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Basic nutritional investigation

Effects of Japanese diet in combination with exercise on visceral fat accumulation

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ABSTRACT

Objectives: In our previous study, we showed that among Japanese diets from different time periods, the 1975 Japanese diet has the greatest health benefits and is the most effective to prevent obesity. In addition, exercise is also effective to reduce obesity. Therefore, we conducted a human clinical trial combining the 1975 Japanese diet and exercise and, as a result, found a reduction in body weight, visceral fat, and serum lipids. However, the mechanism of this phenomenon was not determined. Therefore, in this study, we examined this mechanism in mice using a diet that was similar to that used in the human trial.
Methods: The modern and 1975 Japanese diets were cooked, lyophilized, powdered, and fed freely to 5 wk old male C57 BL/6 J mice for 8 wk. In addition, the mice exercised on a treadmill.
Results: Total white adipose tissue weight decreased significantly due to the interaction between the 1975 Japanese diet and exercise. A histologic examination revealed that the hypertrophy of adipocytes was suppressed. To clarify this mechanism, the mRNA levels for lipid metabolism-related genes in epididymal adipose tissue were measured, and the mRNA level of hormone sensitive lipase (*Hsl*), which is related to lipolysis, was found to be significantly increased after intake of the 1975 Japanese diet combined with exercise. In the gut microbiota analysis, the Bacteroidetes to Firmicutes ratio, which is decreased in obese people, was increased by the 1975 Japanese diet and exercise. At the genus level, there was an increase in butyrate-producing bacteria as a result of the 1975 Japanese diet intake and exercise.
Conclusions: A combination of the 1975 Japanese diet and exercise increased lipolysis in white adipose tissue and increased butyrate-producing bacteria in gut microbiota, and thereby suppressed fat accumulation.

Introduction

The average life expectancy of the Japanese population continues to increase, and Japanese people have been known for their healthy longevity since Japan became the country with the longest life expectancy in the 1980s [1]. This longevity is partly due to the influence of the Japanese diet, which has less fat and calories than that of the Western diet, and contains a lot of seafood and vegetables. The components of the Japanese diet have been widely studied, but there is little research on the influence of the diet itself on health. In a previous study, we found that the Japanese diet reduced stress compared with the American diet, activated the metabolism, and suppressed obesity [2]. Also, given that the Japanese diet changes over time, we fed mice Japanese diets from 1960, 1975, 1990, and 2005, and found that the 1975 Japanese diet (JD) suppressed obesity most effectively and had a high health benefit that did not depend on the protein/lipid/carbohydrate energy balance [3,4].

The beneficial characteristics of the JD were divided into five elements on the basis of our previous study (Suppl Fig. 1) [5]. The first characteristic is variety, which means that there are many types of food items used. The second one is cookery, meaning that many dishes were prepared using simmering, steaming, and raw cooking methods. The third characteristic is food items for the high use frequency of soy products, fish (shellfish), vegetables (pickles), fruit, green tea, seaweed, and mushrooms. The fourth characteristic is condiment for the high use frequency of soup stock “Dashi” and fermented seasoning (soy sauce, miso, vinegar, mirin, and sake). The final characteristic is form and covers the form of rice and soup that is prepared per meal. As indicated, the benefit of the JD was shown in this study.

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Exercise is also effective to prevent the progression of obesity. The main effects of exercise are the reduction of body fat, improvement of lipid metabolism, lowering of blood pressure, and improvement of glucose tolerance [6]. Because the JD and exercise each provide health benefits, we expected that if they were combined, the health benefit would be enhanced. Therefore, we conducted a human clinical trial combining the JD and exercise. We found that body weight, visceral fat, serum triacylglycerol (TG), and serum low-density lipoprotein cholesterol were reduced with the intake of the JD in addition to moderate exercise [5]. However, the mechanisms of the effects of the combined use of the JD and exercise as well as the influence on the liver and adipose tissue have yet to be clarified.

In this study, we investigated the effect of a combination of the Japanese diet and exercise in mice and examined the mechanisms for the findings in our human trial. In addition, in recent years, the development of obesity and related metabolic diseases has been related to the gut microbiota [7] because a lack of exercise and an unbalanced diet affect its composition [8,9]. Approximately 100 trillion microbes live in the human intestine [10]. The gut microbiota varies depending on environmental variables such as eating habits, antibiotic medications, pathogens, and lifestyle, and is thought to have various effects on the host [11]. Many studies have examined the effect of diet on the gut microbiota, but the relationship between exercise and the gut microbiota has not been widely examined. Therefore, in this study, we also investigated the effect of a combination of the Japanese diet and exercise in mice on changes in the gut microbiota.

