Antiobesity-, and glucose metabolism -, and lipid metabolism-improving effects and active components in the mycelia and fruiting bodies of *Cordyceps militaris* (Vuill.) Fr. fermented on pupae of silkworms (*Bombyx mori*)

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Abstract

Cordyceps militaris (Vuill.) Fr. (CM) is an entomopathogenic fungus that has traditionally been used as a herbal medicine, particularly as a tonic, throughout Asia. In this study, we show for the first time that not only the fruiting body (FB) but also mycelia (pupa part; PM) of *C. militaris* fermented in silkworm (*Bombyx mori*) pupa (SPCM) can reduce body weight, visceral fat, liver fat, blood glucose, cholesterol, insulin, and leptin levels in a high-fat diet-induced obese mouse model. Pair-feeding examination revealed that appetite suppression somewhat contributed to the effectiveness of SPCM. Ultraperformance liquid chromatography–high-resolution tandem mass spectrometry analysis and feature-based molecular networking analysis revealed that the PM contained a molecular network of beauveriolides, including beauveriolides I and III, that are potent inhibitors of cholesterol synthesis, whereas the FB contained a network of spermidines that exhibit antiobesity effects and improve glucose and lipid metabolism.

Introduction

Cordyceps militaris (Vuill.) Fr. (CM), an entomopathogenic fungus, is a medicinal mushroom belonging to Cordycipitaceae in the phylum Ascomycota. CM is parasites on the larvae and pupae of Lepidoptera, causing mycelium within their bodies and forming fruiting bodies (FB, mushrooms) on their body surface. More than 400 species of *Cordyceps* sp. have been identified worldwide (Sung et al., 2007), of which CM is the most well-studied *Cordyceps*, along with *Ophiocordyceps sinensis* (synonym, *Cordyceps sinensis*) in the family Ophiocordycipitaceae. *Cordyceps* sp. has been traditionally used in Asian folk medicine as a tonic (Jędrejko, Lazur, & Muszyńska, 2021). Nowadays, extracts of CM and their constituents were reported to have various pharmacological activities, including tonic, anti-inflammatory, antioxidant, anti-aging, antitumor, immunomodulatory, antibacterial, antimicrobial, antiviral, antiprotozoal, insecticidal, antifibrotic, steroidogenic, hypoglycemic, lipid-lowering, antiobesity, antidiabetic, antiangiogenic, anti-HIV, antimalarial, antifatigue, neuroprotection, hepatoprotection, renoprotection, and lung protection (Das, Masuda, Sakurai, & Sakakibara, 2010).

Among them, numerous studies have reported that CM improves glucose and lipid metabolism and have antiobesity effects in animal experiments (studies on mushroom extracts; Choi et al., 2012, Choi et al., 2014, Kime et al., 2014, Yu et al., 2015, Li, et al., 2022, Jang et al., 2022 and Zhao et al., 2023; studies on mushroom polysaccharides, Lee et al., 2021, Yu et al., 2021, Huang et al., 2022, Huang et al., 2023 and Yu et al., 2023; studies on cordycepin, Niu et al., 2010, An et al., 2018, Li et al., 2018 and Gong, Li, Wan & Sha, 2021). The large number of reports suggest that CM is a promising functional food candidate that improves glucose and lipid metabolism and has antiobesity effects. However, the range of the effects varies among studies. For example, a study on commercially available mixed powder of FB and mycelia, administered to high-fat diet (HFD)-fed mice (Yu et al., 2015), and a study on commercially available hot water extract of FB, administered to a spontaneous type 2 diabetes model db/db mice (Choi et al., 2012) have shown reductions in blood glucose, blood cholesterol, and triglyceride (TG), but not body weight. Conversely, in a trial in which 50% ethanol extract of FB fermented with rice bran, administered to HFD obese mice, body weight gain was reduced compared with the control group, in addition to a decrease in blood cholesterol, TG, and visceral and liver fat levels (Kim et al., 2014). Moreover, among commercial extracts of CM fermented on artificial substrates, those with high myriocin contents have lowering effects on blood glucose and visceral and liver fat and suppress weight gain, whereas those with low myriocin contents have no such effects (Li, et al., 2022). These studies suggested that the effects of CM vary depending on the fermentation and extraction methods.

Because CM is one of the most commercially important *Cordyceps* species along with *O. sinensis,* various fermentation systems using various artificial media, such as beech wood meal,

rice bran, wheat bran, husked rice, wheat grains, and liquid media, have been developed (Das, Masuda, Sakurai, & Sakakibara, 2010, Kontogiannatos, Koutrotsios, Xekalaki, & Zervakis, 2021). Following the natural ecology of the CM, fermentation in silkworm pupae has also been developed. However, its effects on abnormal glucose and fat metabolism and obesity have not been clarified. In *O. sinensis,* FB together with the insect part (filled with mycelium) are used as a crude drug. In CM fermented on silkworm pupae (SPCM), not only the FB but also the insect part could be used.

In this study, SPCM were evaluated to ameliorate glucose and lipid metabolism disorders and obesity in an HFD-fed mouse model and 3T3-L1 preadipocyte cell line. The results show that not only the FB but also the insect part with mycelium (PM) has improving effects on glucose and lipid metabolism and obesity. Feature-based molecular networking analysis based on nontargeted analysis charts using high-resolution-electrospray ionization-mass spectrometry (HRESIMS) and MS/MS spectra revealed characteristic compound networks in PM and FB, respectively.

2. Materials and methods

2.1 Reagents

Dulbecco's modified Eagle medium–high glucose (DMEM), dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), human recombinant insulin (insulin), penicillin–streptomycin solution, Cell Counting Kit-8 (CCK-8), methanol, chloroform, acetic acid, LabAssay ™ Cholesterol, LabAssay ™ Triglyceride, and LabAssay ™ NEFA were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo Japan). Mouse/Rat leptin (ELISA) kit and Mouse/Rat insulin ELISA kit were purchased from Morinaga Institute of Biological Science, Inc. (Kanagawa Japan). Mouse/Rat adiponectin ELISA kit was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo Japan). AdipoRed was purchased from LONZA K.K. (Tokyo Japan)

2.2. Fermentation of *C. militaris* and sample preparation

CM, collected in the Kyushu University Forest (Miyazaki Prefecture Kyushu, Japan) located in Kyushu Mountain Range, was verified by DNA analysis. CM was maintained on an agar medium (10 g of peptone, 20 g of sucrose, 10 g of yeast extract, 0.1 g of chloramphenicol, and 17 g of powdered agar per liter).

The inoculation of pupae with the fungus was performed as follows. A piece of cutout disc of the agar medium with CM was transferred in the liquid medium (same composition of the agar medium minus agar) and incubated at 24°C for 72 h with shaking. The culture medium was filtered to remove clumps of the mycelia, and the precipitate obtained by centrifugation was suspended in pure water to 1.2×10^8 cells/mL. Live pupae of silkworms (hybrid race; Kin-Shu

× Sho-Wa) reared on an artificial diet (SilkMate; Nosan Corporation Kanagawa, Japan) at Amami Sericulture Co, Ltd., were used as culture substrates. Each silkworm pupa was injected with 10 μL of cell suspension and then incubated at room temperature of approximately 20℃ for 43 days. Silkworm pupae that developed FBs (SPCM) were lyophilized and used in the experiments (Fig. 1). Lyophilized SPCM or its FB and PM were ground into powder using a metal cone crusher (multibead shocker, Yasui Kikai Corporation, Osaka Japan).

