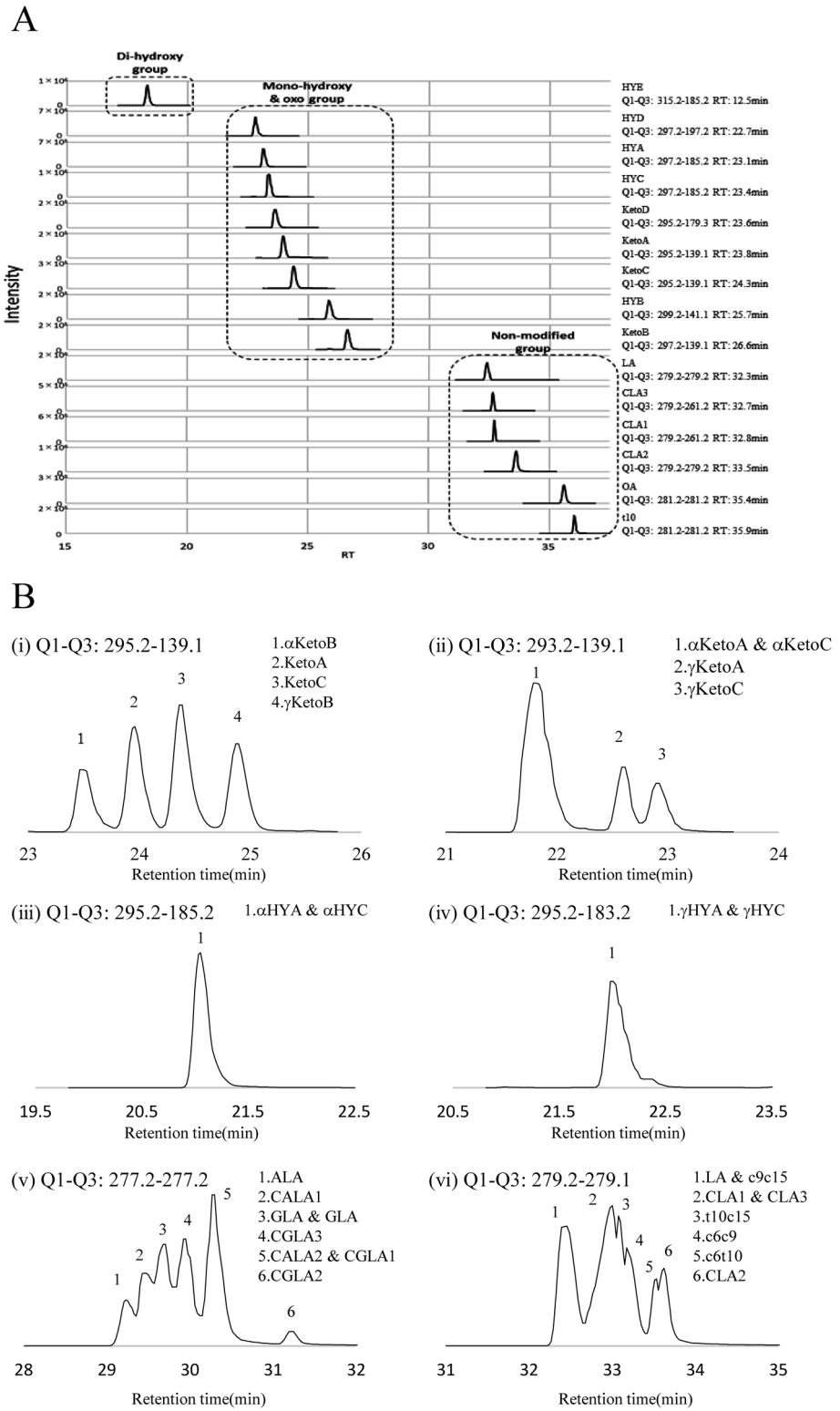

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of C18 fatty acid metabolites. A. Chromatograms of all LA-derived metabolites. B. Chromatograms of C18 fatty acid metabolites detected with the same MS parameters.
(i) Q1−Q3: 295.2−139.1, (ii) Q1−Q3: 293.2−139.1, (iii) Q1−Q3: 295.2−185.2, (iv) Q1−Q3: 295.2−183.2, (v) Q1−Q3: 277.2−277.2, (vi) Q1−Q3: 279.2−279.2.