Materials and methods

Preparation of test diets

To compare the influence on visceral fat accumulation of the JD and the modern Japanese diet (MD) combined with exercise, two test diets similar to those in the human trial were prepared [5]. A menu for 28 d (84 meals) of the MD was created in accordance with the 2015 National Health and Nutrition Survey with the guidance of a registered dietitian (Suppl. Table 1). A menu for 28 d (84 meals) with the five beneficial features of the JD was created on the basis of the MD with the guidance of a dietitian (Suppl. Fig. 1; Suppl. Table 2). Each cooked diet was lyophilized using a vacuum freeze-dryer (FD-550 R; Tokyo Rikakikai) and homogenized by pulverization and stirring. The products were used as test diets in mice.

The nutrient composition (protein, lipid, carbohydrate, moisture, ash, and energy) of the prepared diet was measured as follows: Protein was measured by a modified Dumas method (Sumigraph NC analyzer NC-220 F; Sumika Chemical Analysis Service); fat by acid digestion; moisture by vacuum oven drying; ash by direct ashing; and carbohydrate by subtracting the fat, protein, moisture, and ash contents from the total amount [12,13]. The energy content was calculated using modified Atwater factors (4, 9, and 4 kcal/g for protein, fat, and carbohydrate, respectively) [12].

Animals

All animal procedures were performed in accordance with the Animal Experiment Guidelines of the Tohoku University, and the animal protocol was approved by the Animal Use Committee at the Tohoku University (2017 AgA-015) [14,15]. C57 BL/6 J mice (4 wk old, male; Japan CLEA) were used for the experiments. During the acclimatization period of 1 wk, 50 mice were fed a powdered CE-2 diet (Japan CLEA) and performed running exercise under preliminary test conditions. At 5 wk of age, all mice were divided into five groups of 10 mice each with nearly equal average body weight between the groups. The mice were fed the MD without exercise (group MD), JD without exercise (group JD), MD with exercise (group MD + E), JD with exercise (group JD + E), and CE-2 without exercise (group CO).

The mice were given free access to their respective diets and distilled water and were housed in a group of five animals per cage in a breeding room at room temperature (24 ± 1°C with a 12-h light/12-h dark cycle). The test period was 8 wk, body weight was measured once a week, and food intake was measured twice a week. At 13 wk of age, the mice were sacrificed by decapitation after fasting for 10.5 h and blood samples were collected. The brain, heart, lungs, liver, spleen, pancreas, kidneys, gastrocnemius muscle, soleus muscle, and white adipose tissues were removed and weighed. The blood samples were centrifuged (4°C; 3000 rpm; 15 min) to obtain serum, and the serum and organs were stored at −80°C until the time of the analysis.

Exercise protocol

Exercise was performed using a treadmill (MK-680; Muromachi Kikai), and movement on the treadmill was carried out from 17:00 h to 20:00 h (just before the start of the dark period, which is the active period of the mouse). The exercise conditions during the acclimatization period were 18 m/min, 10 min/d, 3 d/wk at 0° inclination. After 5 wk of age, the MD + E and JD + E groups performed running exercises under conditions of 20 m/min, 50 min/d, 5 d/wk at 0° inclination from 5 to 6 wk of age; 20 m/min, 50 min/d, 5 d/wk at 5° inclination (condition 1) from 6 to 7 wk of age; and 20 m/min, 50 min/d, 5 d/wk at 10° inclination (condition 2) from 7 to 8 wk of age. From 8 wk of age until the end of the study, the mice alternated between conditions 1 and 2 every other week. The mice were able to do all exercise menus; therefore, they were loaded with exercise of same strength.

Biochemical analyses in serum and liver

The serum and liver biochemical parameters were measured as described in previous reports [16,17]. TG and total cholesterol (TC) levels in serum and the liver were measured using TG and TC Wako E-tests (Wako Pure Chemical), respectively. Phospholipids (PL), non-esterified fatty acid (NEFA), glucose, alanine aminotransferase (ALT), and aspartic acid aminotransferase in serum were measured using a PL C-test Wako, NEFA C-test, Wako glucose CII test, and Wako transaminase CII test (Wako Pure Chemical), respectively. Insulin, leptin, and adiponectin in the serum were measured using a mouse insulin-measuring kit, mouse/rat leptin-measuring kit (both Morinaga Institute of Biological Science), and a mouse/rat adiponectin enzyme-linked immunosorbent assay kit (Otsuka Pharmaceuticals). PL in the liver was measured using the method described by Rouser et al. [16–18]. A microplate reader (Infinit F 200; Tecan, Japan) was used for the absorbance and fluorescence measurements.

Histologic analysis

For the histologic analysis, the liver and epididymal adipose tissue were fixed in 10% formalin and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, stained with hematoxylin and eosin (H&E), and observed using a microscope (BZ-9000; Keyence) [19]. The adipocyte size was calculated by counting the number of cells in a constant view that was selected at random (10 times per mouse) [20].