2.3. SPCM extraction

The powdered form of PM, FB, and whole SPCM were extracted with 20 times the volume of methanol, pure water at 80℃ (hot water), or pure water at room temperature (water). For methanol extraction, the mixture was shaken at room temperature for 24 h, and the supernatant obtained by centrifugation was dried with a centrifugal evaporator (EZ-2 Plus, ATS Genevac, UK). For hot water extraction, the mixture was stood at 80℃ for 3 h, and the supernatant obtained by centrifugation was lyophilized. For water extraction, the mixtures were sonicated at 25℃ for 2 h, and the supernatant obtained by centrifugation was then lyophilized.Commercially available powdered *O. sinensis* and freeze-dried and powdered silkworm pupae (the substrate of *C. militaris* fermentation) were extracted in the same way as water extraction.

The methanol extracts were dissolved in dimethylsulfoxide (DMSO) and used for cell experiments and LC-MS/MS analysis. The hot water extracts were dissolved in water and used for cell experiments. The water extracts were dissolved in 0.1% DMSO and used for cordycepin analysis.

2.4. Animal experiments

2.4.1 Pair-feeding study

Five-week-old male ddY mice, purchased from Japan SLC, Inc. (Shizuoka, Japan), were used in this study. Mice were kept at 25℃ with a 12-h light/dark cycle. The study was approved by the Kumamoto University Animal Care and User Committee. The study schedule is shown in Fig. 2. The mice were housed in four per cage and fed a rodent diet CE-2 (CLEA Rodent Diet CE-2; Nippon Clare Corporation, Tokyo). From day 12 after arrival (day-41), mice were reared individually and fed with powdered HFD (60% CE-2, 30% powdered beef fat, and 10% dextrin) or powdered CE-2 (normal diet; NOD) using a powder feed feeder.

On day 40 after switching to the diet (day-1), the HFD-fed animals were divided into the following nine groups based on body weight: HFD group, W0.5 group that fed HFD containing 0.5% of the whole (PM + FB) SPCM powder, W1 group that fed HFD containing 1% of the whole SPCM powder, PM group that fed HFD containing 0.75% of PM powder, FB group that fed HFD containing 0.25% of FB, and four feeding control groups, where the feeding amount

was matched to the corresponding dosing group. The feeding amount of the feeding control groups were determined as follows. The 24-h food intake of each animal in the dosing group was measured daily, and the ratio of the food intake to that of the initial value (immediately before the start of treatment) was then calculated daily for each dosing group. Then, to determine the daily feeding amount, the food intake ratio of each dosing group was multiplied by the amount of the initial food intake of each individual in the corresponding feeding control group. To make it easier for the mice to eat small amounts of food, the feeding control group was fed in small plastic cups placed inside a powder food feeder.

Body weights and food intake were measured every 3–5 days before the start of administration and daily during the administration period. The last weight measurement was taken on day 17, and blood was drawn from the tail vein of the mice on day 18. Blood was collected in two heparin-coated capillaries per individual and centrifuged at 15 000 x g for 5 min within 1 h. The plasma was stored at −80℃. On days 19 and 20, mice were exsanguinated by severing the inferior vena cava under isoflurane anesthesia, and the liver and fat around the testis and kidney were collected, frozen in liquid nitrogen, and weighed.

2.4.2 Measurement of blood glucose, blood lipids, and blood hormones

Using a glucose dehydrogenase-based glucose measuring device (Oshiete-gluco; ForaCare Japan Co., Ltd., Tokyo Japan), blood glucose measurements were performed on blood drained from the tail vein injured with a razor. Blood cholesterol, triglyceride (TG), and nonesterified fatty acid (NEFA) were measured by colorimetric methods using LabAssay ™ Cholesterol, LabAssay ™ Triglyceride, and LabAssay ™ NEFA, respectively, and insulin, leptin, and adiponectin levels were measured by enzyme-linked immune-sorbent assay (ELISA) using Mouse/Rat insulin ELISA kit, Mouse/Rat leptin (ELISA) kit and Mouse/Rat adiponectin ELISA kit, respectively in accordance with their instructions.

2.4.3 Determination of liver TG content

Liver TG content was measured using the Bligh and Dyer method (Blight & Dyer, 1956) with modifications. In brief, a portion was taken from the liver that had been stored at −80℃ and crushed to powder using a Multibead shocker. Approximately 100 mg of them was measured in a tube. The liver was kept frozen up to here. Acetic acid (0.1 mol/L) of 1.5 mL was added to the tube, mixed well, and then left for 10 min at room temperature. Then, 1 mL of the mixture was transferred to a glass spit tube, and 2.5 mL of methanol and 1.25 mL of chloroform were added and stirred well. The mixture was centrifuged at 3000 rpm for 5 min, and 3.8 mL of the supernatant was then transferred to a glass spit tube, to which 1 mL of chloroform and 1 mL of water were added in that order and then stirred well. The mixture was centrifuged at 3000 rpm

for 10 min, and 1 mL of the lower layer was taken off and blow-dried. The dried solid was dissolved in 50 μL of isopropanol (lipid extract) and used for TG determination.

TG was measured using LabAssay ™ Triglyceride with a modification of the protocol supplied with the kit. In brief, 6 μL of the lipid extract was transferred into each well of a 96-well microplate, which was filled with 200 μL of the chromogenic reagent. Then, the plate was left at room temperature for 15 min, and the absorbance was measured at 600 nm using a plate reader (Cytation 5; Agilent Technologies, Ltd., USA). A standard curve was prepared using the standard solution supplied with the kit, and the TG level of the sample was calculated from the equation of the standard curve. The TG level in the liver was expressed per g wet weight of the liver. Measurements were taken on four individuals, excluding the heaviest and lightest

2.5. Cell experiment

2.5.1. Cell culture and evaluation of the inhibitory effect of adipocyte differentiation

The 3T3-L1 mouse pre-adipocyte cell line was transferred from the JCRB cell bank (Japanese Collection of Research Bioresources Cell Bank). Cells were cultured under a humidified atmosphere with 5% CO_2 at 37℃. The cells were seeded in 96-well microplates at a concentration of 2.5×10^4 cells/well and cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. The medium was replaced every other day until confluent. The day after confluence (DAY 0), the medium was replaced with differentiation media (10% FBS supplemented DMEM containing 1 μM dexamethasone, 500 μM IBMX, and 10 μg/mL insulin) to induce differentiation (Fig. 6A). At the same time as differentiation was induced, the PM and FB extracts were added. From day 2, DMEM with 10% FBS, 10 μg/mL human insulin, and the extracts were replaced every other day. On day 5, after the differentiation of the control group (no extract added) was confirmed (as indicated by pronounced fat droplet accumulation in the cells), the medium was removed, and the cells were washed with phosphate-buffered saline (PBS). To evaluate cell viability, 105 μL of DMEM containing 10% CCK-8 was added and incubated for 4 h, after which the absorbance at 450 nm was measured in a plate reader. DMEM containing CCK-8 was removed, each well was washed with PBS, and 200 μL of PBS and 5 μL of AdipoRed reagent (LONZA) were added. After 10 min, fluorescence intensity was measured in a plate reader (ex/em = 355/460 nm).

