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Research article

Japanese mushroom consumption alters the lipid metabolomic profile of high-fat diet-fed mice

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ABSTRACT

Mushrooms are familiar ingredients in Japanese cuisine and large numbers are consumed in Japan. Recently, we reported that the consumption of Japanese mushrooms suppressed the accumulation of visceral fat. The purpose of this study was to examine the alteration of lipid metabolism by Japanese mushrooms consumption in high-fat diet (HFD) mice. Multivariate analysis of serum, liver, adipose tissue, cecal contents, large intestinal and fecal lipids showed differing compositions in the mice that had consumed HFD or HFD supplemented with 3% freeze-dried mushroom mixture (HFMD). There were higher concentrations of diacylglycerol in the adipose tissue, non-esterified fatty acids in the serum, and triacylglycerol in the feces of the HFMD group. These results suggest that mushroom consumption promotes the degradation of lipids in visceral fat and limits the absorption of food lipids. Moreover, the HFMD group demonstrated higher concentrations of phospholipids, some of which contained odd-chain fatty acids. Thus, we speculated that the alteration of lipid metabolism in mice such that mushroom consumption prevent obesity progression, as demonstrated by metabolomic analysis.

1. Introduction

Obesity is a risk factor for many diseases, including cardiovascular disease, type 2 diabetes, cancer, and depression (Dandona et al., 2005; Faith et al., 2002; Hursting et al., 2012). The numbers of obese men and women have increased rapidly worldwide since the 1970s, and many researchers have investigated means of resolving this risk factor (NCD-Risk, 2016). To prevent or reduce obesity, the imbalance between energy intake and expenditure must be ameliorated using dietary and/or lifestyle interventions (Hofbauer, 2002). Japan is known to have fewer obese people than Western countries, and Japanese cuisine is considered to be one of the explanations for this difference (Iwagaki et al., 2017; T Tsuduki et al., 2008). A distinctive feature of Japanese cuisine is the predominant use of ingredients such as vegetables, fish, soy beans, and mushrooms. In particular, various types of mushrooms are often used in Japanese cuisine, and about 16 g of mushrooms are consumed per person each day in Japan ("National Health and Nutrition Survey Report, 2017"). Mushrooms contain many useful nutrients, such as dietary fiber, vitamin B_1, vitamin B_2, vitamin B_3 (niacin), vitamin B_6, vitamin B_9 (folic acid), and vitamin D (Valverde et al., 2015). Furthermore, they have been reported to have anti-obesity (Handayani et al., 2011; Iuchi et al., 2015; Mizutani et al., 2010; Yeh et al., 2014), immunomodulatory (Vetvicka and Vetvickova, 2014), anti-tumor (Masuda et al., 2013), anti-atherosclerotic (Mori et al., 2008), and anti-diabetic effects (Hong et al., 2007).

Our previous study showed that the consumption of a mixture of five types of Japanese mushrooms (*Flammulina velutipes*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Grifola frondosa*, and *Pleurotus eryngii*) suppressed fat accumulation by inhibiting fatty acid synthesis and promoting lipolysis in the perinephric adipose tissue of mice (Shimizu et al., 2018). The weight of perinephric adipose tissue is significantly reduced during starvation compared to other adipose tissues (Hegarty and Kim, 1981; Suzuki and Koyanagi, 1968). Therefore, we hypothesized that perinephric adipose tissue might be more affected than other adipose tissue due to the inhibition of lipid absorption by mushrooms. In addition, it caused the proliferation of short-chain fatty acid (SCFA)- and some lactic acid-producing bacteria, possibly because of the dietary fiber content of the mushrooms. Moreover, this mushroom consumption increased the activity of hormone-sensitive lipase (HSL), which regulates lipolysis in adipocytes, and suppressed the expression of sterol regulatory element-binding factor 1 c (SREBP-1c), which regulates fatty acid synthesis, in the perinephric adipose tissue (Shimizu et al., 2018). These

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findings are consistent with mushroom consumption suppressing visceral fat accumulation. However, whereas many studies have reported anti-obesity effects of mushrooms, few studies have analyzed the effects of the consumption of a mixture of mushrooms on obesity-related metabolism.

Recently, methods have been established for the comprehensive metabolomic analysis of blood, cells, tissues, feces, and food, which have permitted the elucidation of the effects of numerous interventions on metabolism (Beckonert et al., 2007; Wishart, 2008). Furthermore, it is likely that the metabolomic analysis of lipid species will lead to the identification of markers of pathologic features of lifestyle-related diseases that involve abnormalities of lipid metabolism, such as obesity and type 2 diabetes (Nam, Choi, Jung, Jung, Choi, Ryu, et al., 2015; Zhao et al., 2013). Lipids are major components of biologic membranes and play important functional roles, for example as energy sources and in signal transduction. Differences in the diet are reflected in differences in lipid metabolite concentrations (M. J. Kim, Yang, Kim, Ahn, Lee, Kim, et al., 2013; Nam, Choi, Choi, Kim, Kim, Jung, et al., 2018); for example, standard and high-fat diet consumption are associated with significant differences in the concentrations of lipid metabolites in the serum and liver (H.-J. Kim, Kim et al., 2010).

Our previous study showed that mushroom consumption is an effective strategy for the prevention of obesity (Shimizu et al., 2018). In the present study, we compared the serum, liver, adipose tissue, cecal, large intestinal, and fecal lipid profiles of mice consuming a normal diet, a high-fat diet, or a high-fat diet supplemented with the same mixture of mushrooms, to examine the effect of mushroom consumption on lipid metabolism in initial stage of obesity.

2. Materials and methods

2.1. Preparation of the mushroom mixture

Preparation of the mushroom mixture and its nutritional composition were described in our pervious study (Shimizu et al., 2018). We used five types of mushrooms (*F. velutipes, H. marmoreus*, *L. edodes*, *G. frondosa* and *P. eryngii*) that consumed the most in Japan ("Survey on production forest products production statistics 2014"). *F. velutipes* was obtained from Mashgarden Corp. (Miyagi, Japan), and *H. marmoreus*, *L. edodes*, *G. frondosa* and *P. eryngii* were obtained from Hokuto Corp. (Nagano, Japan). The fresh fruiting bodies of the mushrooms were boiled in water for 10 min, and then the hot water-treated mushrooms and the water were freeze-dried. The self-sufficiency rate of mushrooms is very high in Japan. For this reason, we hypothesized the Japanese mushrooms consumption is approximately equal to the domestic production. The freeze-dried mushroom powders were mixed according to the proportions of Japanese mushrooms produced, according to the Ministry of Agriculture, Forestry, and Fisheries of Japan ("Survey on production forest products production statistics 2014").

2.2. Experimental diets

The mushroom mixture was mixed with a high-fat diet (Western Diet-D12079B, Research Diet Inc.). The high-fat diet contains 21 g of lipid per 100 g. Three test diets were prepared as follows: a normal diet (control; 98121701, Research Diet Inc.); a high-fat diet; and the high-fat diet containing the mushroom mixture at 3% w/w (ND, HFD, and HFMD groups, respectively). The intake of the test diet containing the