Messenger RNA expression analysis

For real-time quantitative reverse transcriptase (qRT) polymerase chain reaction (PCR), total RNA was isolated from the liver using an RNeasy Mini Kit (Qiagen), eluted with 30 μl of RNase-free water, and stored at −80°C until use. Total RNA

Table 1
Primer pairs used for the real-time quantitative reverse transcriptase polymerase chain reaction analysis

Genbank identification number	Gene name		Primer sequence(5′ to3′)
NM_133904	*Aco*	Forward	CGCTCACCAACAGTAAGGTGG
		Reverse	GCTTGGCAGGGAGTTCCTC
NM_009948	*Cpt1*	Forward	ATTCTGTGCGGCCCTTATTGGAT
		Reverse	TTTGCCTGGGATGCGTGTAGTGT
NM_009949	*Cpt2*	Forward	CCTGCTCGCTCAGGATAAACA
		Reverse	GTGTCTTCAGAAACCGCACTG
NM_007988	*Fas*	Forward	CCTGGATAGCATTCCGAACCTG
		Reverse	TTCACAGCCTGGGGTCATCTTTGC
NM_008062	*G6 pdx*	Forward	TGGGTCCACCACTGCCACTTTTG
		Reverse	ATTGGGCTGCACACGGATGACCA
NM_001039507	*Hsl*	Forward	TTCTCCAAAGCACCTAGCCAA
		Reverse	TGTGGAAAACTAAGGGCTTGTTG
M29546	*Me*	Forward	GAAAGAGGTGTTTGCCCATGA
		Reverse	AATTGCAGCAACTCCTATGAGG
NM_011144	*Pparα*	Forward	AACATCGAGTGTCGAATATGTGG
		Reverse	AGCCGAATAGTTCGCCGAAAG
NM_011146	*Pparγ*	Forward	GGAAGACCACTCGCATTCCTT
		Reverse	TCGCACTTTGGTATTCTTGGAG
NM_011480	*Srebp1 c*	Forward	GGAGACATCGCAAACAAGC
		Reverse	TGAGGTTCCAAAGCAGACTG

Aco, acetyl-Coenzyme A oxidase 1, palmitoyl; *Cpt1*, carnitine palmitoyl transferase1 beta; *Cpt2*, carnitine palmitoyl tlansferase 2; *Fas*, fatty-acid synthase; *G6 pdx*, glucose-6-phosphate dehydrogenase X-linked; *Hsl*, lipase, hormone sensitive; *Me*, malic enzyme; *Pparα*, peroxisome proliferator activated receptor alpha; *Pparγ*, peroxisome proliferator activated receptor gamma; *Srebp1 c*, sterol regulatory element binding transcription factor 1.

was isolated from epididymal adipose tissue using an RNeasy Lipid Tissue mini kit (Qiagen), eluted with 35 μl of RNase-free water, and stored at −80°C until use [20,21]. Complementary DNA was made from total RNA using Prime Script RT Master Mix and subjected to PCR amplification using SYBR Premix Ex Taq (Perfect Real Time, Takara Bio) and gene-specific primers (Table 1).

For the PCR, a Thermal Cycler Dice Real Time System (Takara Bio) was used and amplification was performed with an activation step at 95°C for 10 s followed by 40 cycles at 95°C for 5 s (denaturation) and 60°C for 31 s (extension) and a dissociation stage at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s for each gene. The messenger RNA (mRNA) expression level was measured by the fluorescence intensity of SYBR green. The threshold cycle (Ct value) was determined, and the mRNA level was calculated relative to that for β-actin.

Gut microbiota analysis

For the analysis of the gut microbiota, pooled feces for 2 d before sacrifice were collected and DNA was extracted from the feces using a DNeasy PowerSoil Kit (Qiagen) for 16S ribosomal RNA region-specific primers to which an adapter sequence for high-speed sequence analysis was added (341 F [5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′], 806R [5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACH VGG GTW TCT AAT-3′]). Tks Gflex DNA Polymerase (Takara Bio) and 2x Gflex PCR Buffer (Mg^{2+}, dNTP plus; Takara Bio) were used to PCR-amplify a specific region on the basis of this DNA. The PCR conditions were one cycle at 94°C for 1 min, 28 cycles at 98°C for 10 s, 50°C for 15 s, and 68°C for 15 s.