parameters were not well separated (Fig. 2B). For example, LA-derived KetoA, KetoC, ALA-derived αKetoB and GLA-derived γKetoB were well-separated, whereas ALA-derived αKetoA, αKetoC, GLA-derived γKetoA and γKetoC were not separated under the defined LC conditions (Fig. 2B-i, ii). In addition, fatty acids with a hydroxy group at position 10 and two double bond, such as αHYA, αHYC, γHYA and γHYC were not separated (Fig. 2B-iii, iv). There were eight compounds each of C18:2 and C18:3 fatty acids, with LA and c9c15, CLA1 and CLA3, CALA2 and CGLA1, and CALA3 and GLA being inseparable combinations (Fig-2B-v, vi).

Quantification performance and method validation

The quantification performance, including sensitivity, linearity, accuracy, and precision, of C18 fatty acid metabolites was investigated using synthetic standards (Table 3). These metabolites showed excellent calibration linearity with good correlation coefficients ($r>0.99$) (Fig. S3). The lower limit of detection was between 0.03 and 10 pg/μL for each C18 fatty acid metabolite. Deuterium-labeled compounds such as EPA-d5, 15-HETE-d8 and LTB_4-d4 were used as internal standards to calibrate the deviation introduced by sample pretreatment processes and instrument fluctuation. The accuracy and precision of the method were investigated using QC samples of different concentrations. The accuracies of low-, medium-, and high- QC samples were between 80% and 118.7%, and the RSDs of precision were less than 15% for all metabolites.

Quantification of C18 fatty acid metabolites in feces of antibiotics-treated mice

LC-MS/MS-based targeted lipidomics was applied to the biological samples. HYA and related fatty acids including LA-, ALA- and GLA-derived metabolites in mice feces were successfully monitored (Fig. 3). By comparing these with the samples from antibiotics-treated mice, we could illuminate a series of fatty acid metabolites, including HYA and related metabolites uniquely produced by commensal microbiota.

Profiling of fatty acid metabolites under different dietary conditions

We also measured the levels of fatty acid metabolites in mice fed with diets containing Saff, which is a good source of the substrate LA and free of ALA and GLA, Lin, which is a good source of ALA, or Eve, which specifically contains GLA. In addition, we measured metabolite profiles in feces as well as in the small intestine (absorptive organs), blood (plasma) transporting lipids throughout the body, and kidney and liver (metabolic organs), to confirm that metabolites produced by the gut bacteria could also be detected in the body. The fatty acid composition of each diet group is summarized in Fig. 4A. Quantitative results of these metabolites are provided in Tables 4, S1, and S2, and are summarized as a heat map by Z-score (Fig. 4B). The lipidomic profile in feces reflected different dietary conditions. In the feces of Lin-fed mice, ALA-derived metabolites (αHYA, αHYC, αHYD, and αHYE) were predominant, whereas that of Eve-fed mice contained enhanced levels of GLA- and LA-derived metabolites (γHYA, γHYB, γHYC, γKetoB, CGLA1, CGLA2, c6c9, c6t10, KetoA, KetoC, CLA1, and CLA3). In contrast, LA-derived metabolites with a modified group at position 13 (HYD, HYE, KetoD) were frequently detected in Saff-fed mice feces. The lipidomic profile in SI was similar to that in feces; however, LA-derived metabolites KetoA and KetoC, ALA-derived metabolite αHYE, and other compounds present in feces were not detected in SI. Oxo fatty acids, except for KetoB, KetoD, and dihydroxy fatty acids, were not detected in the plasma, liver, and kidney. The ALA-derived metabolites (e.g., αHYA, αHYC, and αHYD) and GLA-derived metabolites (e.g., γHYA and γHYC) detected in these tissues showed characteristic profiles, each dependent on diet. In contrast, LA-derived metabolites showed different profiles in each tissue and were largely unaffected by diet.