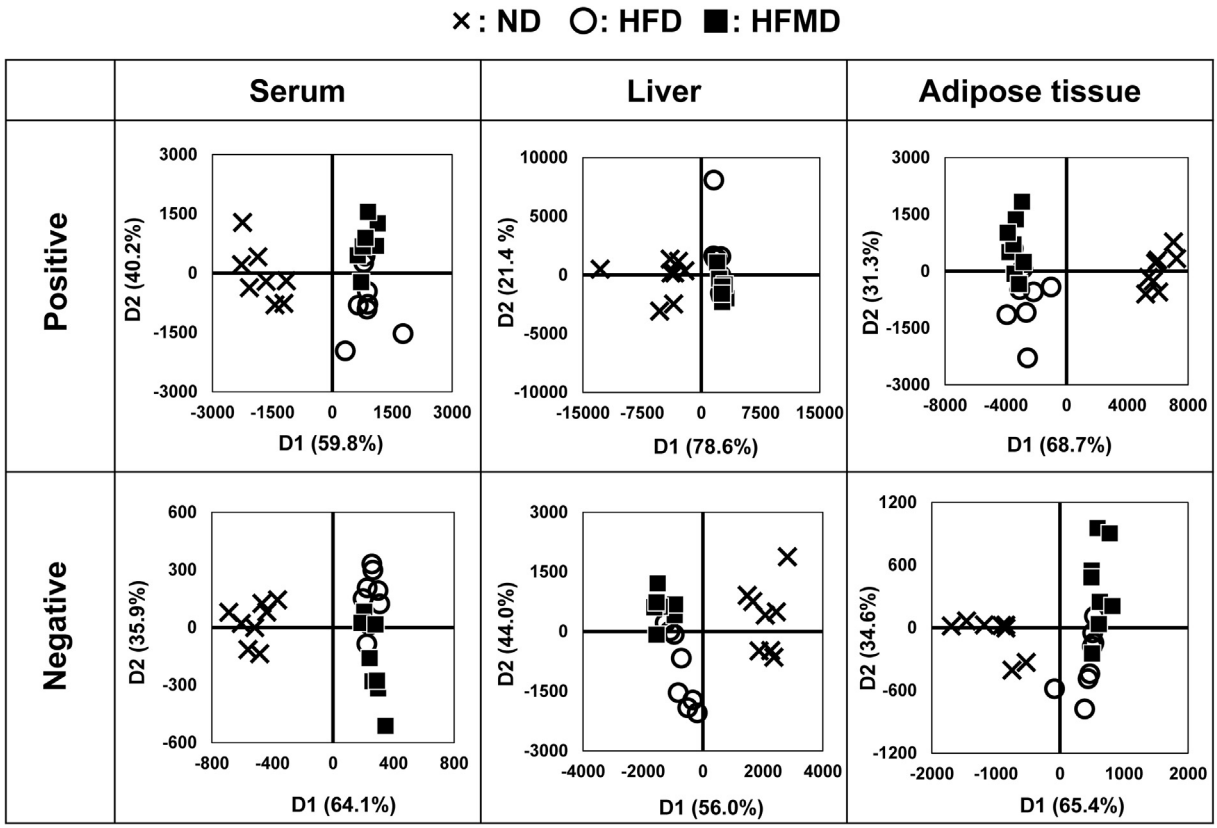

Figure 1. Multivariate analysis of the serum, liver, and adipose tissue metabolites of mice fed an ND, HFD, or HFMD. PCA-DA score plots obtained from LC-QTOF-MS spectra in positive and negative modes. ND: normal diet, HFD: high-fat diet, HFMD: high-fat diet plus mushroom mixture.

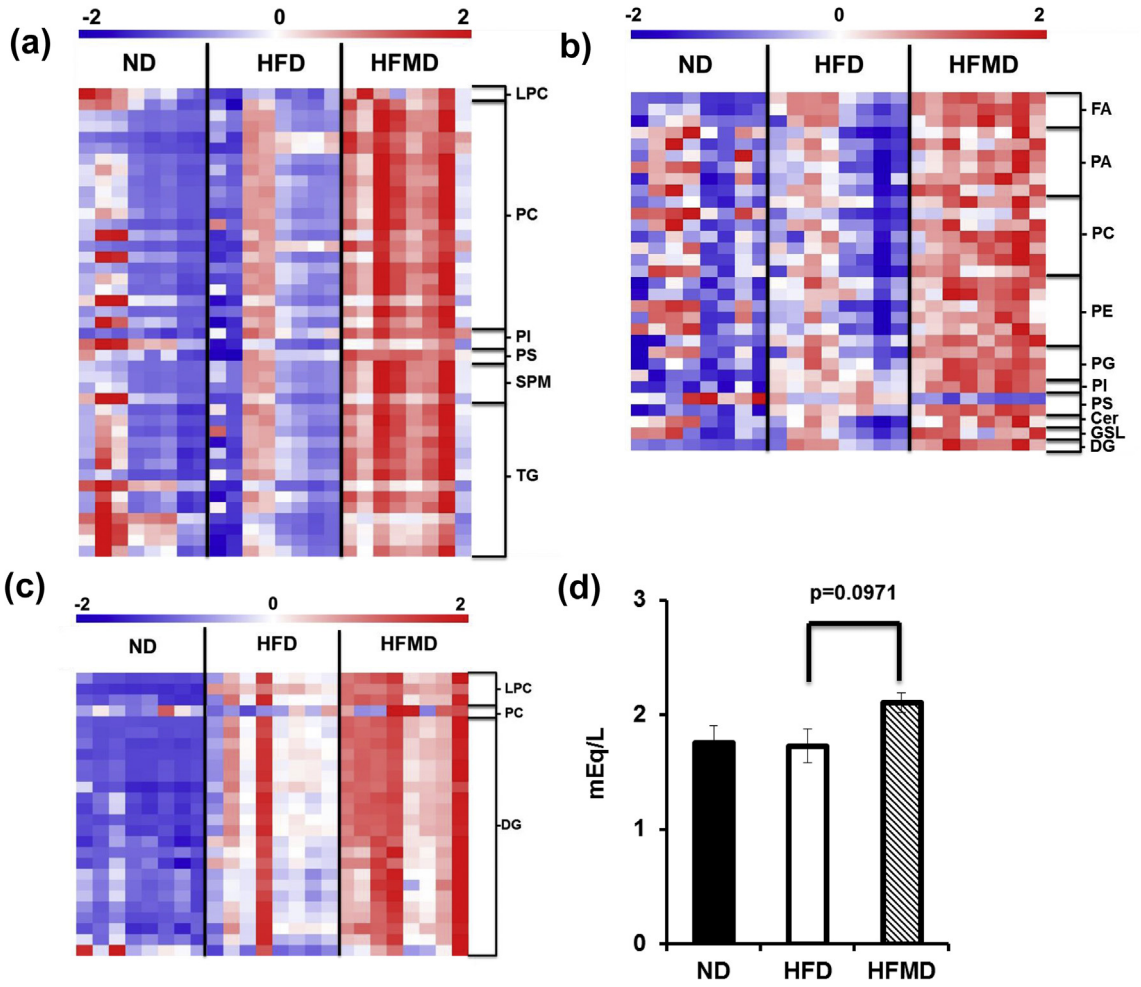

Figure 2. Heatmaps of the lipid metabolites identified in serum (a), liver (b), and adipose tissue (c). Each value in the heatmaps is a colored representation of calculated z-score-transformed raw data for the serum, liver, or adipose tissue lipid metabolites that demonstrated significant differences among the groups. Red and blue colors indicate higher and lower metabolite concentrations, respectively. ND: normal diet, HFD: high-fat diet, HFMD: high-fat diet plus mushroom mixture. LPC: lysophosphatidylcholine, PC: phosphatidylcholine, PI: phosphatidylinositol, PS: phosphatidylserine, SPM: sphingomyelin TG: triacylglycerol, FA: fatty acid, PA: phosphatidic acid, PE: phosphatidyl ethanolamine, PG: phosphatidyl glycerol, Cer: ceramide, GSL: glycosphingosaccharide, DG: diacylglycerol. Effect of mushroom consumption on serum NEFA concentration (d). Values are mean ± SE; n = 8.

mushroom mixture by the mice was calculated to be equivalent to a human intake of ~600 g of fresh mushrooms per day. To render the macronutrient composition and energy content of the HFD and HFMD equivalent, the contributions of the mushrooms were quantified and these amounts replicated in the HFD diet using an equivalent control mixture, comprising casein as the protein source, soy oil as the lipid source, corn starch as the carbohydrate source, pectin as the water-soluble dietary fiber source, and cellulose as the insoluble dietary fiber source, with the remaining mass being made up using cellulose (Shimizu et al., 2018). The final energy content per 100 g of each test diet was ND: 391 kcal, HFD: 460 kcal, and HFMD: 460 kcal.

2.3. Animals

All animal procedures were performed in accordance with the Animal Experimentation Guidelines of Tohoku University, and the animal protocol was approved by the Animal Use Committee at Tohoku University (Registration ID No. 2016AgA-009). Four-week-old male C57BL/6J mice (mean body mass: 18 g) were obtained from SLC, Inc. (Shizuoka, Japan). The mice were housed for the duration of the study under a 12 h/12 h light/dark cycle in a temperature and humidity-controlled room, and fed a standard rodent chow (CE-2, CLEA Japan, Inc., Tokyo, Japan) during a 1-week acclimation period. When 5 weeks old, the mice were randomly allocated to the three dietary groups (n = 8 per group; four mice per cage), and each group received ND, HFD, or HFMD and water *ad libitum* for 4 weeks. At the end of this 4-week period, blood samples were collected following decapitation under isoflurane anesthesia. Liver, perinephric adipose tissue, large intestine, and cecal contents were removed. Serum samples and organs were stored at −80 °C until use. Feces were collected after 4 weeks of test diet consumption, just after a routine cage change, pooled for each group, and stored at −80 °C until analyzed.