For the metagenome analysis, a tag sequence was added to the amplified nucleotide sequence using a Nextera XT Index Kit (Illumina). Nextera XT Index Primer 1, Nextera XT Index Primer 2, Tks Gflex DNA Polymerase, and 2x Gflex PCR Buffer (Mg^{2+}, dNTP plus; Takara Bio) were added to the extracted DNA, and PCR was performed with one cycle at 98°C for 1 min and 8 cycles at 98°C for 10 s, 60°C for 15 s, and 68°C for 15 s. A homology search and lineage classification analysis using the 16S ribosomal RNA database were performed for the obtained sequence (Illumina MiSeq). The analysis of the gut microbiota was entrusted to Takara Bio.

Statistical analysis

The results are expressed as mean ± standard error. The effect of the JD and exercise showed the effect of the diet, exercise, and interaction between the two among four groups (excluding the CO group) by using two-way analysis of variance (ANOVA). For the parameters that showed a significant difference, a one-way ANOVA with Tukey-Kramer post hoc test was used among all five groups (including the CO group). A difference was considered significant at P.

Results

Test diet

The JD was based on the MD but with five strengthened characteristics (Suppl. Fig. 1; Suppl. Table 3). The number of food items per serving was 1.14 times higher; the frequency of boiling, steaming, and raw cooking was 1.26 times higher; the content of soy products, fish and shellfish, vegetables (pickles), fruits, green tea, seaweed, and mushrooms was 1.30 times higher; the use of soup and fermented seasonings (soy sauce, miso, vinegar, mirin, and liquor) was 1.43 times higher; and meals taken in the form of rice and soup were 1.42 times higher in the JD compared with the MD. In terms of diet composition and energy content, there was less lipid per weight in the JD than in the MD (Table 2).

Table 2
Diet compositions of powder CE-2 and test diets (g/100 g)

	CE-2	MD	JD
Protein	25.13	16.7	17.8
Fat	4.9	12.2	8.6
Carbohydrate	54.3	67.3	69.7
Energy (kcal/100 g)	344	412	407

JD, 1975 Japanese diet; MD, modern Japanese diet.
The MD and JD prepared according to the menu were freeze-dried, powdered, and fed to the mice as a test diet. The measured value was obtained by measuring the whole diet after cooking used in this study.

Growth parameters

Growth parameters were measured to investigate how differences in diet and exercise influence body weight and organ weights (Table 3). The final body weight was decreased by exercise. The final body weight in the MD group was significantly higher than that in the CO group, and those in the MD + E and JD + E groups were significantly lower than those in the MD and JD groups, respectively. There was no significant difference in initial body weight, food intake, and energy intake in the respective pairs of groups.

Table 3
Body weight, food intake, and tissue weights

	CO	MD	JD	MD + E	JD + E	Interaction
Initial body weight (g)	19.46 ± 0.40	19.44 ± 0.36	19.54 ± 0.34	19.51 ± 0.34	19.45 ± 0.35	
Final body weight (g)	29.07 ± 0.48†‡	31.96 ± 0.68*	30.96 ± 0.43*	27.42 ± 0.55‡	27.75 ± 0.65‡	E
Food intake (g/d)	3.58 ± 0.10	2.71 ± 0.14	2.96 ± 0.19	2.71 ± 0.12	2.68 ± 0.09	
Energy intake (kcal/d)	12.32 ± 0.36	11.16 ± 0.58	12.04 ± 0.78	11.16 ± 0.49	10.89 ± 0.37	
Tissue weight (g/100 g body weight)						
Brain	1.64 ± 0.01	1.59 ± 0.02	1.61 ± 0.03	1.67 ± 0.02	1.64 ± 0.04	E
Heart	0.52 ± 0.03	0.49 ± 0.01	0.52 ± 0.02	0.50 ± 0.01	0.54 ± 0.04	
Lung	0.90 ± 0.05	1.04 ± 0.07	1.02 ± 0.07	0.91 ± 0.09	0.88 ± 0.10	
Liver	3.93 ± 0.12*	3.82 ± 0.07*	3.88 ± 0.07*	3.27 ± 0.09†	3.29 ± 0.08†	E
Pancreas	0.89 ± 0.04	0.81 ± 0.03	0.84 ± 0.04	0.74 ± 0.04	0.81 ± 0.04	
Spleen	0.27 ± 0.02*	0.28 ± 0.01*	0.27 ± 0.01*	0.21 ± 0.02†	0.26 ± 0.02†	E
Kidney	1.26 ± 0.06	1.14 ± 0.03	1.20 ± 0.03	1.16 ± 0.04	1.22 ± 0.08	
Muscle						
Gastrocnemius muscle (total)	0.99 ± 0.04	0.95 ± 0.01	1.04 ± 0.04	1.03 ± 0.03	1.05 ± 0.06	
Soleus muscle (total)	0.15 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	
White adipose tissue						
Mesenteric	0.58 ± 0.08†‡	1.41 ± 0.15*	0.89 ± 0.10†	0.44 ± 0.07‡	0.54 ± 0.08†‡	D, E, D × E
Perinephric	0.48 ± 0.07†	1.46 ± 0.20*	0.72 ± 0.10†	0.40 ± 0.08†	0.21 ± 0.02†	D, E
Epididymal	1.39 ± 0.22†	3.40 ± 0.33*	1.46 ± 0.18†	1.09 ± 0.12†	1.02 ± 0.14†	D, E, D × E
Total	2.46 ± 0.35†	6.27 ± 0.67*	3.00 ± 0.35†	1.93 ± 0.23†	1.64 ± 0.17†	D, E, D × E