Discussion

In this study, comprehensive profiling and quantification of fatty acid metabolites were performed using LC-MS/MS. We prepared a series of synthetic standards to optimize the quantitative targeted analysis of these metabolites. The separation performance was also improved by examining the mobile phase and gradient conditions.

Several combinations of compounds that could not be separated even with the method developed in this study, and their quantitative analysis is an issue that needs to be improved in the future. In addition to the new method developed in this study, it is a possible that all compounds can be quantified by combining this method with LC conditions specific for the separation of target compounds or other analytical method, such as gas chromatography or

Table 3. Quantification performance in profiling of C18 fatty acid metabolites

Group	Name	Linearity (R^2)	Dynamic range (pg/μL)	LOD (pg/μL)	Accuracy (%)			Precision (%)		
					LQC	MQC	HQC	LQC	MQC	HQC
LA	LA	0.99	1−1000	0.3	114.7	81.6	91.9	14.8	6.7	0.9
	HYA	0.99	0.1−1000	0.03	104.6	101.9	94.1	4.5	4.5	3.8
	HYB	0.99	1−1000	0.3	119.6	116.6	118.3	2.5	3.6	6.5
	HYC	0.99	0.3−1000	0.1	105.7	109.6	102.6	6.3	3.6	3.2
	HYD	0.99	1−1000	0.3	101.1	96.6	92.2	1.6	2.9	2.9
	HYE	0.99	1−1000	0.3	118.4	95.8	97.0	7.0	4.8	1.7
	KetoA	0.99	1−1000	0.3	84.5	88.3	101.4	13.3	6.8	4.6
	KetoB	0.99	1−1000	0.3	114.3	94.5	97.3	7.3	6.7	5.4
	KetoC	0.99	1−1000	0.3	106.0	103.8	100.8	5.0	5.3	2.1
	KetoD	0.99	1−1000	0.3	111.6	112.8	101.5	7.3	5.8	1.1
	CLA1	0.99	30−1000	10	N.D.	119.8	117.9	—	8.2	4.8
	CLA2	0.99	1−1000	0.3	95.8	80.1	80.8	15.5	3.9	5.2
	CLA3	0.99	30−1000	10	N.D.	90.9	100.2	—	11.1	2.8