2.4. Serum, tissue, and fecal lipid extraction

Serum (10 μL), liver (60 mg), perinephric adipose tissue (10 mg), cecal contents (60 mg), large intestine (60 mg), and feces (60 mg) were transferred to 1.5 mL-tubes containing 3.0 mm zirconium beads and homogenized twice using a Micro Smash™ MS-100 (Tomy Digital Biology Co., Ltd., Tokyo, Japan) at 4,800 rpm in 1.0 mL methanol for 30 s. After homogenization, the mixtures were sonicated for 5 min and centrifuged for 1 min at 21,130 × g at 4 °C. The supernatants were filtered using a 0.2 μm PTPE filter (Agilent Technologies, California, USA) and transferred to vials (GL Sciences, Tokyo, Japan).

2.5. Conditions for the metabolomic analysis

Liquid chromatography (LC)-electrospray ionization (ESI)- mass spectrometry (MS)/MS analyses were carried out on a SCIEX X500R Q-

Table 1. Lipid profiles of the serum.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
LysoPC (17:0)	C25H52NO7P	3.48	527.3738	527.382	15	[M+NH4]+	1.00	0.67	1.10	0.003
PC (37:2)	C45H86NO8P	5.8	817.6354	817.6429	9	[M+NH4]+	1.00	0.81	1.37	0.004
PC (37:5)	C45H96NO7P	6.27	794.5828	794.5694	16	[M+H]+	1.00	2.27	6.07	0.000
PC (37:6)	C45H78NO8P	5.92	792.5377	792.6058	20	[M+H]+	1.00	1.79	5.08	0.000
PC (38:3)	C46H86NO8P	6.15	829.6223	829.6429	24	[M+NH4]+	1.00	2.96	4.76	0.001
PC (38:4)	C46H84NO8P	5.66	827.6113	827.6273	19	[M+NH4]+	1.00	2.32	4.00	0.002
PC (38:7)	C46H78NO8P	6.24	821.5878	821.5803	9	[M+NH4]+	1.00	1.54	4.31	0.000
PC (38:8)	C46H76NO8P	6.43	819.5911	819.5647	32	[M+NH4]+	1.00	1.14	2.84	0.002
PC (39:4)	C47H86NO8P	5.95	824.5984	824.6164	21	[M+H]+	1.00	1.39	3.42	0.001
PC (39:5)	C47H84NO8P	6.32	822.6022	822.6007	1	[M+H]+	1.00	1.77	4.64	0.000
PC (39:6)	C47H82NO8P	6.24	820.5684	820.5851	20	[M+H]+	1.00	1.52	4.20	0.000
PC (39:7)	C47H80NO8P	6.07	818.5912	818.5694	1	[M+H]+	1.00	0.94	2.47	0.001
PC (40:0)	C48H100NO6P	6.36	835.7472	835.7626	18	[M+NH4]+	1.00	2.59	4.99	0.006
PC (40:0)	C48H96NO8P	5.38	846.7387	846.731	6	[M+H]+	1.00	0.61	1.50	0.028
PC (40:1)	C48H96NO7P	6.03	830.7011	830.6997	1	[M+H]+	1.00	2.52	4.20	0.001
PC (40:1)	C48H96NO7P	5.3	847.7405	847.7263	16	[M+NH4]+	1.00	0.60	1.54	0.018
PC (41:0)	C49H100NO7P	6.46	863.776	863.7576	21	[M+NH4]+	1.00	2.87	6.88	0.000
PC (41:4)	C49H90NO8P	6.32	852.6482	852.6477	0	[M+H]+	1.00	1.23	2.85	0.002
PC (42:0)	C50H102NO7P	6.46	860.7477	860.7467	1	[M+H]+	1.00	1.63	3.47	0.001
PC (42:0)	C50H100NO7P	5.36	875.7695	875.7576	13	[M+NH4]+	1.00	0.47	1.07	0.033
PC (42:6)	C50H88NO8P	5.41	862.6176	862.632	16	[M+H]+	1.00	2.71	6.33	0.000
PC (43:1)	C51H100NO8P	5.76	903.7354	903.7525	18	[M+NH4]+	1.00	0.71	1.43	0.023
PI (39:3)	C48H87O13P	4.28	903.5645	903.5957	34	[M+H]+	1.00	2.04	3.05	0.000
PI (40:1)	C49H95O12P	5.6	924.6744	924.6899	16	[M+NH4]+	1.00	0.31	0.76	0.016
PS (42:8)	C48H78NO10P	4.3	877.5396	877.5702	34	[M+NH4]+	1.00	0.99	1.48	0.006
SPM (39:1)	C44H89N2O6P	6.23	795.6276	795.635	9	[M+Na]+	1.00	2.24	6.17	0.000
SPM (39:2)	C44H87N2O6P	6.15	793.6027	793.6194	21	[M+Na]+	1.00	1.94	5.48	0.000
SPM (41:1)	C46H93N2O6P	6.25	823.6584	823.6663	9	[M+Na]+	1.00	1.77	4.99	0.000
SPM (44:0)	C49H101N2O6P	6.3	845.7277	845.747	22	[M+H]+	1.00	0.42	1.18	0.034
TG (45:6)	C56H102O6	6.58	888.7847	888.8015	18	[M+NH4]+	1.00	1.68	3.16	0.002
TG (50:1)	C53H100O6	6.43	850.7652	850.7858	24	[M+NH4]+	1.00	1.16	2.72	0.002
TG (51:3)	C54H100O5	7.13	851.7685	851.7463	26	[M+Na]+	1.00	1.41	2.61	0.014
TG (52:1)	C55H104O6	5.78	878.7918	878.8171	28	[M+NH4]+	1.00	1.04	2.28	0.003
TG (53:2)	C58H102O5	6.71	879.8001	879.78	23	[M+H]+	1.00	1.12	2.12	0.009
TG (54:3)	C58H90O6	6.67	905.6626	905.663	0	[M+Na]+	1.00	1.02	1.95	0.006
TG (54:9)	C57H92O6	6.72	890.7607	890.7232	42	[M+NH4]+	1.00	2.40	4.67	0.002
TG (55:1)	C58H110O6	6.44	925.7832	925.8195	39	[M+Na]+	1.00	0.32	0.77	0.015
TG (55:8)	C58H96O6	6.29	907.235	906.7545	31	[M+NH4]+	1.00	1.05	2.14	0.011
TG (55:11)	C57H106O6	6.87	904.8074	904.8328	28	[M+NH4]+	1.00	1.01	1.70	0.027
TG (56:4)	C61H104O6	6.37	950.7929	950.8171	25	[M+NH4]+	1.00	0.26	0.62	0.038
TG (56:5)	C59H104O6	6.46	926.8168	926.8171	0	[M+NH4]+	1.00	0.39	0.87	0.017
TG (57:11)	C60H94O6	6.13	928.7317	928.7388	7	[M+NH4]+	1.00	0.43	1.07	0.035
TG (58:7)	C59H108O6	5.8	930.8196	930.8484	30	[M+NH4]+	1.00	0.70	1.38	0.024

TOF mass spectrometer (Framingham, U.S.A.). Separations were performed on an iHILIC®-Fusion (100 × 2.1 mm) column (Hilicon, Umeå, Sweden). The column temperature was maintained at 40 °C and the autosampler temperature at 5 °C. Ten-microliter samples were injected. The binary gradient system included 10 mM ammonium formate in a water: acetonitrile mixture (95:5, v/v; solvent A) and 10 mM ammonium formate in a water: acetonitrile mixture (5:95, v/v; solvent B). The gradient profile was 95% A–5% B at 0–5 min, 5% A–95% B at 5–10 min, and 95% A–5% B at 10–15 min, and the flow rate was maintained at 0.2 ml/min. The mass spectrometer was operated in positive and negative ion modes, and data acquired in the mass range 50–1000 *m/z*. The total ion chromatogram was acquired using the following operation parameters: capillary voltages of +5,500 V and -4,500 V for the positive and negative modes, respectively, a nebulizer pressure of 413.8 kPa (60 psi), a drying gas pressure of 413.8 kPa (60 psi), a curtain gas pressure of 206.9 kPa (30 psi), a source temperature of 350 °C, a declustering potential of ±80 eV, and a collision energy of ±10 eV for single MS and ±35 eV for MS/MS.