Cell viability was calculated as follows: Cell viability (%) = {1-(absorbance of the extract-added group/absorbance of the control group)} × 100. The fat accumulation rate was calculated as an index of cell differentiation: Fat accumulation rate (%) = {1- (fluorescence intensity of the extract-added group/fluorescence intensity of the control group)} × 100.

2.5.2. Evaluation of the inhibitory effect of fat accumulation

After induction of differentiation (DAY0) as in the differentiation study, the cells were further cultured in DMEM (supplemented with 10% FBS and 10 μg/mL insulin) which was replaced every other day (Fig. 6D). After the fat droplets in the cells were confirmed at DAY4 (differentiation confirmation), the extracts were added. The cells were then cultured in DMEM (supplemented with 10% FBS, insulin 10 μg/mL, and the extract), which was replaced every other day. On DAY7, cell viability and fat accumulation rates were calculated.

2.6. Feature-based molecular networking analysis

Analysis was carried out on methanol extracts. The LC-MS/MS measurement and feature-based molecular networking analysis were performed as previously described (Aron et al., 2020, Nothias et al., 2020, Kimura et al., 2023). In brief, high-resolution-electrospray ionization-mass spectrometry and MS/MS spectra were measured by UPLC-HRMS using a Waters Acquity UPLC system connected to a Waters Xevo G2-XS Qtof mass spectrometer as previously described (Kimura et al., 2023). Raw LC-MS/MS data were transformed into an mzML file using Waters2mzML-1.2.0, and the peak list was generated through MZmine 3.9.0. The list was then exported to the Global Natural Products Social Molecular Networking-compatible format to create the feature-based molecular network. The network was visualized using Cytoscape version 3.10.0.

2.7. Determination of cordycepin

A water extract of PFCM dissolved in 0.1% DMSO and diluted 1000-fold in pure water was used. HPLC (EXTREMA; JASCO Corporation, Tokyo, Japan) was used for the analysis. Samples of 2 μL each were submitted to a CAPCELL PAK ADME-HR S2 column (2.1 150 mm, OSAKA SODA Co., Ltd., Osaka, Japan) and eluted with a gradient comprising water (solvent A) and acetonitrile solution containing 0.1% formic acid (solvent B). The gradient profile was as follows: 0.5% B in 1 min, 0.5%–5% B in 5 min, 5% B in 1 min, and 5%–0.5% B in 2 min. The flow rate was adjusted to 0.6 mL/min, and the column temperature was maintained at 40℃. The peak area of the constituents in the chromatogram was determined and used as an indicator of content.

2.8 Statistical analysis

Data were analyzed using the t-test. Statistical significance was set at $P< 0.05$ and $P< 0.01$.

3. Results

3.1 Animal experiments

3.1.1. Food intake and body weight

The food intake (Fig. 3) was almost unchanged relative to the initial value until day 10 in the HFD group, whereas it decreased in a concentration-dependent manner by 0.85-fold and 0.73-fold on the day after the test commenced in the W0.5 and W1 groups, respectively (Fig. 3A). In PM and FB groups (Fig. 3B), food intake also decreased 0.87 times from the initial value on the day after the test commenced. The daily food intake of the treated groups recovered to 0.95 times to the initial value on some days but remained lower than that of the HFD group throughout the test period. The average daily food intake ratios to the initial value for 17 days of the test period were significantly lower in any treated group than in the HFD group ($P < 0.01$) (Fig. 3C).

The average initial body weight of approximately 45 g in the HFD fed mice was significantly higher in the NOD fed mice of 41 g ($P < 0.05$). Throughout the test period (Fig. 3), the body weight of the HFD group steadily increased, reaching 1.080 ± 0.042 times the initial value (48.5 ± 6.6 g) on day 16, whereas that of the W0.5 group remained nearly the same as the initial value until the last weight measurement on day 17 (1.007 ± 0.025 times, 45.2 ± 3.5 g). The body weight of the W0.5C group (pair-feeding control for the W0.5 group) was nearly the same as W0.5 group until day 8, but then remained slightly higher than that of the W0.5 group until the last day (day 17; 1.022 ± 0.033 times, 45.8 ± 3.2 g). The body weight of the W1 group decreased 0.99-fold on the day after the test started, which fell to 0.945 times on day 7 and remained almost at that level until day 17 (0.964 ± 0.037, 42.1 ± 1.7 g, Fig. 3D). The body weight of the W1C group also decreased in nearly the same manner as that of the W1 group until day 8, but then remained slightly higher than that of the W1 group until the last day (0.987 ± 0.029; 44.2 ± 3.3 g, Fig. 3B). In the PM group (Fig. 3D), the body weight remained nearly the same until the end of the test, with slight increase and decrease compared with the initial value, and on the day 17, last day of the study, the body weight (45.9 ± 4.3 g) was 1.02 ± 0.034 times the initial value. The body weight of the pair-feeding control group for the PM group (PMC group) remained similar to that of the PM group up to day 8; however, thereafter, the values were slightly higher than in the treated group and were 1.04 ± 0.023 times higher (46.6 ± 4.3 g) on day 17. However, the difference between the two groups was not significant. The ratio of the body weight to the initial value in the FB group was also lower than that in the HFD group throughout the test period, and on day 17, it was 1.037 ± 0.033 times. However, the difference from the HFD group was not significant (Fig. 3D). The body weight of the FBC group remained lower than that of the treatment group throughout the study period; however, the difference between the two was not significant, except for day 4.

3.1.2. Visceral and liver fat accumulation

The visceral fat weight (sum of the testicular and perirenal fat weight) ratio to the body weight was significantly higher in the HFD group (6.1% ± 1.6%) than in the NOD group (2.4% ± 1.5%) ($p < 0.05$) (Fig. 4A). The ratios in the W0.5 group (3.8 ± 1.8), W1 group (2.5 ± 0.7), and their feeding controls (W0.5C, 4.2% ± 1.3%; W1C, 4.2% ± 0.7%) on day 19 or 20 were all significantly lower than those in the HFD group. In the administration groups, the values were lower than their corresponding fooding control groups, and the difference between the W1 and W1C groups was significant ($p < 0.01$).The visceral fat weight ratio in the PM (4.0% ± 1.3%) and FB (4.1% ± 1.4%) groups and their food intake controls (PMC, 5.0% ± 0.8%; FBC, 4.3% ± 1.5%) were also lower than those in the HFD group, with a significant difference for PM relative to HFD ($p < 0.05$).