	OA	0.99	10−1000	3	116.2	116.9	93.9	15.0	2.8	4.1
	t10	0.99	3−1000	1	99.0	105.5	87.8	8.3	7.5	5.1
ALA	ALA	0.99	1−1000	0.3	94.0	80.5	83.1	10.8	12.5	6.6
	αHYA	0.99	0.3−1000	0.1	104.8	101.5	99.4	9.0	1.9	5.3
	αHYB	0.99	1−1000	0.3	115.8	104.5	113.1	13.8	6.2	3.5
	αHYC	0.99	0.3.−1000	0.1	110.5	103.3	105.3	1.5	5.3	2.5
	αHYD	0.99	0.1−1000	0.03	100.9	101.8	104.2	1.0	5.3	3.0
	αHYE	0.99	0.1−1000	0.03	107.7	114.0	111.7	0.6	1.2	0.9
	αKetoA	0.99	1−1000	0.3	97.5	109.5	102.3	19.1	5.8	0.6
	αKetoB	0.99	1−1000	0.3	98.4	108.7	96.2	2.8	3.5	0.6
	αKetoC	0.99	1−1000	0.3	116.5	116.6	112.5	1.6	4.6	5.5
	αKetoD	0.99	3−1000	1	98.1	100.7	95.6	1.3	6.3	3.6
	CALA1	0.99	3−1000	1	83.2	81.2	80.4	5.2	5.8	8.8
	CALA2	0.99	1−1000	0.3	81.3	80.3	81.1	0.2	8.2	5.0
	CALA3	0.99	1−1000	0.3	88.2	82.4	80.0	6.4	10.8	3.8
	c9c15	0.99	1−1000	0.3	117.3	109.0	91.7	6.3	4.6	5.5
	t10c15	0.99	1−1000	0.3	118.7	108.9	103.3	6.7	4.1	3.1
GLA	GLA	0.99	1−1000	0.3	89.0	80.8	81.1	2.2	3.1	5.4
	γHYA	0.99	0.3−1000	0.1	97.1	89.6	100.4	5.0	6.9	6.5
	γHYB	0.99	0.3−1000	0.1	101.4	101.2	98.0	6.3	2.2	4.1
	γHYC	0.99	3−1000	1	102.2	93.6	91.8	13.1	9.7	4.4
	γHYD	0.99	0.3−1000	0.1	95.4	89.8	94.7	5.4	4.7	1.6
	γHYE	0.99	0.3−1000	0.1	103.7	105.9	112.1	4.1	5.6	2.6
	γKetoA	0.99	3−1000	1	107.5	112.9	118.0	2.8	3.2	6.5
	γKetoB	0.99	0.3−1000	0.1	95.4	96.5	89.3	3.7	5.5	2.2
	γKetoC	0.99	1−1000	0.3	82.4	80.9	81.5	2.9	8.4	7.4
	γKetoD	0.99	1−1000	0.3	109.6	101.8	95.4	3.5	3.8	3.2
	CGLA1	0.99	1−1000	0.3	88.2	81.6	80.7	4.8	2.1	0.8
	CGLA2	0.99	3−1000	1	95.7	80.6	80.1	14.9	7.0	6.6
	CGLA3	0.99	1−1000	0.3	113.5	104.3	110.0	1.3	3.7	2.5
	c6c9	0.99	30−1000	10	N.D.	88.7	83.5	—	2.1	3.0
	c6t10	0.99	3−1000	1	90.4	85.3	81.1	2.0	7.4	6.5