CO, CE-2 without exercise; D, diet; E, exercise; JD, 1975 Japanese diet; MD, modern Japanese diet.
Values are mean ± standard error; n = 8 to 10. The results of two-way analysis of variance were expressed by the effect of D, the effect of E, and the interaction between the two.
*†‡Significantly different means at $P < 0.05$.

Brain weight was increased by exercise load but with no significant differences among the five groups. There were also no significant differences in the heart, lung, pancreas, kidney, gastrocnemius, and soleus muscle weights. Liver weight was decreased by exercise, and liver weights in the MD + E and JD + E groups were significantly lower than those in the CO, MD, and JD groups. Spleen weight was also decreased by exercise and significantly lower in the MD + E group compared with the other groups. Mesenteric adipose tissue weight was decreased by the JD, exercise, and the interaction between the two. This weight was significantly higher in the MD group than in the CO group, significantly

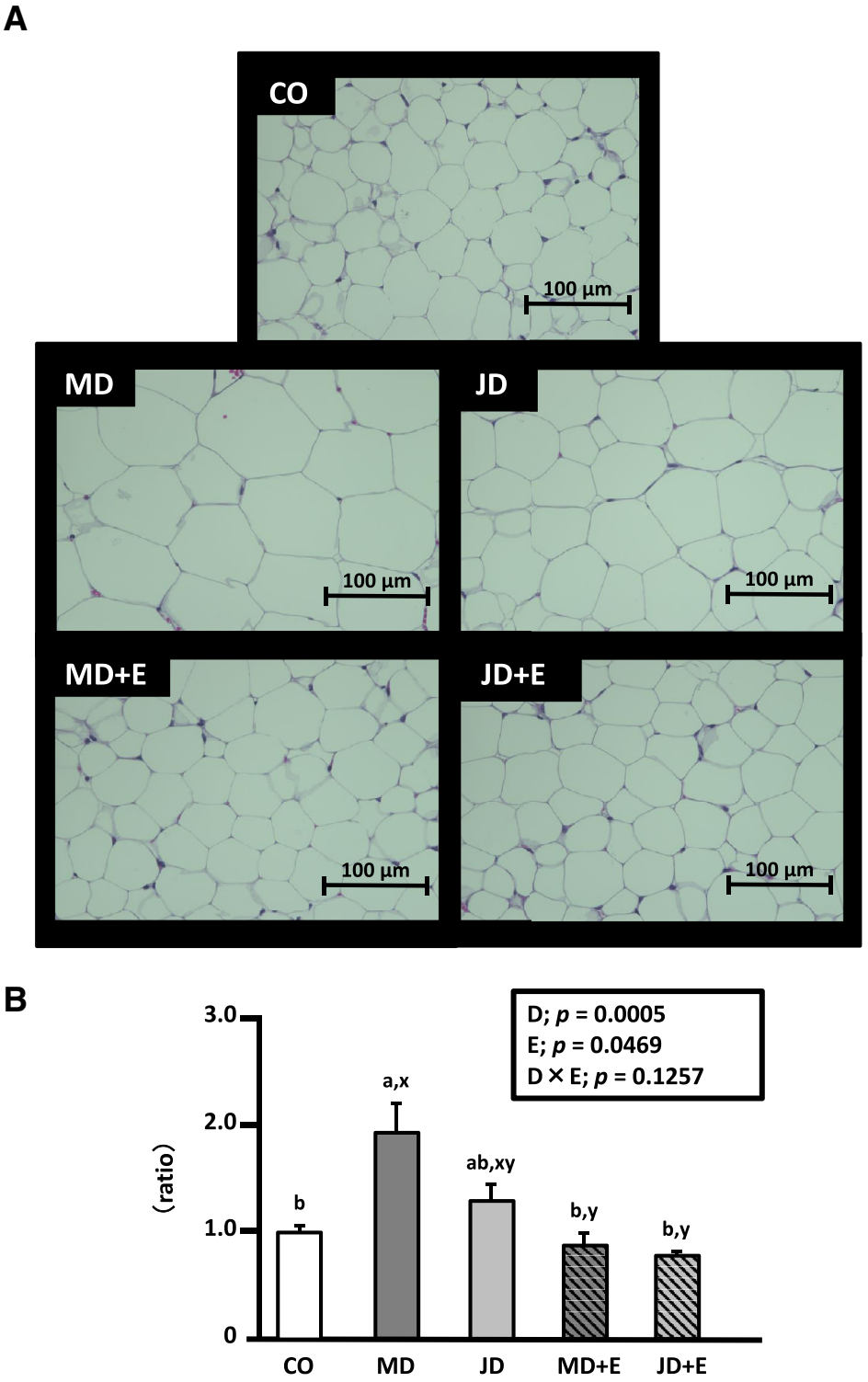

Fig 1. Effects of test diet and exercise on white adipose tissue in mice. (A) Epididymal adipose tissue sections from representative mice in each group (hematoxylin and eosin, scale bar = 100 μm). (B) Average size of adipocytes in epididymal adipose tissue. Values are mean ± standard error, n = 4. Different superscript letters indicate significantly different means at $P < 0.05$. Two-way analysis of variance showed the effects of diet (D), exercise (E), and interaction between diet and exercise (D × E).

lower in the JD, MD + E, and JD + E groups than in the MD group, and significantly lower in the MD + E group compared to with JD group.

Perinephric adipose tissue weight was also decreased by the JD and exercise and was significantly higher in the MD group than in the CO group and lower in the JD, MD + E, and JD + E groups compared with the MD group. Epididymal adipose tissue weight was decreased by the JD, exercise, and the interaction between the two. This weight was significantly higher in the MD group than in the CO group and significantly lower in the JD, MD + E, and JD + E groups than in the MD group. The total white adipose tissue weight was decreased by the JD, exercise, and the interaction between the two. This weight was significantly higher in the MD group than in the CO group and significantly lower in the JD, MD + E, and JD + E groups compared with the MD group. These results show that the white adipose tissue weight was decreased by the intake of the JD and exercise.