The liver fat content in the HFD group (15.9 ± 10.38 mg/g) was significantly higher ($p < 0.05$) than that in the NOD group (1.84 ± 1.29 mg/g). The liver fat contents of the pair-feeding control groups were 10.75 ± 5.59, 16.96 ± 11.16, 13.83 ± 9.13, and 11.69 ± 7.82 mg/g for W0.5C, W1C, PMC, and FBC groups, respectively, which were not significantly different from that in the HFD group. Conversely, the liver fat contents of the administration groups were 2.22 ± 2.11, 2.66 ± 1.27, 2.38 ± 1.01, and 4.7 ± 4.71 mg/g for the W0.5, W1, PM, and FB groups, respectively, which were significantly lower than those of the HFD group, except for the FB group.

3.1.3. Blood parameters

The blood glucose level on day 18 (Fig. 5A) was 167.1 ± 21.7 mg/dL in the HFD group, which showed an increasing trend against 141.2 ± 19.0 mg/dL in the NOD group ($p < 0.057$). In the W0.5 and W1 groups, the blood glucose levels decreased in a concentration-dependent manner to 137.9 ± 14.3 and 124.3 ± 13.6 mg/dL, respectively, both significantly lower than that in the HFD group ($p < 0.05$). On the contrary, the blood glucose levels of the corresponding pair-feeding controls of 152.6 ± 12.6 mg/dL for W0.5C and 144.3 ± 23.1 mg/dL for W1C tended to be lower than those in the HFD group but higher than those in the treatment groups. The blood glucose level of the PM group (139.2±15.7 mg/dL) was significantly lower than that of the HFD group ($p < 0.05$) and PMC group (158.2 ± 9.7 mg/dL) ($p < 0.01$) The blood glucose level of the FB group (153.5 ± 12.0 mg/dL) also tended to be lower than that of the FBC (162.5 ± 13.3 mg/dL) and HFD groups; however, the difference was not significant.

Blood TG levels (Fig. 5B) tended to be lower in the HFD group (124.0 ± 49.4 mg/dL) than in the NOD group (158.3 ± 61.8 mg/dL). The blood TG values in the W0.5, W1, PM, and FB groups were 129 ± 56.2, 110.8 ± 35.2, 145.3 ± 57.7, and 142.8 ± 45.9 mg/dL, respectively, and these were not significantly different from those in the HFD group. The values in the pair-feeding controls for each group were W0.5C, 91.8 ± 44.1; W1C, 78.3 ± 23.3; PMC, 120.3 ±

40.6; and FBC, 83.3 ± 10.4 mg/dL, respectively, all of them were lower than those in the NOD and HFD groups.

The blood NEFA level (Fig. 5C) was significantly higher ($p < 0.05$) in the HFD group (1.3 ± 0.30 mEq/L) than in the NOD group (0.86 ± 0.37 mEq/L). The values for the treatment and corresponding feeding control groups were not significantly different from those for the HFD group, except that the value in the PMC group (1.82 ± 0.38 mEq/L) was significantly higher than that in the HFD group ($p < 0.05$).

The blood insulin levels (Fig. 5E) tended to be higher in the HFD group (4.34 ± 2.97 ng/mL) than in the NOD group (1.48 ± 0.80 ng/mL) ($p < 0.064$), whereas the levels in the W0.5 (2.64 ± 3.14 ng/mL) and W1 (1.67 ± 0.95 ng/mL) groups were lower than those in the HFD group. The difference between the W1 and HFD groups was significant. Insulin levels were even lower in the pair-feeding control groups (W0.5C, 1.05 ± 0.43; W1C, 0.75 ± 0.17 ng/mL), which were lower than that in the NOD group.

The blood insulin levels in the PM and FB groups were 2.24 ± 2.4 and 3.93 ± 3.45 ng/mL, respectively, which were lower than those of the HFD group; however, the difference was not significant. The values for each feeding control group were 1.69 ± 0.89 ng/mL for the PMC group and 1.45 ± 0.61 ng/mL for the FBC group, both tending to be lower than the corresponding treatment groups.

The blood leptin levels (Fig. 5E) were significantly higher ($p < 0.05$) in the HFD group (10.3 ± 6.30 ng/mL) than in the NOD group (3.73 ± 3.50 ng/mL). The levels in the treatment groups and their pair-feeding controls were all lower than those in the HFD group. For HFD, the differences were significant in the W0.5 group (3.59 ± 1.99 ng/mL), W1 group (2.53 ± 1.50 ng/mL), and its pair-feeding controls: the W1C group had 4.35 ± 1.27 ng/mL, and the PM group had 3.62 ± 1.61 ng/mL. The leptin levels in the W1 and PM groups were significantly lower than the levels in their control (W1C) group (PMC group, 7.17 ± 2.91 ng/mL).

The blood adiponectin levels (Fig. 5F) were not significantly different between any of the groups.

3.2. Inhibitory effects of adipocyte differentiation and fat accumulation on 3T3-L1 cells

Hot water and methanol extracts of PM and FB inhibited the differentiation of 3T3-L1 mouse pre-adipocytes into adipocytes (Fig. 6C) and the accumulation of fat (Fig. 6F) in a dose-dependent manner at concentrations that did not show cytotoxicity (Fig. 6B, E). Both activities tended to be stronger in FB than in the PM in the same concentrations in the same extraction solvents.

3.3. Compounds in the PM and FBs of SPCM

3.3.1. Feature-based molecular networking analysis

Feature-based molecular networking analysis was performed using LC-MS/MS data (Fig 6). Beauveriolides (BVDs) and steroids were detected in PM and whole. Among BVDs, BVDs I or III with a peak at m/z 488.3137 $[M+ H]^+$ were the main compounds (Table S1). Five unidentified compounds specific to PM and whole were also detected. A molecular network of spermidines was identified in the FBs. In FBs, three unidentified molecular groups and one single compound were also detected. Betaine lipids DGTSA and galactosylceramide (GalCel), as well as two unidentified compound groups and seven single compounds, were detected in FB and PM.

3.3.2. Cordycepin content

The peak area of the chromatogram of cordycepin by HPLC analysis was three times larger in FB than in PM. No peak of cordycepin was detected in commercially available *O. sinensis* and uninfected silkworm pupae.

4. Discussion

4.1 Antiobesity, glucose metabolism-, and lipid metabolism-improving effects of SPCM

Oral administration of whole, FB, and PM of SPCM with food on HFD-induced obesity with glucose and fat metabolism abnormal mice, significantly reduced their body weight (Fig. 3), visceral fat weight, liver TG content (Fig. 4), blood glucose, blood cholesterol, blood insulin, and blood leptin levels (Fig. 5) compared with the untreated HFD group. Similar effects were observed in the PM and FB groups, respectively. PM and FB extracts inhibited adipocyte differentiation and fat accumulation in 3T3-L1 cells (Fig. 6). These results suggested that not only the FB but also the PM has antiobesity-, glucose metabolism-, and lipid metabolism-improving effects.