2.6. Data processing

Alignment of the detected peaks was performed according to their *m/z* values and the retention times were normalized using MarkerView™ Software (version 1.2.1, AB SCIEX). Principal component analysis (PCA)-Discriminant analysis (DA) results were processed using MarkerView™ Software. Assignment of the spectral peaks was performed using METLIN (https://metlin.scripps.edu) and the Human Metabolome Database (HMDB; http://www.hmdb.ca/). Heatmaps were generated using MultiExperiment Viewer Ver. 4.9.0 (Mev, http://www.tm4.org/mev/).

Table 2. Lipid profiles of the liver.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
FA (C14:0)	C14H28O2	1.46	227.199	227.2017	11	[M-H]-	1.00	1.91	2.58	0.001
FA (C16:0)	C16H30O2	3.57	253.2171	253.2173	0	[M-H]-	1.00	1.27	1.73	0.002
FA (C18:0)	C18H28O2	3.26	299.2041	299.1981	19	[M+Na]+	1.00	1.16	1.65	0.015
PA (36:0)	C39H77O7P	4.86	687.5372	687.5334	5	[M-H]-	1.00	0.63	0.99	0.007
PA (37:1)	C40H77O8P	3.51	715.527	715.5283	1	[M-H]-	1.00	0.90	1.93	0.030
PA (39:6)	C42H71O8P	3.92	793.5023	793.5025	0	[M+CH3COO]-	1.00	0.95	1.12	0.017
PA (40:6)	C43H73O7P	4.74	791.5429	791.5232	21	[M+CH3COO]-	1.00	0.84	1.08	0.004
PA (41:6)	C44H75O8P	4.53	761.4958	761.5127	22	[M-H]-	1.00	1.01	1.17	0.038
PA (42:6)	C45H77O8P	3.56	835.532	835.5495	20	[M+CH3COO]-	1.00	0.84	1.54	0.022
PC (29:1)	C37H72NO8P	4.69	688.4947	688.4923	3	[M-H]-	1.00	1.28	1.72	0.006
PC (29:2)	C37H70NO8P	4.63	686.4806	686.4766	5	[M-H]-	1.00	0.40	0.74	0.012
PC (31:2)	C39H74NO8P	3.55	714.5019	714.5079	8	[M-H]-	1.00	0.91	1.67	0.042
PC (32:4)	C40H72NO8P	4.89	724.5239	724.4923	43	[M-H]-	1.00	1.02	1.25	0.001
PC (37:5)	C45H80NO8P	4.71	792.5501	792.5549	6	[M-H]-	1.00	0.98	1.15	0.007
PC (37:7)	C45H76NO8P	4.63	788.5252	788.5236	2	[M-H]-	1.00	1.00	1.32	0.000
PC (39:0)	C47H94NO8P	3.35	830.6514	830.6644	15	[M-H]-	1.00	0.78	1.12	0.008
PE (36:4)	C41H74NO8P	4.69	738.5071	738.5079	1	[M-H]-	1.00	1.16	1.33	0.009
PE (36:5)	C41H72NO8P	3.95	736.4962	736.4923	5	[M-H]-	1.00	1.18	1.44	0.003
PE (38:6)	C43H74NO8P	4.65	762.5068	762.5079	1	[M-H]-	1.00	0.84	1.02	0.009
PE (38:7)	C43H72NO8P	4.54	760.4918	760.4923	0	[M-H]-	1.00	0.95	1.10	0.033
PE (40:6)	C45H78NO8P	4.81	790.5384	790.5392	0	[M-H]-	1.00	0.86	1.09	0.005
PE (40:8)	C45H74NO8P	4.5	786.5039	786.5079	5	[M-H]-	1.00	1.18	1.36	0.003
PG (21:0)	C27H55O9P	1.47	555.3507	555.3656	26	[M+H]+	1.00	0.99	1.37	0.045
PG (30:0)	C36H73O9P	4.63	739.5117	739.5131	1	[M+CH3COO]-	1.00	1.16	1.33	0.007
PG (38:2)	C44H83O9P	6.05	845.5998	845.5913	10	[M+CH3COO]-	1.00	1.61	2.04	0.006
PI (36:4)	C45H79O13P	4.01	857.5168	857.5186	2	[M-H]-	1.00	1.14	1.22	0.008
PS (40:4)	C46H82NO10P	4.41	898.5585	898.5815	25	[M+CH3COO]-	1.00	0.84	0.55	0.019
PS (44:8)	C48H78NO10P	3.99	858.5231	858.5291	6	[M-H]-	1.00	1.14	1.25	0.002
Cer (40:0)	C40H81NO4	4.79	638.609	638.6093	0	[M-H]-	1.00	0.84	1.21	0.014
GSL (44:2)	C50H95NO8	2.94	836.6816	836.6985	20	[M-H]-	1.00	0.87	1.41	0.030
DG (30:1)	C33H62O5	4.77	556.5027	556.4935	16	[M+NH4]+	1.00	4.61	9.10	0.001

2.7. Biochemical analyses of serum and feces

Free fatty acid concentrations were measured using a NEFA C test kit (Wako Pure Chemical Industries, Osaka, Japan). The lipid compositions of feces were determined as described previously (Tsuyoshi Tsuduki, Yamamoto, Hatakeyama and Sakamoto, 2015). Triacylglycerol (TG) concentration was measured using a commercial enzymatic kit (Wako Pure Chemical, Osaka, Japan).

2.8. Statistical analysis

All statistical analyses were performed using KaleidaGraph (Hulinks Inc., Tokyo, Japan). Results are expressed as mean ± standard error (SE). Data were analyzed using one-way ANOVA, followed by the Tukey test. Differences was considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. Multivariate analysis of serum, liver, and adipose tissue after mushroom consumption

Lipid profiles of the serum, liver and adipose tissue in high-fat diet-fed mice can reflect the course of obesity development (Anjos et al., 2019; Nam et al., 2018). As shown in our previous study, there were no significant differences in body mass gain and energy intake among the groups. However the high-fat plus mushroom diet group showed significantly lower perinephric adipose tissue mass than the high-fat diet group (Shimizu et al., 2018). Here, serum, liver, and adipose tissue samples were pre-treated, and the compounds present were comprehensively analyzed using LC-QTOFMS. The analysis used both positive and negative modes, and identified 3,008 and 2,681 peaks in the serum, 2,970 and 2,555 peaks in the liver, and 2,959 and 2,506 peaks in the adipose tissue, respectively. All the peaks for each sample were analyzed by multivariate analysis (PCA-DA) to visualise the clusters and compare the composition of the samples among the ND, HFD, and HFMD groups. The results show that the clusters for each group separated in the positive and negative modes (Figure 1), suggesting that the body composition of the three groups differed as a result of the differing composition of their diets. There were particularly noticeable distances between the clusters of the HFD and HFMD groups in adipose tissue. Therefore, we speculated that mushroom consumption changed lipid metabolism in adipose tissue.