LOD: limit of direction, LOC: low quality control, MQC: medium quality control, HQC: high quality control. The concentrations of the LQC, MQC, and HQC samples were 5, 20, and 200 ng/mL, respectively. N.D.: not detected because concentrations were lower than the limit of detection.

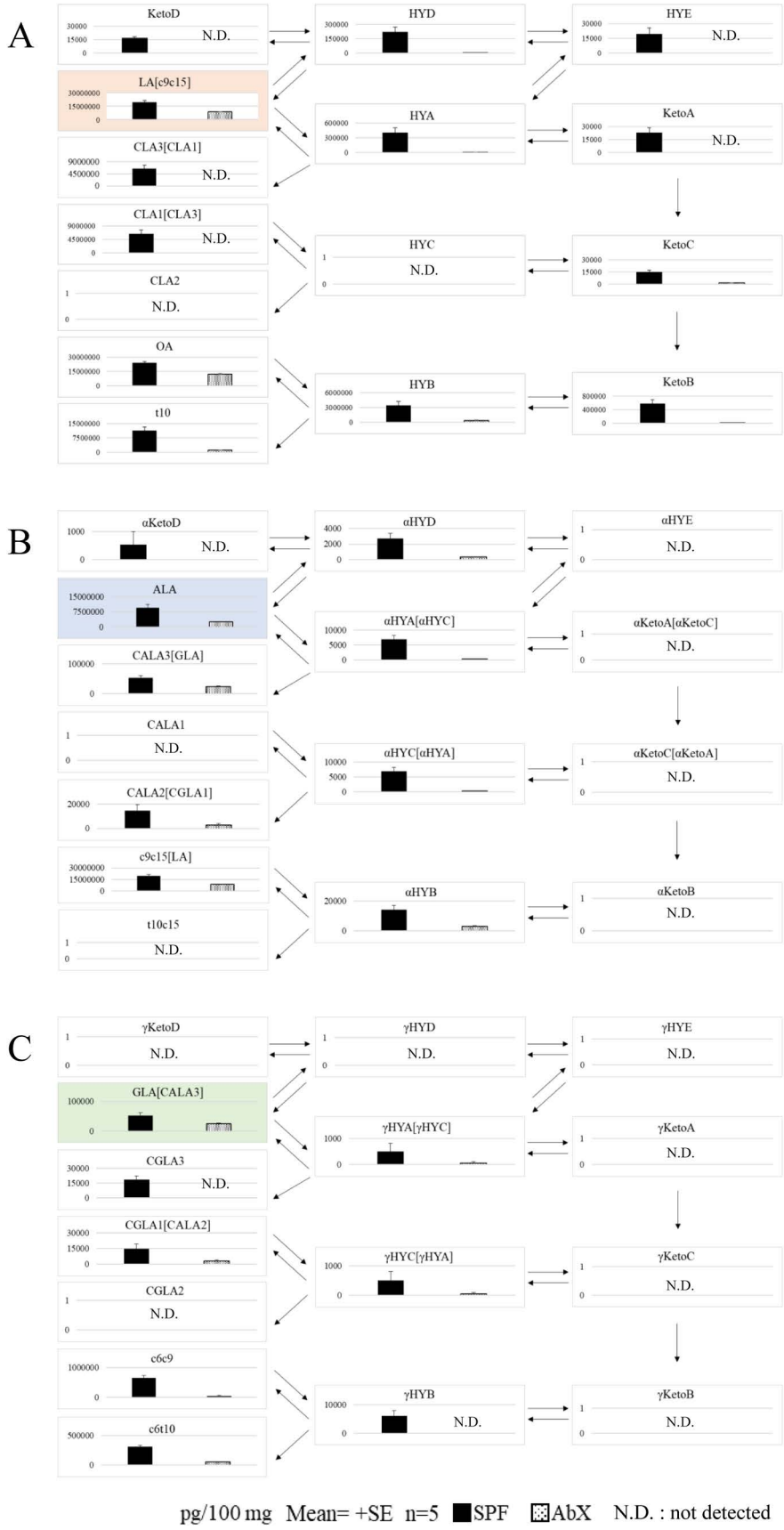

Fig. 3. Detection and quantitative analyses of polyunsaturated fatty acid saturation metabolism intermediates in feces of SPF or AbX-treated mice.

A. in LA-derived metabolism pathway. B. in ALA-derived metabolism pathway. C. in GLA-derived metabolism pathway. Data are presented as means+standard error of the mean (SEM) (n=5).