Unlike previous reports, SPCM showed a decrease in food intake whether with PM or FB (Fig. 3). Compared with previous reports in which, extract of CM (Kime et al., 2014, Li, et al., 2022, Choi et al., 2014, Jang et al., 2022, Zhao et al., 2023, Choi et al., 2012, and Yu et al., 2015) or isolated compounds from CM (Huang et al., 2022, Huang et al., 2023, Lee et al., 2021, Yu et al., 2021, and Yu et al., 2023, Gong, Li, Wan & Sha, 2021, Niu et al., 2010, An et al., 2018, and Li et al., 2018) were used, in the present study, lyophilized and powdered SPCMs were used for oral administration, which means that all compounds in the mushroom were ingested by mice. Compounds that have not been extracted by the methods reported so far, or compounds produced by using silkworm pupa as a culture substrate, may have a suppressive effect on feeding. Pair-feeding trials were conducted to determine the extent to which reduced food intake was responsible for the effects of SPCM. The body weights of the feeding control

group decreased to the same extent as the treatment group (Fig. 3B, 3D). These results suggest that food intake suppression is one of the factors responsible for the weight-lowering effect of SPCM. In the feeding control group, visceral fat (Fig. 4), blood glucose, blood TG, blood cholesterol, blood insulin, and blood leptin levels were also reduced compared with those in the HFD group, but to a lesser extent than in the treatment group except for TG and insulin levels. (Fig. 5). In the liver fat content (Fig. 4), no decrease was found in the feeding control groups, whereas significant reduction in the administration groups was found when compared with that in the HFD group. These results indicate that the food intake-reducing effect contributes to the effect of SPCM, but to a limited extent. Nevertheless, as appetite suppression is one of the targets of obesity treatment (Brandfon et al., 2023), elucidating the mechanisms and components involved in appetite suppression by SPCM may lead to the development of new drugs. Various factors are associated with appetite control, such as gut hormones, neuropeptides, and brain amines (Druce & Bloom, 2006). Thus, more studies are needed to elucidate the appetite suppression mechanism of SPCM.

In contrast to previous reports (ex; Yu et al., 2015), no reduction in blood TG levels in the treated group was noted in the present study, even though hepatic TG accumulation was significantly reduced in the treatment group and TG synthesis in the liver was thought to be reduced. The strain of mice and the conditions under which the blood was collected may have contributed to the fact that plasma TG was not reduced in the treated group. Most studies on the antiobesity and glucose and lipid metabolism improving effects of CM have mainly used C57B/6J mice and collected blood samples under fasting conditions (ex; Kim et al, 2014). In the present study, ddY mice were used, and blood was collected under free feeding conditions. The ddY mice show a greater increase in blood TG levels in the oral lipid tolerance test and a slower rate of decrease in TG levels owing to the small increase in lipoprotein lipase (LPL) activity (1.2 times) after fat loading. Conversely, C57B/6J mice show a 4.3-fold increase in LPL activity after fat loading, which are much higher than that of ddY mice (Yamazaki, Kishimoto, & Ezaki, 2012). Blood drawing under ad libitum feeding conditions and the use of ddY mice with low TG clearance capacity may have been influenced by TG levels in administration groups, which did not reduce.

4.2. Active compounds of SPCM

The feature-based molecular networking analysis based on LC-MS/MS data detected molecular networks of different compounds in PM and FB, same of which have been reported to improve glucose and lipid metabolism and to have antiobesity effects.

The cyclodepsipeptide beauveriolides (BVDs), detected in the PM, have been reported in 28 species (Yin, et al., 2020), among which BVD I and III are known potent inhibitors of sterol O-

acyltransferase 1 (SOAT1) (previously acyl -CoA: cholesterol acyltransferase1 (ACAT1) isozyme) (Namatame et al., 1999a, 1999b, Namatame, Tomoda, Ishibashi, & Omura, 2004, Ohshiro et al., 217). SOAT1 catalyzes the formation of cholesterol esters by transferring an acyl group to the 3-position hydroxyl group of cholesterol using long-chain acyl-CoA as a substrate and is considered to play an important role in cholesterol metabolism in vivo. Therefore, enzyme inhibitors are considered candidates for hypercholesterolemic drugs (Tomoda & Ohomura, 2007). From mycelia of CM fermented in Sabouraud dextrose broth, eight species of BVDs including BVD I and III were reported (Yin et al., 2020). In the same paper, however, in fruiting bodies fermented in Chinese tussah silk moth (*Antheraea pernyi*) pupae or rice medium did not contain BVDs detectable by HPLC, consistent with our results that BVDs were detected in the PM of SPCM and not in the FB. On the contrary, Wang et al. (2020) reported that BVD I and III were identified in the fruiting body of CM fermented in a potato dextrose broth; however, the content of the compounds in the mycelium was at trace levels. Thus, the content of BVDs in the mycelium and fruiting body varied depending on the culture conditions.

Steroids were also detected in PM (Fig. 6). Steroids are important constituents of fungi, and the major compound species are ergosterol derivatives (Zhabinskii, Drasar & Khripach, 2022). From the CM, ergosterol (Das, et al., 2010) and its derivatives, ergosta-7, 9 (11), and 22-trien-3β-ol (EK100) (Hsieh, et al., 2021) have been reported. Because ergosterol and its derivatives have a cholesterol-lowering effect (Das & Kumar, 2021), the steroids detected in the PM in this study may contribute to its cholesterol-lowering effect.

In this study, assuming a dry weight ratio of PM to FB in the whole of SPCM, their concentrations in the feed were set at PM = 0.75% and FB = 0.25%, respectively. Therefore, if they have equal contributions to the activity, the effects on both treatment groups should be the same. However, the effect of PM tended to be stronger than that of FB (Figs. 3–5), and the effect of lowering cholesterol was particularly stronger in the PM group than in the FB group (Fig. 5D). BVDs and steroids contents may be factors that contribute to the pronounced cholesterol-lowering effect of PM. In the previous report (Jędrejko, Lazur, & Muszyńska, 2021), the mycelia of the CM contains approximately 10 times higher lovastatin than FB. However, no lovastatin was identified in SPCM.

Spermidines were detected in the FB of SPCM. Spermidines are polyamines found in the cells of organisms (Shi et al., 2022). In an HFD-induced obesity mouse model, it has been reported to reduce body weight and subcutaneous and visceral fat, reduce TG and total cholesterol concentrations in the liver and serum, improve insulin resistance, and suppress gene expression involved in the fatty acid synthesis pathway by activating hepatic AMP-activated protein kinase (AMPK) (Gao et.al., 2018). Spermidine also reduces fasting blood glucose levels

and improves glucose utilization associated with improved fat oxidation and reduced white fat mass (Sadasivan et.al, 2014).

Cordycepin, a typical bioactive component of *Cordyceps* sp., was detected in the FB and PM of SPCM, and its content was three times higher in the FB than that of PM (Table 1). The administration of cordycepin to HFD induced obese mice leads to the suppression in body weight gain and visceral and hepatic fat accumulation and downregulates the factors involved in fatty acid synthesis in the liver, namely, sterol regulatory element-binding protein 1c, acetyl-CoA carboxylase, steroyl-CoA desaturase 1, liver X receptor α, and fatty acid translocase CD36, whereas the expression of factors involved in β-oxidation, namely, AMPK, p-AMPK, carnitine palmitoyltransferase I, and PPARα are upregulated (Gong et al. 2021). Studies using 3T3-L1 cells have also reported that cordycepin inhibits fatty acid synthesis by inhibiting mTOR1 via Akt inhibition and AMPK activation and by suppressing C/EBPβ and PPARγ expression (Takahashi et al., 2012).