3.2. Effect of mushroom consumption on the lipid metabolite composition of serum, liver, and adipose tissue

We examined whether mushroom consumption altered the lipid profiles of high-fat diet-fed mice. Heatmaps indicate the lipid metabolite concentrations that were significantly higher or lower in the HFMD group than in the HFD group (Figure 2). Analysis of these serum lipid profiles showed that mushroom consumption increased the relative concentrations of lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM), and triacylglycerol (TG) (Figure 2a, Table 1). Analysis of the liver lipid profiles showed that mushroom consumption increased the relative

Table 3. Lipid profiles of the adipose tissue.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
LysoPC (16:0)	C24H50NO7P	4.95	513.3782	513.3663	23	[M+NH4]+	1.00	9.39	14.88	0.000
LysoPC (18:1)	C26H52NO7P	6.99	522.3557	522.3554	0	[M+H]+	1.00	9.01	10.53	0.026
LysoPC (22:6)	C30H50NO7P	5.05	568.3405	568.3398	1	[M+H]+	1.00	2.22	2.86	0.008
PC (42:0)	C50H100NO8P	7.04	872.7028	872.7114	9	[M-H]-	1.00	0.92	1.26	0.005
DG (28:0)	C31H60O5	4.82	530.4808	530.4779	5	[M+NH4]+	1.00	11.82	18.79	0.000
DG (28:1)	C31H58O5	4.69	528.4636	528.4622	2	[M+NH4]+	1.00	7.99	12.54	0.000
DG (30:0)	C33H64O5	5.07	541.4814	541.4827	2	[M+H]+	1.00	5.79	8.33	0.000
DG (30:1)	C33H62O5	4.96	539.4685	539.467	2	[M+H]+	1.00	7.28	9.90	0.005
DG (30:2)	C33H60O5	4.69	554.4778	554.4779	0	[M+NH4]+	1.00	5.35	7.74	0.003
DG (31:0)	C34H66O5	4.81	555.4825	555.4983	28	[M+H]+	1.00	7.61	11.18	0.002
DG (31:1)	C34H64O5	4.99	570.508	570.5092	2	[M+NH4]+	1.00	3.37	4.27	0.017
DG (32:0)	C35H68O5	5.15	569.5128	569.514	2	[M+H]+	1.00	1.96	2.68	0.001
DG (32:2)	C35H64O5	5.44	587.4649	587.4646	0	[M+Na]+	1.00	1.93	2.54	0.001
DG (32:5)	C35H58O5	5.12	559.4345	559.4357	2	[M+H]+	1.00	5.73	8.09	0.000
DG (33:2)	C36H66O5	5.05	596.5249	596.5248	0	[M+NH4]+	1.00	2.58	3.33	0.008
DG (34:0)	C39H74O5	5.48	640.5826	640.5874	7	[M+NH4]+	1.00	1.90	2.36	0.014
DG (34:5)	C37H62O5	4.98	585.4299	585.4524	38	[M-H]-	1.00	4.45	7.45	0.000
DG (36:1)	C41H68O5	6.41	641.5112	641.514	4	[M+H]+	1.00	1.74	2.17	0.015
DG (36:2)	C39H67D5O5	5.23	614.5064	614.4916	24	[M-H]-	1.00	1.51	2.46	0.000
DG (36:4)	C39H68O5	5.2	615.5103	615.4994	17	[M-H]-	1.00	1.88	3.03	0.001
DG (36:5)	C39H66O5	5.22	613.5025	613.4837	30	[M-H]-	1.00	1.55	2.47	0.000
DG (36:6)	C39H64O5	5.01	611.4849	611.4681	27	[M-H]-	1.00	2.11	3.06	0.001
DG (37:6)	C40H66O5	5.1	625.5028	625.4837	30	[M-H]-	1.00	2.73	3.96	0.001
DG (38:4)	C37H72O5	5.1	597.5287	597.5453	27	[M+H]+	1.00	2.23	2.91	0.012
DG (38:6)	C41H68O5	5.3	639.5175	639.4994	28	[M-H]-	1.00	1.39	1.97	0.001
DG (38:7)	C41H66O5	5.1	637.5012	637.4837	27	[M-H]-	1.00	0.73	1.03	0.013

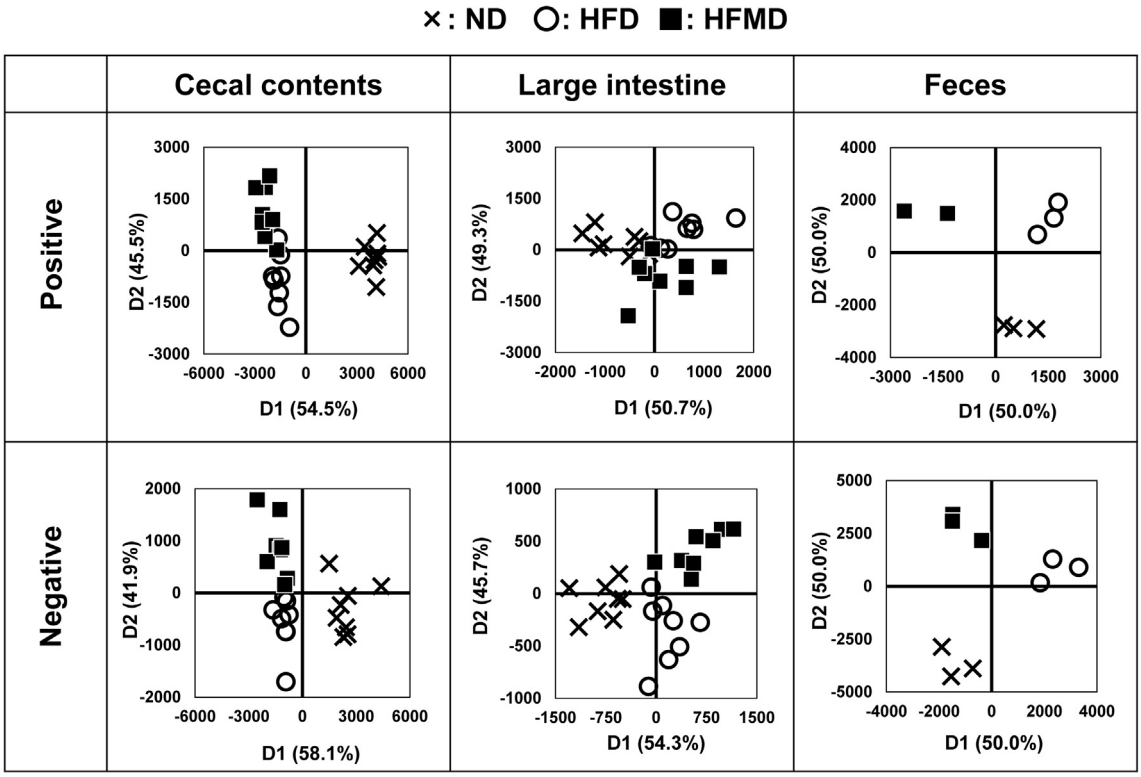

Figure 3. Multivariate analysis of the cecal content, large intestinal, and fecal metabolites of mice fed ND, HFD, or HFMD. PCA-DA score plots obtained from LC-QTOF-MS spectra in positive and negative modes. ND: normal diet, HFD: high-fat diet, HFMD: high-fat diet plus mushroom mixture.