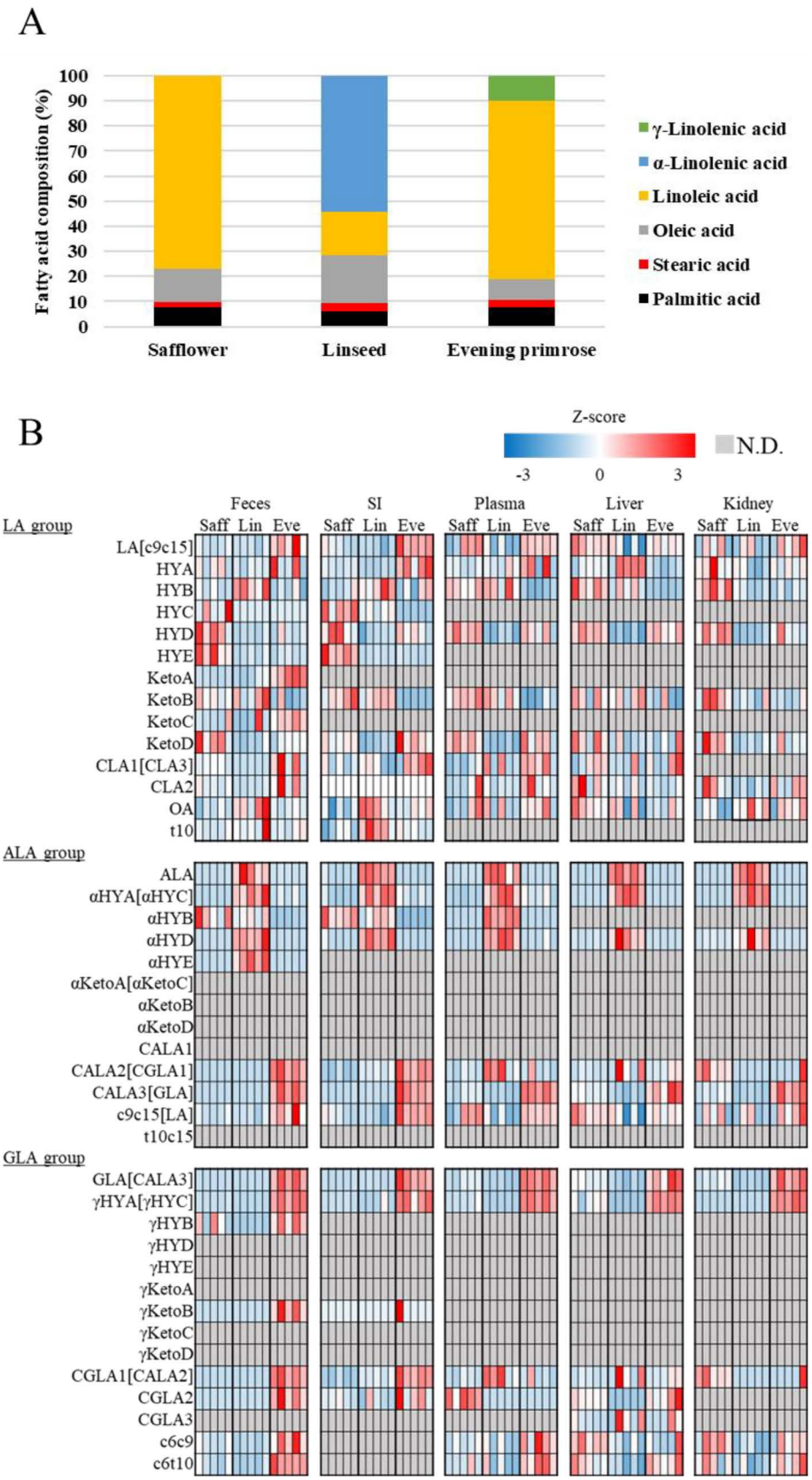

Fig. 4. C18 fatty acid metabolite profile in Lin-mice and Saff-mice and Eve-mice. A.
Fatty acid composition of each oil. B. Heat map of C18 fatty acid metabolites. Heat map of C18 fatty acid metabolites detected in the intestine following Z-score transformation of the raw data. Hierarchical clustering was performed on both animals and C18 fatty acid metabolites. Red color indicates Z-scores>0, blue color indicates Z-scores<0, white color indicates Z-scores=0. Gray color indicates not detected (N.D.).

Table 4. Concentration of C18 fatty acid metabolites (ng/100 mg) in feces of Saff-, Lin-, and Eve-mice