Galactosylceramide (GaCl) and DGTS and DGTA, detected in both the PM and FB have not been reported to have antiobesity-, glucose metabolism-, and lipid metabolism-improving effects on mammals.

In this study, different compounds and compound networks were detected in the PM and FB, respectively. Previous studies have also reported that the content of bioactive compounds differs between the mycelia and the fruiting body of CM (Jędrejko et al., 2021, Jędrejko et al., 2022). Consumption of the whole SPCM provides a greater variety of active components than consumption of the mycelium alone. Therefore, additive effects between active components are then expected.

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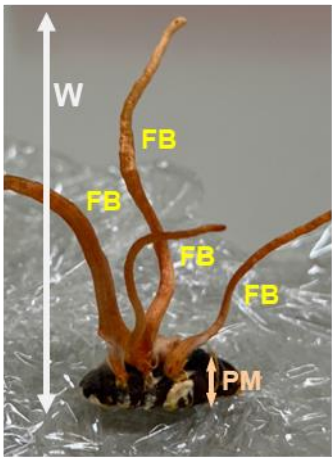

Fig. 1 Freeze-dried *Cordyceps militaris* (Vuill.) Fr. fermented by silkworm pupae. W, whole; PM, mycelium on pupae; FB, fruiting body

Fig. 2. Schedule of animal experiment
Five-week-old ddY mice were fed a high-fat diet (HFD) for 41 days to induce obesity and abnormal glucose and lipid metabolism. Then, the mice were fed with HFD containing SPCM of whole (W), pupae with mycelium (PM), and fruiting body (FB). The food restricted groups corresponding to each SPCM administration groups were also set.

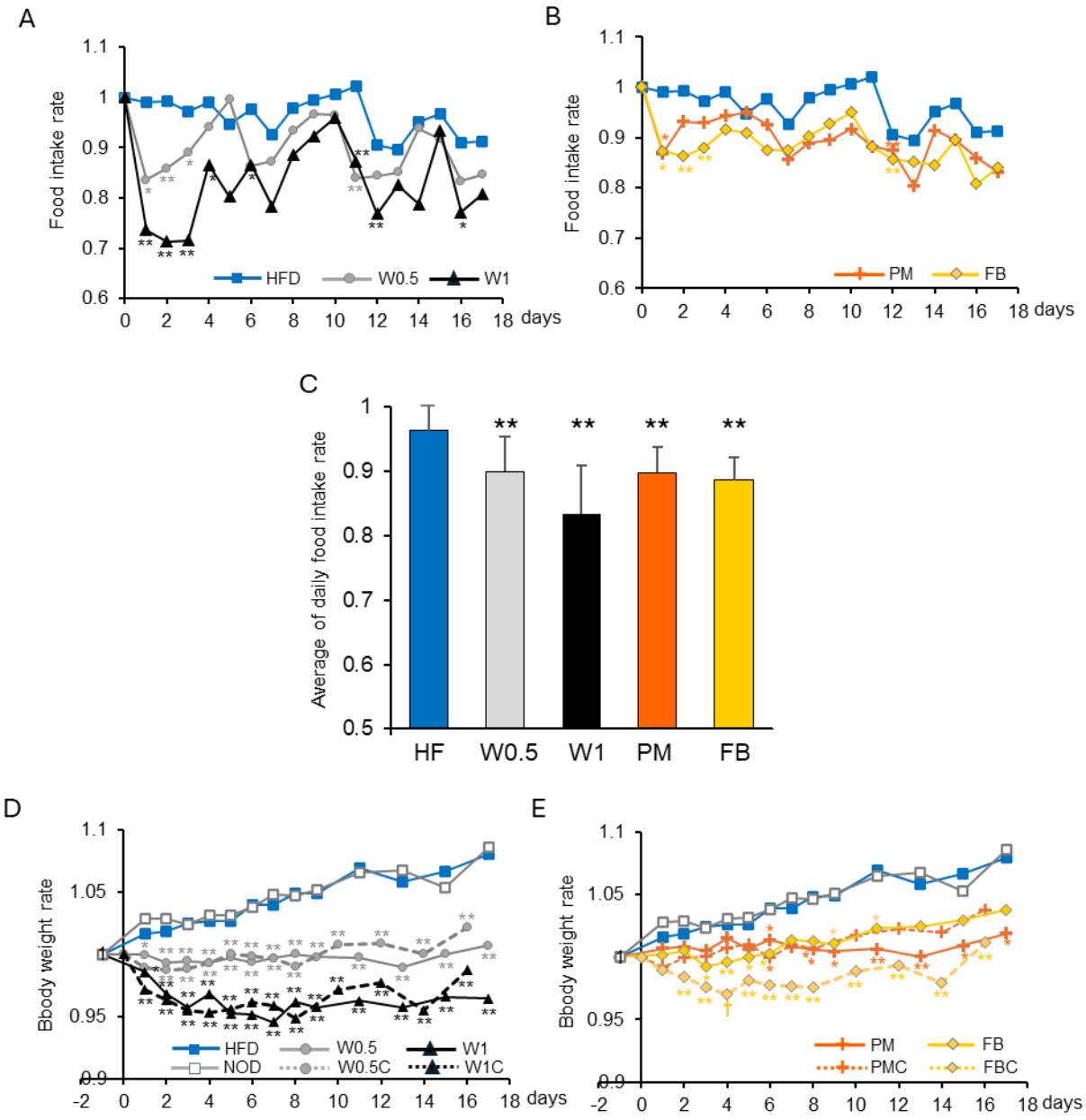

Fig. 3. The food intake and body weight rate relative to the initial values (immediately before SPCM administration). Food intake ratio of (A) the HFD, whole 0.5% (W0.5), whole 1% (W1) groups, (B) PM 0.75% (PM), and FB 0.25% (FB) groups. (C) Average daily food intake rate for 17 days. Body weight rate of (D) the W0.5 and W1 groups and their pair-feeding control groups (W0.5 vs W0.5C, W1 vs W1C) and (E) the PM and FB groups and their pair-feeding control groups (PM vs PMC, and FB vs FBC)