Histologic examination of epididymal adipose tissue

Strong effects of diet and exercise on epididymal adipose tissue weight were found; therefore, this tissue was stained with H&E and adipocytes were observed (Fig. 1). The size of the adipocytes was the largest in the MD group and the smallest in the JD + E group (Fig. 1A). The adipocyte area was decreased by the JD and exercise. This area was significantly higher in the MD group than in the CO group and significantly lower in the MD + E and JD + E groups than in the MD group (Fig. 1B). These results show that adipocyte hypertrophy in epididymal adipose tissue was strongly suppressed by the JD and exercise.

Messenger RNA expression analysis in epididymal adipose tissue

The significant differences in epididymal adipose tissue weight and adipocyte size prompted an analysis of mRNA levels of lipid metabolism-related genes in epididymal adipose tissue (Table 4). The mRNA level for fatty-acid synthase (*Fas*), which is related to fatty-acid synthesis, was increased by the JD and was higher in the JD + E group than in the MD and MD + E groups. There were no significant differences in mRNA levels for malic enzyme (*Me*), glucose-6-phosphate dehydrogenase X-linked (*G6 pdx*), and sterol regulatory element binding transcription factor 1 (*Srebp1 c*), which are also related to fatty-acid synthesis. The mRNA level for *Hsl*, which is related to lipolysis, was decreased by the JD and exercise and lower in the MD group compared with the CO and JD + E groups. The mRNA level for peroxisome proliferator activated receptor gamma (*Pparγ*), which controls the differentiation and proliferation of adipocytes, was decreased by exercise load and lower in the MD group than in the CO and MD + E groups. These results suggest that the intake of the JD and exercise promote fatty-acid synthesis, fatty-acid degradation, and adipocyte differentiation and proliferation.

Table 4
Messenger RNA expression level of lipid metabolism-related gene in epididymal adipose tissue

	CO	MD	JD	MD + E	JD + E	Interaction	Gene function
Fas	1.00 ± 0.16*†	0.74 ± 0.08†	1.07 ± 0.14*†	0.70 ± 0.11†	1.43 ± 0.12*	D	Fatty-acid synthesis
Me	1.00 ± 0.18	1.00 ± 0.14	1.11 ± 0.22	1.18 ± 0.18	1.60 ± 0.34		
G6 pdx	1.00 ± 0.09	1.45 ± 0.22	1.45 ± 0.26	1.70 ± 0.20	1.54 ± 0.31		
Srepb1 c	1.00 ± 0.23	0.65 ± 0.12	0.99 ± 0.16	0.75 ± 0.13	1.06 ± 0.14		
Hsl	1.00 ± 0.18*	0.39 ± 0.06†	0.57 ± 0.08*†	0.68 ± 0.09*†	0.98 ± 0.16*	D, E	Neutral lipolysis
Pparγ	1.00 ± 0.18*	0.48 ± 0.08†	0.66 ± 0.08*†	0.85 ± 0.08*	0.79 ± 0.11*†	E	Differentiation