Group	Compound	Feces		
		Safflower	Linseed	Evening primrose
LA	LA [c9c15]	1969.54±693.32	1530.46±685.34	6439.68±3376.55
	HYA	12.57±5.27	9.01±1.92	18.14±13.58
	HYB	231.06±58.45	517.2±139.04	180.04±105.93
	HYC	3.35±4.74	N.D.	N.D.
	HYD	126.27±59.11	20.54±11.49	38.71±30.05
	HYE	17.21±8.02	3.27±1.77	3.43±1.44
	KetoA	1.86±0.33	2.1±1.5	10.34±2.61
	KetoB	135.41±19.32	159.32±49.07	95.48±38.93
	KetoC	6.22±4.37	6.47±8.88	12.45±2.81
	KetoD	219.57±94.57	54.01±35.79	102.92±30.96
	CLA1 [CLA3]	574.7±243.39	75.27±22.02	1875.56±1621.83
	CLA2	52.95±28.05	16.38±11	142.82±131.15
	OA	5835.1±973.01	10302.02±3075.43	6769.36±637.15
	t10	267.5±115.37	662.49±690.82	346.2±160.99
ALA	ALA	49.66±15.85	1451.39±709.8	99.31±24.57
	αHYA [αHYC]	0.2±0.07	4.55±1.74	0.67±0.47
	αHYB	11.6±6.58	6.83±2.7	0.57±0.72
	αHYD	0.44±0.12	19.01±6.84	0.16±0.05
	αHYE	N.D.	0.42±0.15	N.D.
	αKetoA [αKetoC]	N.D.	N.D.	N.D.
	αKetoB	N.D.	N.D.	N.D.
	αKetoD	N.D.	N.D.	N.D.
	CALA1	N.D.	N.D.	N.D.
	CALA2 [CGLA1]	29.33±11.68	30.25±14.18	1575.47±381.15
	CALA3 [GLA]	64.44±21.66	63.93±29.77	2554.45±689.82
	c9c15 [LA]	1969.54±693.32	1530.46±685.34	6439.68±3376.55
	t10c15	N.D.	N.D.	N.D.
GLA	GLA [CALA3]	64.44±21.66	63.93±29.77	2554.45±689.82
	γHYA [γHYC]	0.57±0.31	N.D.	9.05±1.64
	γHYB	1.57±1.47	0.02±0.03	2.95±1.05
	γHYD	N.D.	N.D.	N.D.
	γKetoA	N.D.	N.D.	N.D.
	γKetoB	N.D.	N.D.	0.43±0.21
	γKetoC	N.D.	N.D.	N.D.
	γKetoD	N.D.	N.D.	N.D.
	γHYE	N.D.	N.D.	N.D.
	CGLA1 [CALA2]	29.33±11.68	30.25±14.18	1575.47±381.15
	CGLA2	N.D.	N.D.	93.89±46.07
	CGLA3	N.D.	N.D.	N.D.
	c6c9	80.84±37.26	32.68±18.1	249.85±114.51
	c6t10	90.82±41.83	50.08±28	286.09±46.37

Data are expressed as the mean±SE, n=5. N.D.: not detected because concentrations were lower than the limit of detection.
[ ]: not separated metabolite.

supercritical fluid chromatography, which have different separation characteristics from LC. The developed method was applied to mouse feces, where we successfully detected a series of HYA and related fatty acid metabolites produced by the gut microbiota. The estimated concentration of HYA and KetoA in mouse feces was 0.4 and 0.06 μM, respectively. As these metabolites are bioactive in the micromolar range[3-6,12-14], the established method using LC-MS/MS appears to be sensitive enough to monitor endogenous levels of HYA and related fatty acid metabolites in biological systems.

Additionally, we applied this method to mouse tissues under different dietary conditions. Dietary LA, ALA, and GLA were metabolized by the gut microbiota, and the metabolite levels showed a good correlation with the type of dietary oils. In contrast, fatty acid metabolites with a modified group at position 13 in Saff-fed mice showed a substrate amount-independent profile, suggesting that differences in intestinal microbiota are influential. The attribution of metabolites was inferred from the different type of dietary oil ingested. The supply of these metabolites to the body is likely dependent on their production by gut microbiota and presence in food, as in the case that the consumption of enzymatically modified arabinoxylan increases the amount of intestinal bacteria-derived short-chain fatty acids in feces, and the profile is reflected in plasma[15]. The n-3 and n-6PUFA balance is an important element in nutritional research, and HYA and related fatty acid metabolites produced by gut microbiota may represent a new element worthy of attention.

In conclusion, an LC-MS/MS method was developed for the comprehensive profiling and quantification of HYA and related metabolites produced by the gut microbiota. This optimized method enabled the simultaneous monitoring of metabolites from biological samples. This analytical method will be improved to aid future studies to elucidate the biological significance of bacteria-derived fatty acid metabolites in controlling host health and diseases.

Author Contribution

K. Tsuji, and MA designed the research; K. Tsuji performed most of the experiments, analyzed the data and wrote the manuscript. WS, SK and JO synthesized the compounds. All authors have read and approved the manuscript.

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Conflict of Interest

Kowa Tsuji is an employee of Noster Inc. and is engaged in metabolome analysis services.