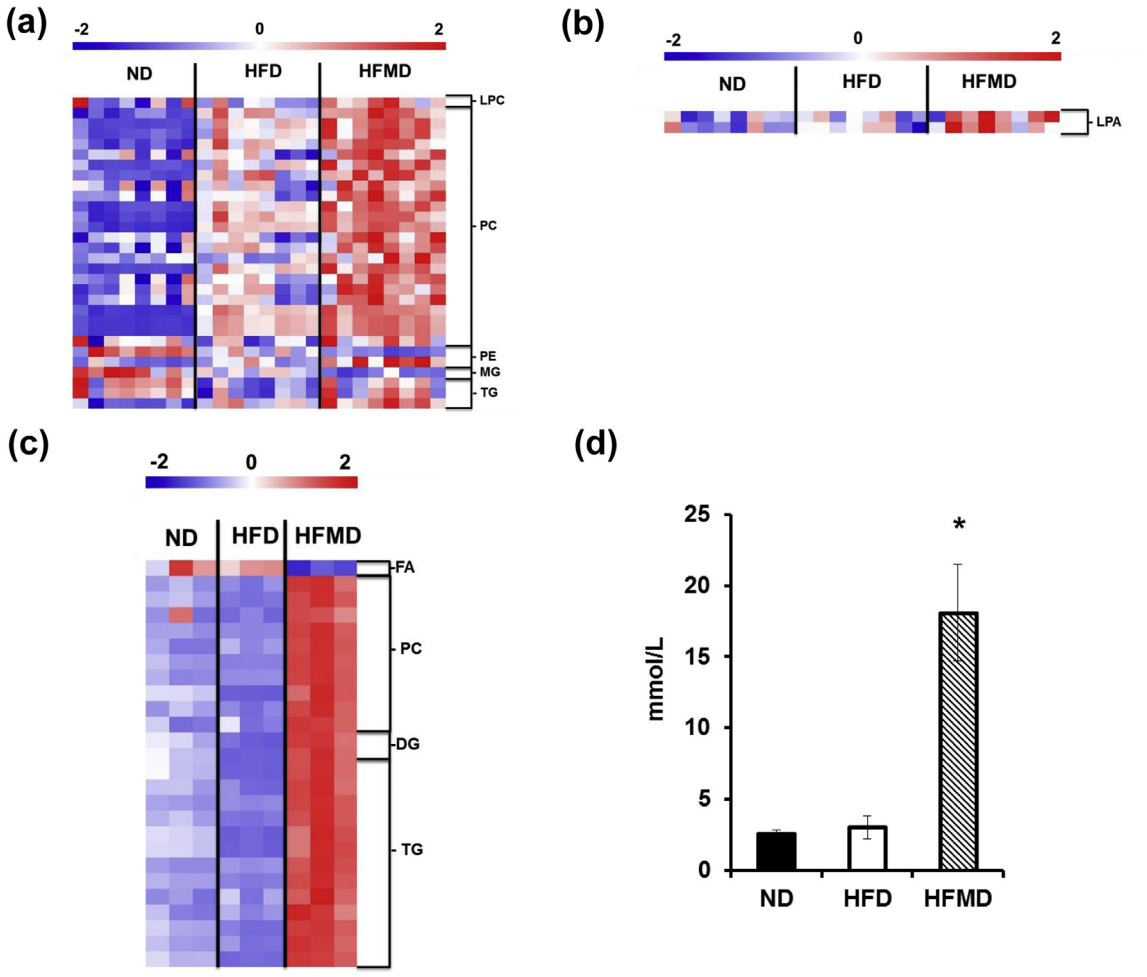

Figure 4. Heatmaps of the lipid species identified in the cecal contents (a), large intestine (b), and feces (c). Each value in the heatmaps is a colored representation of calculated z-score-transformed raw data for each tissue/substance that displayed significant differences among the groups. Red and blue colors indicate higher and lower lipid metabolite concentrations, respectively. ND: normal diet, HFD: high-fat diet, HFMD: high-fat diet plus mushroom mixture. LPC: lysophosphatidylcholine, PC: phosphatidylcholine, PE: phosphatidyl ethanolamine, MG: monoacylglycerol, TG: triacylglycerol, LPA: lysophosphatidic acid, FA: fatty acid, DG: diacylglycerol. Effect of mushroom consumption on fecal triglyceride concentration (d). Values are mean ± SE; n = 8. *: $p < 0.05$ *vs.* the HFD group.

concentrations of fatty acid (FA), phosphatidic acid (PA), PC, phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), PI, ceramide (Cer), glycosphingosaccharide (GSL), and diacylglycerol (DG). Furthermore, mushroom consumption increased the relative concentration of PS (44:8) in the liver, but reduced that of PS (40:4) (Figure 2b, Table 2). Finally, analysis of the adipose lipid profiles showed that mushroom consumption increased the relative concentrations of LPC, PC, and DG (Figure 2c, Table 3). Higher concentrations of LPC in the serum and adipose tissue have also been shown following green tea consumption, which also has an anti-obesity effect (Nam et al., 2018); therefore, LPC may participate in the suppression of fat accumulation by mushroom consumption. Next, we focused on the increase in the DG content of the adipose tissue. TG in adipose tissue is hydrolyzed by hormone-sensitive lipase (HSL), a lipolytic enzyme expressed in adipocytes, generating DG and non-esterified fatty acid (NEFA). NEFA is transported in the blood and used as an energy source in skeletal muscle and heart. To investigate the effect of mushroom consumption on lipolysis in adipose tissue, the concentration of NEFA was measured in the serum. This showed a tendency for NEFA concentration to be higher in the HFMD group than in the HFD group ($p = 0.0971$) (Figure 2d). In addition, our previous study showed that HSL is activated by mushroom consumption (Shimizu et al., 2018). These results imply that mushroom consumption promotes the degradation of TG in adipocytes. Furthermore, we have previously reported that mushroom consumption reduces white adipose tissue mass (Shimizu et al., 2018). Therefore, we speculate that the promotion of triglyceride hydrolysis is involved in the effect of mushroom consumption to suppress visceral fat accumulation.

In addition, DG is known to inhibit insulin signaling in liver and skeletal muscle (Erion and Shulman, 2010). However, there were no significant differences in the serum insulin concentration between the groups, and mushroom consumption tended to reduce serum glucose (Shimizu et al., 2018). Furthermore, adiponectin, which is known to ameliorate insulin resistance (Yamauchi et al., 2001), was present in higher concentrations in the HFMD group than the HFD group. Therefore, the increase in DG concentration in adipose tissue following mushroom consumption is not considered to induce insulin resistance.

3.3. Multivariate analysis of cecal, large intestinal, and fecal lipids after mushroom consumption

Subsequently, we determined the effects of mushroom consumption on the composition of the contents of the lower digestive tract. The collected cecal contents, large intestine, and feces were pre-treated, and the compounds contained in these samples were comprehensively analyzed using LC-QTOFMS. Analyses using both positive and negative modes identified 3,155 and 2,980 peaks in the cecal contents, 3,942 and 2,315 in the large intestine, and 3,271 and 2,175 in the feces, respectively. All the peaks detected for each sample were analyzed by

Table 4. Lipid profiles of the cecal contents.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
LysoPC (22:5)	C30H52NO7P	3.52	570.3576	570.3554	3	[M+H]+	1.00	0.97	1.16	0.010
PC (33:0)	C42H84NO8P	5.49	762.5922	762.6007	11	[M+H]+	1.00	1.25	1.36	0.011
PC (33:0)	C41H82NO8P	6.39	765.6775	765.6116	2	[M+NH4]+	1.00	6.04	9.09	0.000
PC (33:1)	C41H80NO8P	5.8	763.5984	763.596	3	[M+NH4]+	1.00	1.62	2.10	0.000
PC (33:2)	C41H78NO7P	5.49	750.5439	750.5408	4	[M+Na]+	1.00	1.32	1.46	0.008
PC (33:6)	C42H78NO8P	1.48	756.558	756.5538	5	[M+H]+	1.00	0.95	1.39	0.001
PC (34:0)	C42H88NO6P	5.97	751.5441	751.6687	2	[M+NH4]+	1.00	3.75	6.11	0.000
PC (34:3)	C41H72NO8P	4.2	755.542	755.5334	11	[M+NH4]+	1.00	1.56	1.89	0.004
PC (34:5)	C42H74NO8P	1.48	769.5645	769.549	20	[M+NH4]+	1.00	1.00	1.27	0.011
PC (35:1)	C44H86NO7P	1.47	772.6394	772.6215	23	[M+H]+	1.00	1.04	1.67	0.000
PC (35:2)	C43H80NO7P	6.17	776.5525	776.5565	5	[M+Na]+	1.00	2.26	2.93	0.000
PC (35:2)	C43H82NO7P	6.62	778.5692	778.5721	3	[M+Na]+	1.00	1.82	2.07	0.011
PC (35:2)	C43H84NO7P	6.87	780.5835	780.5878	5	[M+Na]+	1.00	2.66	3.14	0.000
PC (35:3)	C43H80NO8P	1.48	787.6072	787.596	14	[M+NH4]+	1.00	0.97	1.60	0.000
PC (35:4)	C43H84NO8P	1.12	774.5993	774.6007	1	[M+H]+	1.00	1.44	1.79	0.008
PC (35:4)	C43H78NO8P	2.81	785.4868	785.5803	23	[M+NH4]+	1.00	1.00	2.74	0.000
PC (36:0)	C44H88NO7P	6.48	791.6874	791.6637	29	[M+NH4]+	1.00	2.73	3.55	0.000
PC (36:2)	C43H82NO7P	1.49	773.4213	773.6167	44	[M+NH4]+	1.00	1.20	1.74	0.000
PC (36:2)	C44H82NO7P	0.83	790.5665	790.5721	7	[M+Na]+	1.00	1.21	1.60	0.010
PC (36:3)	C44H86NO7P	1.49	789.6489	789.648	1	[M+NH4]+	1.00	1.09	1.69	0.000
PC (36:5)	C48H82NO8P	7.11	832.5837	832.5851	1	[M+H]+	1.00	1.69	1.86	0.007
PC (37:7)	C44H82NO8P	6.72	806.5669	806.567	0	[M+Na]+	1.00	6.73	8.47	0.000
PC (40:7)	C45H76NO8P	6.98	807.5824	807.5647	21	[M+NH4]+	1.00	8.75	11.13	0.000
PC (55:0)	C51H102NO8P	7.07	888.7514	888.7416	11	[M+H]+	1.00	0.90	1.09	0.009
PE (33:0)	C38H76NO8P	6.21	704.5177	704.5236	8	[M-H]-	1.00	0.65	0.40	0.049
PE (36:5)	C41H72NO8P	3.77	736.5176	736.4923	34	[M-H]-	1.00	1.38	2.47	0.000
MG (18:4)	C21H34O4	1.18	351.245	351.253	21	[M+H]+	1.00	0.79	0.63	0.007
TG (54:9)	C59H94O6	6.55	916.7583	916.7389	21	[M+NH4]+	1.00	0.54	0.80	0.006
TG (56:7)	C57H92O6	6.42	890.7428	890.7232	21	[M+NH4]+	1.00	1.44	1.79	0.000
TG (56:10)	C59H96O6	6.55	918.7573	918.7545	3	[M+NH4]+	1.00	0.59	0.94	0.001