CO, CE-2 without exercise; D, diet; E, exercise; *Fas*, fatty-acid synthase; *G6 pdx*, glucose-6-phosphate dehydrogenase X-linked; *Hsl*, lipase, hormone sensitive; JD, 1975 Japanese diet; MD, modern Japanese diet; *Me*, malic enzyme; *Srebp1 c*, sterol regulatory element binding transcription factor 1; *Pparγ*, peroxisome proliferator activated receptor gamma.
Values are mean ± standard error; n = 8 to 10. The results of two-way analysis of variance were expressed by the effect of D, the effect of E, and the interaction between the two.
*† Significantly different means at $P < 0.05$.

Table 5
Biochemical parameter of serum

	CO	MD	JD	MD + E	JD + E	Interaction
TG (mmol/L)	1.38 ± 0.06	1.55 ± 0.10	1.39 ± 0.16	1.23 ± 0.15	1.26 ± 0.11	
TC (mmol/L)	2.36 ± 0.06	3.05 ± 0.17	2.91 ± 0.09	2.92 ± 0.08	2.79 ± 0.12	
PL (mmol/L)	70.0 ± 1.3	81.0 ± 2.8	81.4 ± 3.7	79.9 ± 4.3	77.8 ± 4.5	
NEFA (mEq/L)	1.44 ± 0.07	1.60 ± 0.12	1.15 ± 0.16	1.30 ± 0.16	1.10 ± 0.12	D
Glucose (mmol/L)	8.90 ± 0.47*†	7.39 ± 0.27†	10.22 ± 0.68*	7.67 ± 0.69†	9.12 ± 0.78*†	D
Insulin (μg/L)	0.23 ± 0.07	0.15 ± 0.05	0.21 ± 0.03	0.21 ± 0.08	0.26 ± 0.04	
HOMA-IR	1.00 ± 0.29	0.56 ± 0.19	1.02 ± 0.21	0.84 ± 0.29	0.90 ± 0.11	
Leptin (ng/mL)	0.97 ± 0.32‡	3.84 ± 0.70*	3.00 ± 0.67*†	0.47 ± 0.13‡	1.09 ± 0.25†‡	E
Adiponectin (μg/mL)	18.3 ± 2.4	44.7 ± 4.4	42.7 ± 1.9	40.3 ± 4.8	42.7 ± 4.4	
ALT (IU/L)	4.62 ± 0.15*	2.80 ± 0.23†‡	2.77 ± 0.22†‡	3.27 ± 0.26†	2.11 ± 0.28‡	D, D × E
AST (IU/L)	66.7 ± 2.1	58.5 ± 4.2	56.4 ± 5.0	64.2 ± 6.7	49.6 ± 11.5	

ALT, alanine aminotransferase; AST, aspartic acid aminotransferase; CO, CE-2 without exercise; D, diet; E, exercise; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; JD, 1975 Japanese diet; MD, modern Japanese diet; NEFA, non-esterified fatty acid; PL, phospholipids; TC, total cholesterol; TG, triacylglycerol.
Values are mean ± standard error; n = 8 to 10. The results of two-way analysis of variance were expressed by the effect of D, the effect of E, and the interaction between the two.
*†‡Significantly different means at $P < 0.05$.

Table 6
Biochemical parameter of liver

	CO	MD	JD	MD+E	JD+E	Interaction
TG (mmol/L)	14.6 ± 1.6[†]	29.8 ± 2.3[*]	18.1 ± 2.3[†]	18.7 ± 1.3[†]	15.8 ± 3.7[†]	D, E
TC (mmol/L)	3.41 ± 0.32[*†]	4.57 ± 0.30[*]	3.91 ± 0.38[*†]	3.32 ± 0.16[*†]	2.58 ± 0.36[†]	D, E
PL (mmol/L)	31.5 ± 1.5	31.4 ± 0.85	32.1 ± 2.1	32.6 ± 1.8	29.3 ± 2.4	

CO, CE-2 without exercise; D, diet; E, exercise; JD, 1975 Japanese diet; MD, modern Japanese diet; PL, phospholipids; TC, total cholesterol; TG, triacylglycerol.
Values are mean ± standard error; n = 8 to 10. The results of two-way analysis of variance were expressed by the effect of D, the effect of E, and the interaction between the two.
[*†] Significantly different means at $P < 0.05$.

Biochemical parameters in serum

Serum parameters were measured to investigate the effects of diet and exercise on serum lipids and carbohydrates (Table 5). There were no significant differences in serum TG, TC, and PL levels. The serum NEFA level was decreased by JD but with no significant differences among the five groups. Serum glucose was increased by the JD, and glucose found to be significantly higher in the JD group than in the MD and MD + E groups. However, there were no significant differences in the serum insulin level and the Homeostatic Model Assessment of Insulin Resistance (index of insulin resistance).