＊P<0.05、＊＊P＜0.01 vs HFD group　†P<0.05 vs FB

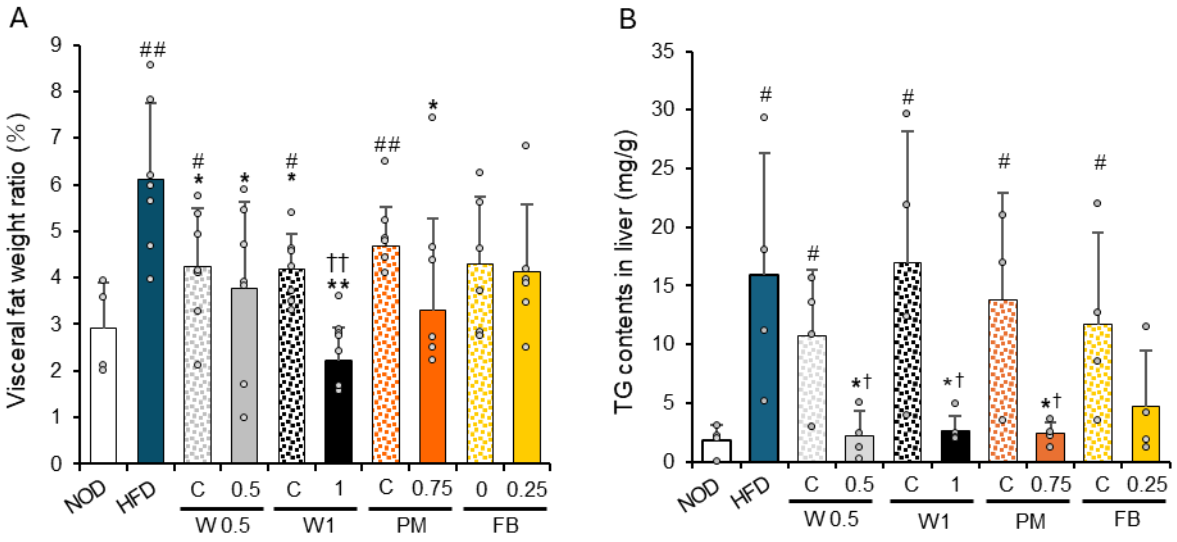

Fig. 4. Visceral fat- and liver triglyceride (TG)-reducing effects of SPCM.

(A) The visceral fat weight (sum of peritesticle and perikidney fat weights) rate to the body weight and (B) liver TG content on days 19 or 20 of SPCM administration. NOD, normal diet group; HFD, high-fat diet group; W0.5, fed HFD containing whole of 0.5% (0.5) and their pair-feeding control (C); W1, containing whole of 1% (1) and their pair-feeding control (C); PM, containing PM of 0.75%(0.75) and their pair-feeding control (C); FB, containing FB of 0.25% and their pair-feeding control (C)

<0.05, # # P＜0.01 vs NOD, ＊P<0.05、 ＊＊P＜0.01 vs HFD group

† P<0.05, †† P<0.01 vs pair feeding group

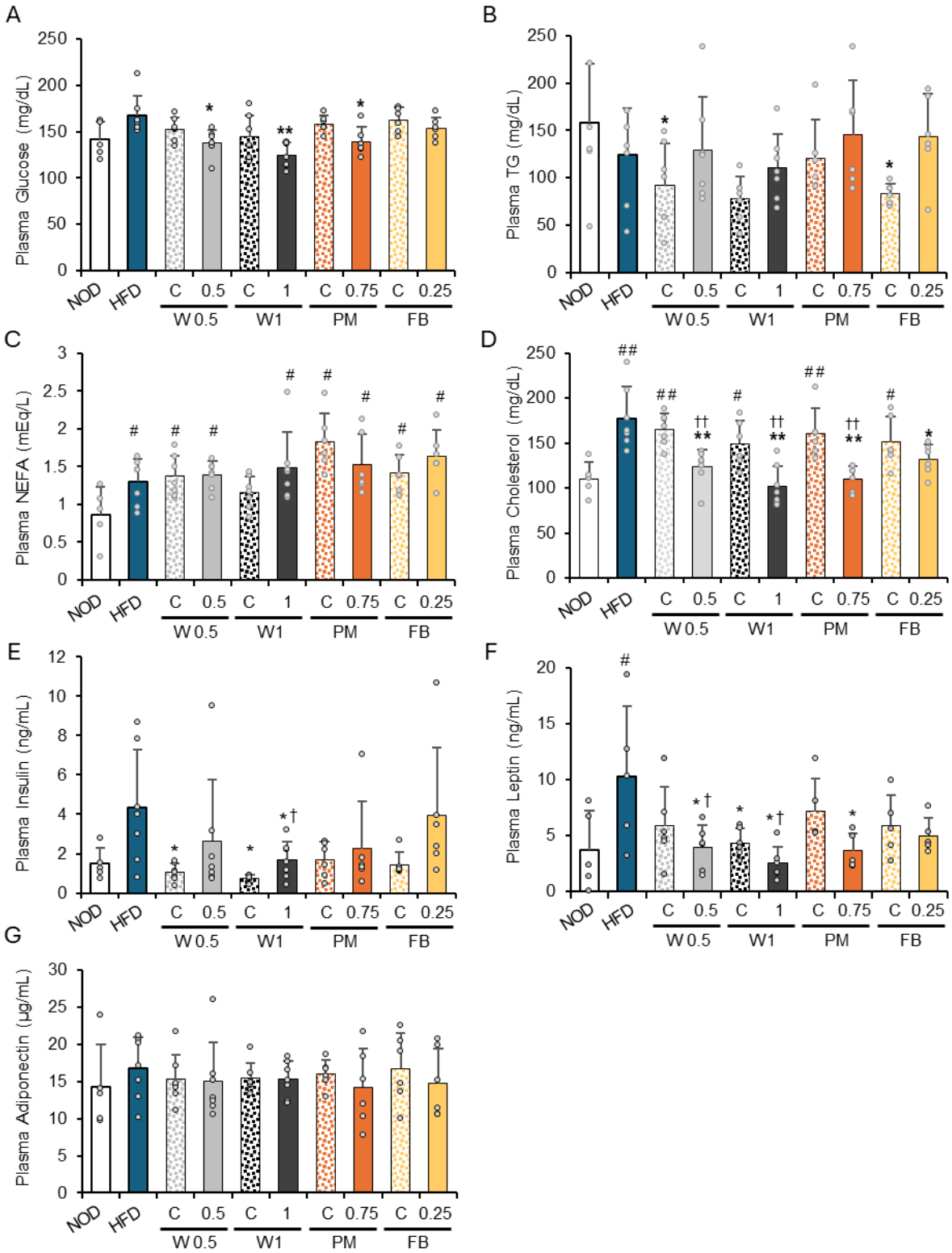

Fig. 5. Blood parameters of glucose and lipid metabolism. (A) Plasma glucose, (B) TG, and (C) NEFA, non-esterified fatty acid, (D) cholesterol, (E) insulin, (F) Leptin, and (G) adiponectin levels on day 18 of SPCM administration. Please refer to Fig. 4 for a description of the horizontal axis labels. # <0.05, # # P＜0.01 vs NOD, ＊P<0.05、 ＊ ＊P＜0.01 vs HFD group