multivariate analysis (PCA-DA) to visualise the clusters and compare the lipid composition among the ND, HFD, and HFMD groups. The results show that the clusters for each group separated in the positive and negative modes (Figure 3), suggesting that the lower digestive tract contents differed in their lipid composition among the three groups, because of differences in the diet consumed. There were particularly noticeable distances among the cluster of three groups in feces.

3.4. Effects of mushroom consumption on the lipid profiles of cecal contents, large intestine, and feces

Heatmaps demonstrate the lipid metabolites that were present in significantly higher or lower concentrations in the HFMD group than the HFD group (Figure 4). Analysis of the lipid profiles of the cecal contents showed that mushroom consumption increased the relative concentrations of LPC, PC, and TG. There were no significant differences in the relative concentration of PE in the large intestine and feces. However, mushroom consumption increased the relative concentration of PE (36:5), and reduced that of PE (33:0) in the cecal contents. Mushroom consumption also reduced the relative concentration of MG in the cecal contents (Figure 4a, Table 4). Analysis of the lipid profiles of the large intestine showed that mushroom consumption increased the relative concentration of lysophosphatidic acid (LPA) (Figure 4b, Table 5). Analysis of the fecal lipid profiles showed that mushroom consumption reduced the relative concentration of FA (C18:2), but increased the relative concentrations of PC, DG, and TG (Figure 4c, Table 6).

We then focused on the increase in phospholipid content of the lower digestive tract. Dietary fiber alters the rate of intestinal cell turnover and viscous dietary fiber exfoliates intestinal cells (Jin et al., 1994; Tasman-Jones et al., 1982), which may aid in the prevention of infection and maintenance of barrier function in intestinal epithelial cells (Cliffe et al., 2005). PC is a major lipid component of cell membranes, and the mushroom-induced increase in PC in the lower digestive tract may be attributed to detached intestinal cells. A mushroom-induced increase in LPA was also identified in the large intestine, and LPA enhances intestinal epithelial wound healing by promoting intestinal epithelial cell

Table 5. Lipid profiles of the large intestine.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
LysoPA (18:0)	C21H45O6P	1.41	423.2776	424.2954	25	[M-H]-	1.00	1.31	2.10	0.0018
LysoPA (20:3)	C23H41O7P	1.38	459.2445	460.259	7	[M-H]-	1.00	1.07	1.43	0.0064

Table 6. Lipid profiles of the feces.

Proposed identity	Molecular formula	Ret. time	Precusor ions (*m/z*)		Difference (ppm)	Aduuct	Relative intensity (ratio; ND = 1)			*p*-value (HD v.s. HDM)
			Observed	Theoretical			ND	HD	HDM	
FA (C18:2)	C18H32O2	10.04	279.2351	279.2329	7	[M-H]-	1.00	0.99	0.55	0.010
PC (33:4)	C41H74NO8P	1.77	757.5478	757.549	1	[M+NH4]+	1.00	0.85	2.74	0.000
PC (34:3)	C42H78NO8P	1.76	756.558	756.5538	5	[M+H]+	1.00	0.46	3.38	0.000
PC (36:0)	C44H88NO8P	1.77	790.6448	790.632	16	[M+H]+	1.00	0.60	1.76	0.009
PC (36:1)	C44H86NO7P	1.79	772.6273	772.6215	7	[M+H]+	1.00	0.72	5.56	0.000
PC (36:2)	C44H86NO8P	1.76	788.6057	788.6164	13	[M+H]+	1.00	1.23	4.36	0.000
PC (36:2)	C44H86NO7P	1.76	789.6369	789.648	14	[M+NH4]+	1.00	0.48	5.30	0.000
PC (37:0)	C45H86NO7P	1.76	806.6388	806.6034	43	[M+Na]+	1.00	0.93	4.62	0.000
PC (37:6)	C45H78NO8P	1.82	809.5899	809.5803	11	[M+NH4]+	1.00	0.16	2.66	0.000
PC (40:3)	C48H90NO7P	1.79	824.7075	824.6528	66	[M+H]+	1.00	0.85	2.04	0.000
PC (41:2)	C49H94NO8P	1.76	873.7073	873.7055	2	[M+NH4]+	1.00	1.08	3.49	0.000
DG (43:2)	C46H86O5	1.79	741.661	741.6367	17	[M+Na]+	1.00	0.42	2.36	0.000
DG (43:3)	C46H84O5	1.79	739.6455	739.6211	32	[M+Na]+	1.00	0.07	2.98	0.000
TG (42:0)	C45H86O6	1.79	740.6467	740.6763	39	[M+NH4]+	1.00	0.14	2.91	0.000
TG (43:3)	C46H82O6	1.77	753.6178	753.6004	23	[M+Na]+	1.00	0.66	2.43	0.000
TG (45:5)	C48H82O6	1.75	755.6386	755.6184	26	[M+H]+	1.00	0.40	3.69	0.000
TG (46:4)	C49H86O6	1.77	771.6324	771.6497	22	[M+H]+	1.00	0.69	7.07	0.000
TG (47:1)	C50H94O6	1.83	808.7073	808.7389	39	[M+NH4]+	1.00	0.22	2.66	0.000
TG (48:0)	C51H98O6	1.83	807.7052	807.7436	48	[M+H]+	1.00	0.18	2.80	0.000
TG (49:8)	C52H84O6	1.76	805.6488	805.6341	18	[M+H]+	1.00	1.09	15.58	0.000
TG (50:6)	C53H90O6	1.81	823.6988	823.681	21	[M+H]+	1.00	0.59	3.91	0.000
TG (52:3)	C53H92O6	1.79	825.7139	825.6967	20	[M+H]+	1.00	1.13	2.42	0.000
TG (54:1)	C57H108O6	1.79	911.7827	911.8038	23	[M+Na]+	1.00	1.03	2.35	0.001
TG (62:11)	C65H104O6	1.79	998.8635	998.8171	46	[M+NH4]+	1.00	0.37	3.57	0.000
TG (63:10)	C66H108O6	1.79	997.8624	997.8218	40	[M+H]+	1.00	0.63	3.61	0.000
TG (63:9)	C66H110O6	1.78	999.8601	999.8375	22	[M+H]+	1.00	0.09	3.73	0.000

migration and proliferation (Sturm et al., 1999). Moreover, mushroom consumption tended to increase the weight and length of the large intestine (Shimizu et al., 2018). Taken together, these findings may suggest that mushroom consumption promotes intestinal turnover and has beneficial effects on maintains homeostasis intestinal epithelial.