Reference

1) Kishino S, Takeuchi M, Park SB, Hirata A, Kitamura N, et al: Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. *Proc Nat Acad Sci USA* 110: 17808−17813, 2013.
2) Hirata A, Kishino S, Park SB, Takeuchi M, Kitamura N, Ogawa J: A novel unsaturated fatty acid hydratase toward C16 to C22 fatty acids from *Lactobacillus acidophilus*. *J Lipid Res*, 56: 1340−1350, 2015.
3) Miyamoto J, Igarashi M, Watanabe K, Karaki S, Mukouyama H, et al: Gut microbiota confers host resistance to obesity by metabolizing dietary polyunsaturated fatty acids. *Nat Commun*, 10: 1−15, 2019.
4) Miyamoto J, Mizukure T, Park SB, Kishino S, Kimura I, et al: A gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, ameliorates intestinal epithelial barrier impairment partially via GPR40-MEK-ERK pathway. *Journal of Biological Chemistry*, 290: 2902−2918, 2015.
5) Hagiwara S, Nagata K, Kasakura K, Sakata F, Kishino S, et al: The anti-inflammatory effect of the gut lactic acid bacteria-generated metabolite 10-oxo-*cis*-6,*trans*-11-octadecadienoic acid on monocytes. *Biochem Biophy Res Commun*, 530: 342−347, 2020.
6) Ohue-Kitano R, Yasuoka Y, Goto T, Kitamura N, Park SB, et al: α-Linolenic acid-derived metabolites from gut lactic acid bacteria induce differentiation of anti-inflammatory M2 macrophages through G protein-coupled receptor 40. *FASEB J*, 32: 304−318, 2018.
7) le Faouder P, Baillif V, Spreadbury I, Motta JP, Rousset P, et al: LC-MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. *J Chromatog B*, *Anal Technol Biomed Life Sci*, 932: 123−133, 2013.
8) Wegner K, Just S, Gau L, Mueller H, Gérard P, et al: Rapid analysis of bile acids in different biological matrices us-

ing LC-ESI-MS/MS for the investigation of bile acid transformation by mammalian gut bacteria. *Anal Bioanal Chem*, 409: 1231−1245, 2017.

9) Dasilva G, Muñoz S, Lois S, Medina I: Non-targeted LC-MS/MS assay for screening over 100 lipid mediators from ARA, EPA, and DHA in biological samples based on mass spectral fragmentations. *Molecules* 24: 2276, 2019.

10) Yasuda S, Okahashi N, Tsugawa H, Ogata Y, Ikeda K, et al: Elucidation of gut microbiota-associated lipids using LC-MS/MS and 16S rRNA sequence analyses. *iScience*, 23: 101841, 2020.

11) Kunisawa J, Arita M, Hayasaka T, Harada T, Iwamoto R, et al: Dietary $\omega 3$ fatty acid exerts anti-allergic effect through the conversion to 17,18-epoxyeicosatetraenoic acid in the gut. *Sci Rep*, 5: 1−8, 2015.

12) Sofyana NT, Zheng J, Manabe Y, Yamamoto Y, Kishino S, et al: Gut microbial fatty acid metabolites (KetoA and KetoC) affect the progression of nonalcoholic steatohepatitis and reverse cholesterol transport metabolism in mouse model. *Lipids*, 55: 151−162, 2020.

13) Goto T, Kim YI, Furuzono T, Takahashi N, Yamakuni K, et al: 10-oxo-12(Z)-octadecenoic acid, a linoleic acid metabolite produced by gut lactic acid bacteria, potently activates PPARγ and stimulates adipogenesis. *Biochem Biophy Res Commun*, 459: 597−603, 2015.

14) Sulijaya B, Takahashi N, Yamada M, Yokoji M, Sato K, et al: The anti-inflammatory effect of 10-oxo-*trans*-11-octadecenoic acid (KetoC) on RAW 264.7 cells stimulated with *Porphyromonas gingivalis* lipopolysaccharide. *J Period Res*, 53: 777−784, 2018.

15) Nielsen TS, Jensen B, Purup S, Jackson S, Saarinen M, et al: A search for synbiotics: Effects of enzymatically modified arabinoxylan and *Butyrivibrio fibrisolvens* on short-chain fatty acids in the cecum content and plasma of rats. *Food Func*, 7: 1839−1848, 2016.