† P<0.05 vs pair feeding group

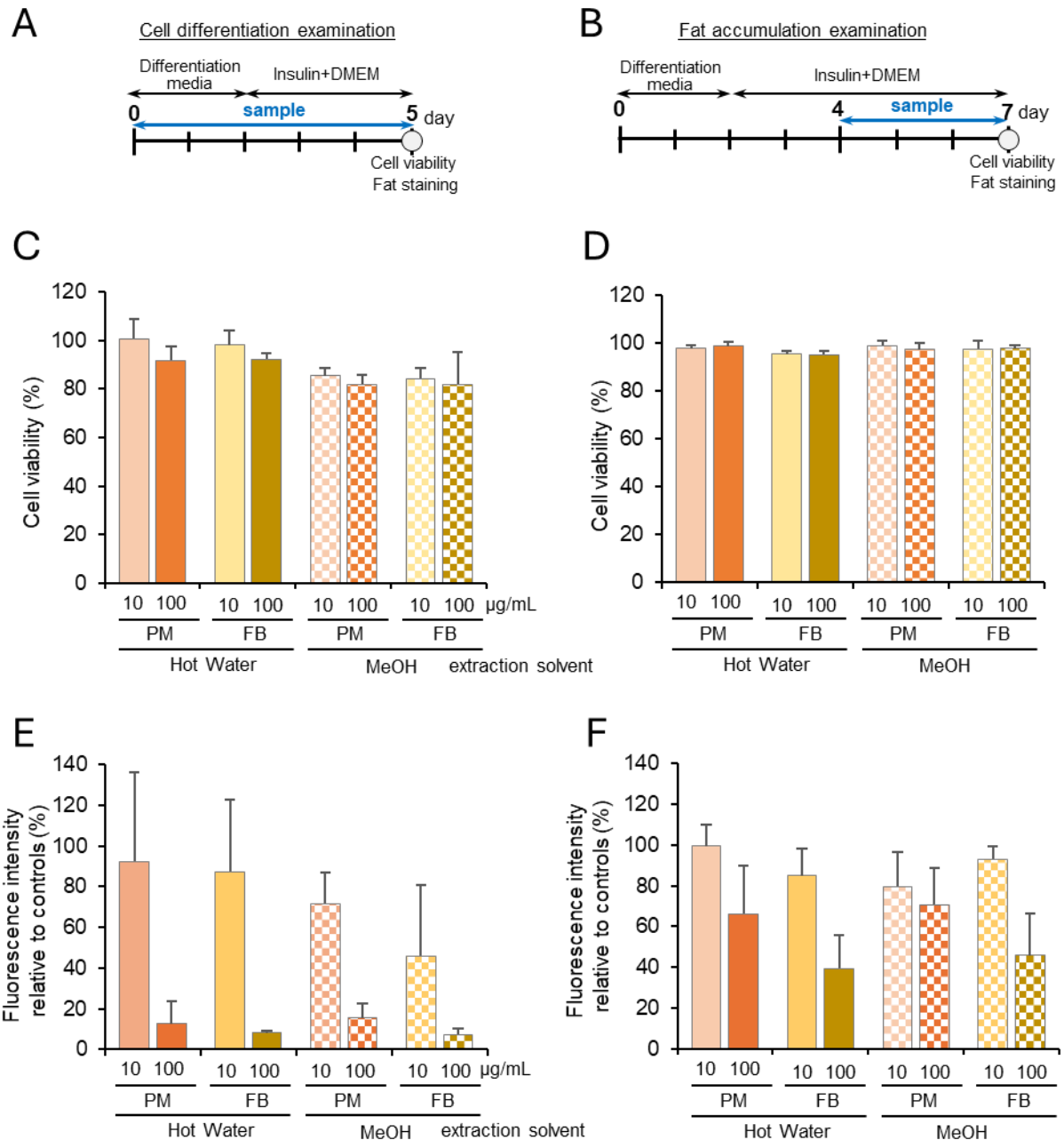

Fig. 6. Effects of SPCM on the differentiation and fat accumulation of 3T3-L1 mouse pre-adipocyte cell line. Experiment schedule for (A) evaluation of cell differentiation and (B) fat accumulation. (C) Cell viability in the experiment for cell differentiation and (D) for fat accumulation. Fluorescence intensity relative to controls as an indicator of (E) adipocyte differentiation and (F) fat accumulation. FB, fruiting body; PM, pupae with mycelium

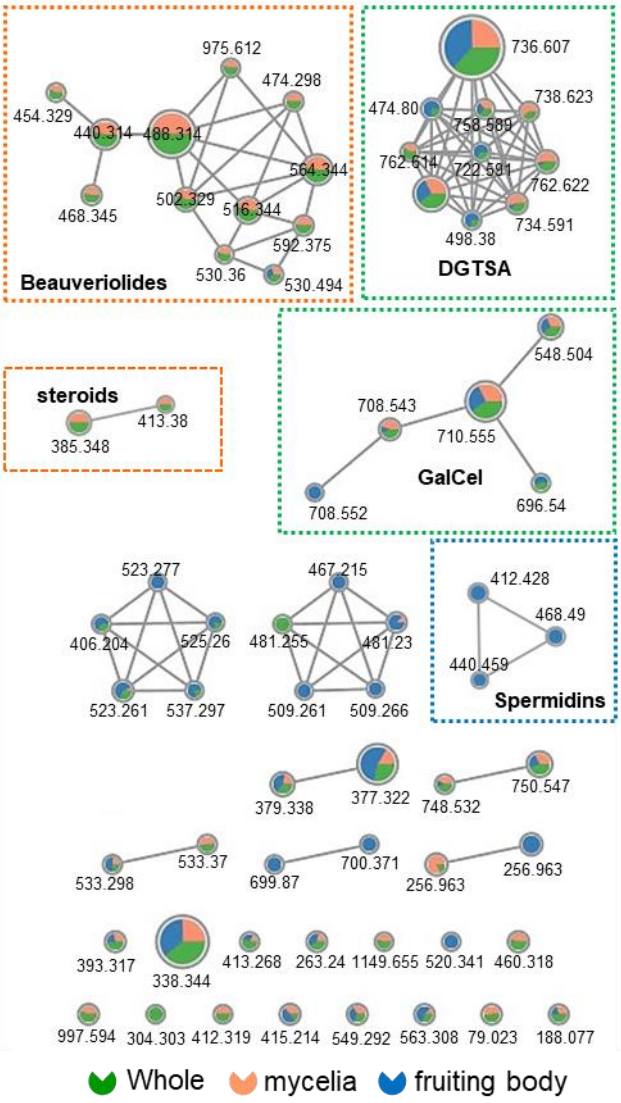

Fig. 7 Molecular networking analysis in the methanol extract of PM, FB, and whole SPCM. The node size represents semiquantitative differences in metabolite concentrations in each extract, and the node colors relate to the presence of each metabolite in different organs. The numbers on the nodes indicate precursor mass. DGTSA represents two types of betaine lipids: 1,2-diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) and 1,2-diacylglyceryl-3-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine (DGTA). GaCel represent galactosylceramidase

Table 1
Comparison of the peak arias of cordycepin

Sample	SPCM		Pupae	*O.sinensis*
	PM	FB	(uninfected)	(market)
Aria	42	135	ND	ND

SPCM : *C.militaris* fermented in silkworm pupae, PM: mycelia (pupae), FB : fruiting body, Pupae: uninoculated silkworm pupae, *O. sinensis* : commercially available powdered *Ophiocordyceps sinensis*