Because mushroom consumption increased the fecal TG concentration, according to the metabolomic analysis, we also measured the total fecal TG content biochemically, and found that it was higher in the HFMD group than in the ND and HFD groups (Figure 4d). The results of the lipid profiling of the lower digestive tract and fecal TG measurement imply that mushroom consumption promotes the excretion of lipids. Because an increase in insoluble dietary fiber has been reported to promote lipid excretion (Hsu et al., 2006; Neyrinck et al., 2009), these effects may be attributable to the fiber content of the mushrooms. However, the same amount of dietary fiber, in the form of cellulose and pectin, was added to the diet of the HFD group. Therefore, specific types of dietary fiber, such as 1,3-β-glucan, chitin, lignin, or cellulose which are present in the mushrooms, may be responsible (Kurasawa et al., 1982). In addition to dietary fiber, the other compounds may participate in promotion of lipid excretion. It was reported that *Pleurotus eryngii* water extract has an inhibitor of pancreatic lipase and suppresses lipid absorption (Mizutani et al., 2010). Although the energy intake was the same for all the groups of mice, less energy-rich lipid was absorbed through the gut of the HFMD group than in the HFD group. We speculate that the lipid stored in the visceral fat may be used to compensate for the resulting energy shortage, such that visceral fat mass is lower in the HFMD group. In addition, the higher serum NEFA concentration and lipid accumulation in the liver, where lipid synthesis occurs, may also be induced to maintain energy homeostasis in the HFMD group. Moreover, the higher concentrations of phospholipids found in the serum and liver likely reflect greater synthesis of lipoproteins, which are required for the transport of lipids and cholesterol around the body (Ockner et al., 1969), and which are also necessary for the maintenance of energy homeostasis in mice consuming mushrooms. It has been reported that a larger lipid concentration in the large intestine and feces disturbs the balance of the gut microbiome, which adversely affects the host (Murphy et al., 2015). However, we have previously reported that the gut microbial composition is altered by mushroom consumption, leading to an increase in the population of some SCFA- and lactic acid-producing bacteria (Shimizu et al., 2018), implying that the amount of TG in the feces is not likely to have adversely affected the gut microbiome.

3.5. Mushroom consumption increases the quantity of PC containing odd-chain fatty acids

Mushroom consumption increased the concentrations of some lipids containing odd-chain fatty acids (Tables 1, 2, 3, 4, 5, and 6). Figure 5 shows that PC containing odd-chain fatty acids was more abundant in the serum, liver, cecal contents, and feces of mice fed HFMD. Because long-chain fatty acids are synthesized from acetyl-CoA, they usually contain an even number of fatty acids. However, it is known that some gut bacteria produce propionic acid from water-soluble dietary fiber, which can be used to synthesize odd-chain fatty acids in the liver (Weitkunat et al., 2017). We hypothesize that the concentrations in the HFMD group increase following the metabolism of the water-soluble dietary fiber contained in the mushrooms by gut bacteria. Propionic acid produced by these bacteria promote energy metabolism in the host (Sa'ad, Peppelenbosch, Roelofsen, Vonk and Venema, 2010). Therefore, we hypothesize that mushroom consumption increases the propionic acid concentration in the body, and that this plays a role in suppressing fat accumulation.

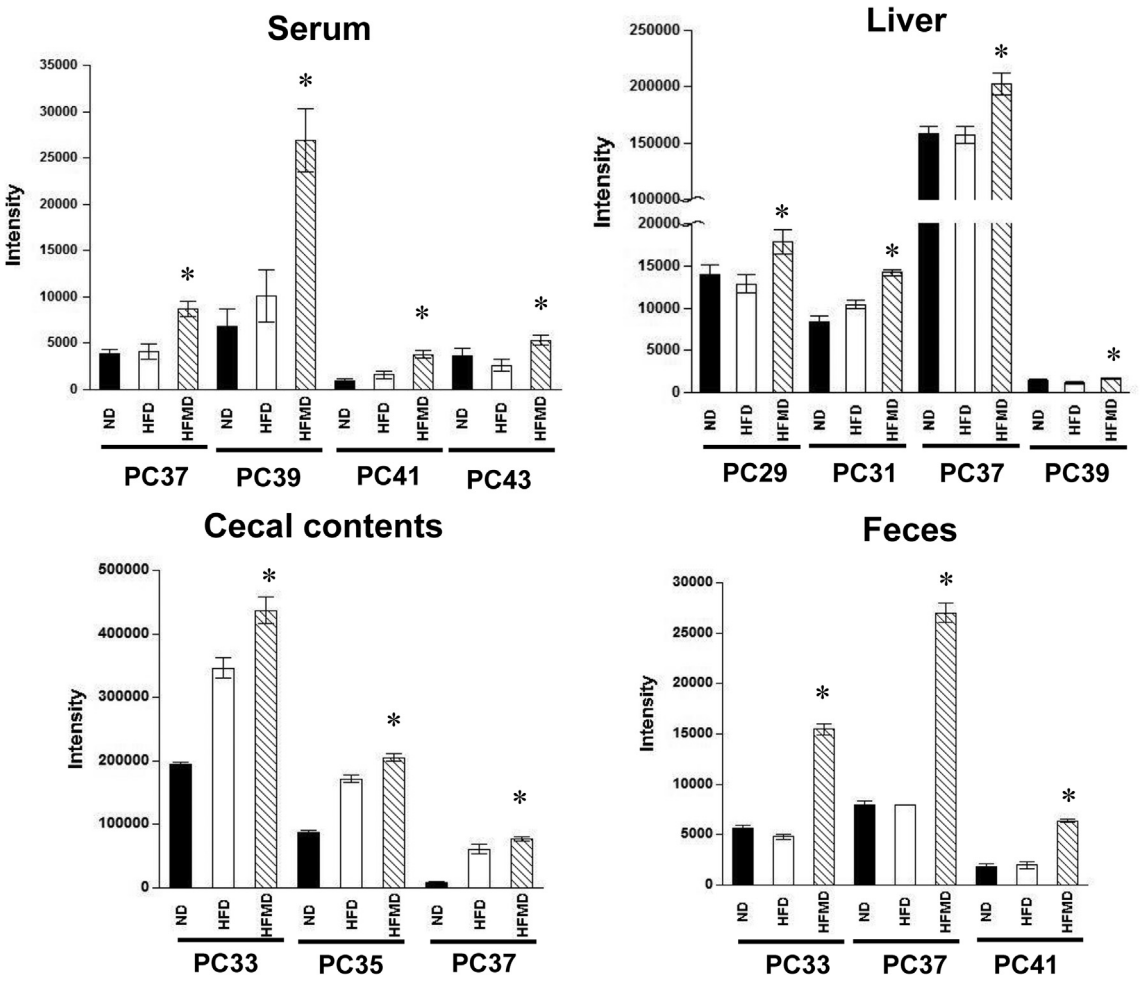

Figure 5. Relative concentrations of PC containing odd-chain fatty acids in the serum, liver, cecal contents, and feces of mice fed ND, HFD, or HFMD. Values are mean ± SE; n = 8. *p < 0.05 vs. the HFD group. PC: phosphatidylcholine, ND: normal diet, HFD: high-fat diet, HFMD: high-fat diet plus mushroom mixture.

4. Conclusions

We aimed to examine how the mushroom consumption alters lipid metabolism in high-fat diet-fed mice. Mushroom consumption resulted in increased phospholipids in serum and liver, increased DG in adipose tissue, and increased TG in feces. The results showed that mushroom consumption promotes the excretion, rather than the absorption of dietary lipids through the gut. The mice that consumed the mushroom mixture were thus relatively energy-deficient and apparently maintained their energy homeostasis by using lipids from their visceral fat stores, reducing visceral fat mass. Thus, a lipid metabolomic analysis has elucidated part of the mechanism underpinning the suppression of fat accumulation by mushroom consumption.

Declarations

Author contribution statement

Takamitsu Shimizu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Koichiro Mori: Analyzed and interpreted the data; Wrote the paper.

Hitoshi Kobayashi: Analyzed and interpreted the data.

Tsuyoshi Tsuduki: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

Three of the authors (Takamitsu Shimizu, Koichiro Mori, and Hitoshi Kobayashi) are salaried employees of the Hokuto Corporation, which cultivated some of the mushrooms used in this study. The remaining author (Tsuyoshi Tsuduki) declares no conflict of interest. We used five types, 4 of which are from our company. However, 33% of the mixture represents a sample from another company. All research funding for this study was provided by the Hokuto Corporation.

Additional information

No additional information is available for this paper.

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