Serum leptin was decreased by exercise, and leptin levels were significantly higher in the MD and JD groups compared with the CO and MD + E groups, and significantly lower in the JD + E group than in the MD group. There was no significant difference in the serum adiponectin level. Serum ALT was decreased by the JD and through an interaction between diet and exercise. Serum ALT was significantly higher in the CO group than in the other four groups and significantly lower in the JD + E group than in the MD + E group. There was no significant difference in serum aminotransferase. These results show that the JD and exercise did not significantly affect serum lipid and glucose metabolic parameters but improved liver function.

Biochemical parameters in the liver

Liver parameters were measured to investigate how differences in diet and exercise influence liver function (Table 6). The liver TG level was decreased by the JD and exercise, and this level was significantly higher in the MD group than in the CO group and significantly lower in the JD, MD + E, and JD + E groups compared with the MD group. The liver TC level was also decreased by the JD and exercise, and this level was significantly lower in the JD + E group than in the MD group. There were no significant differences in liver PL levels. These results show that the JD and exercise strongly suppress lipid accumulation in the liver.

Histologic observations in the liver

Since there were significant differences in liver TG and TC levels, liver tissue was stained with H&E and hepatocytes were observed (Fig. 2). Significant lipid accumulation was present in the MD group, which is consistent with the increases in liver TG and TC, and lipid accumulation was suppressed by the JD and exercise. These results confirm that the intake of the JD and exercise suppress liver lipid accumulation.

Messenger RNA expression analysis in the liver

The mRNA levels of lipid metabolism-related genes in the liver were measured (Table 7) to examine the mechanism of suppression of lipid accumulation in the liver and improved liver function by the JD and exercise. The mRNA level for *Fas*, which is related to fatty-acid synthesis, was decreased by exercise, but there were no significant differences among the five groups. The mRNA level for *Me*, which is related to fatty-acid synthesis, was decreased by the JD and exercise and significantly lower in the MD + E and JD + E groups than in the CO and MD groups. There were no significant differences in mRNA levels for *G6 pdx* and *Srebp1 c*, which are related to fatty-acid synthesis.

The mRNA level for acetyl-Coenzyme A oxidase 1, palmitoyl *(Aco)*, which is related to fatty-acid β oxidation, was decreased by the JD, exercise, and the interaction between the two, and was significantly lower in the JD + E group than in the CO group and in the JD, MD + E, and JD + E groups compared with the MD group. The mRNA level for carnitine palmitoyl transferase 1 beta (*Cpt1)*, which is related to fatty-acid β-oxidation, was decreased by the JD and exercise and significantly higher in the MD group than in the CO group and in the JD, MD + E, and JD + E groups compared with the MD group. The mRNA level for carnitine palmitoyl tlansferase 2 (*Cpt2)*, which is involved in β-oxidation, was decreased by exercise and the interaction between the JD and exercise and was significantly lower in the MD + E group than in the MD group. The mRNA level for peroxisome proliferator activated receptor alpha (*Pparα)*, which regulates fatty-acid β-oxidation, was affected by an interaction between diet and exercise but with no significant differences among the five groups. These results show that JD intake and exercise suppress both fatty-acid synthesis and β-oxidation in the liver.

Gut microbiota

The gut microbiota analysis was conducted to investigate how differences in diet and exercise influence the intestinal flora (Fig. 3; Suppl. Fig. 2; Suppl. Tables 4 and 5). The bacteria in the *Firmicutes* phylum, which increase due to ingestion of a high-fat diet, were decreased by exercise in mice that ingested the MD and JD. The bacteria in the *Bacteroidetes* phylum, which are reduced by the ingestion of a high-fat diet, were increased by exercise in mice that ingested both diets. A total of 85 bacteria were detected at the genus level.

We also explored the behavior of these genera with respect to white adipose tissue weight, which had a marked relationship with the interaction between diet and exercise. Six genera were identified, and the genus with the highest level in the MD group and the lowest level in the JD + E group (similarly to white adipose tissue weight) was *Bifidobacterium*. In contrast, *Bacillus, Bacteroides, Clostridium, Ruminococcus*, and *Weissella* had the lowest levels in the MD group and the highest levels in the JD + E group. These results show that combined JD intake and exercise have a major influence on the composition of the gut microbiota.

Discussion

In this study, we examined the effect of combined exercise and diet on visceral fat accumulation, using a diet that included the characteristics of the JD. The health benefit of this diet was shown in our previous study. We found that lipid accumulation in white adipose tissue and the liver was strongly inhibited by an interaction between the JD and exercise compared with the MD.

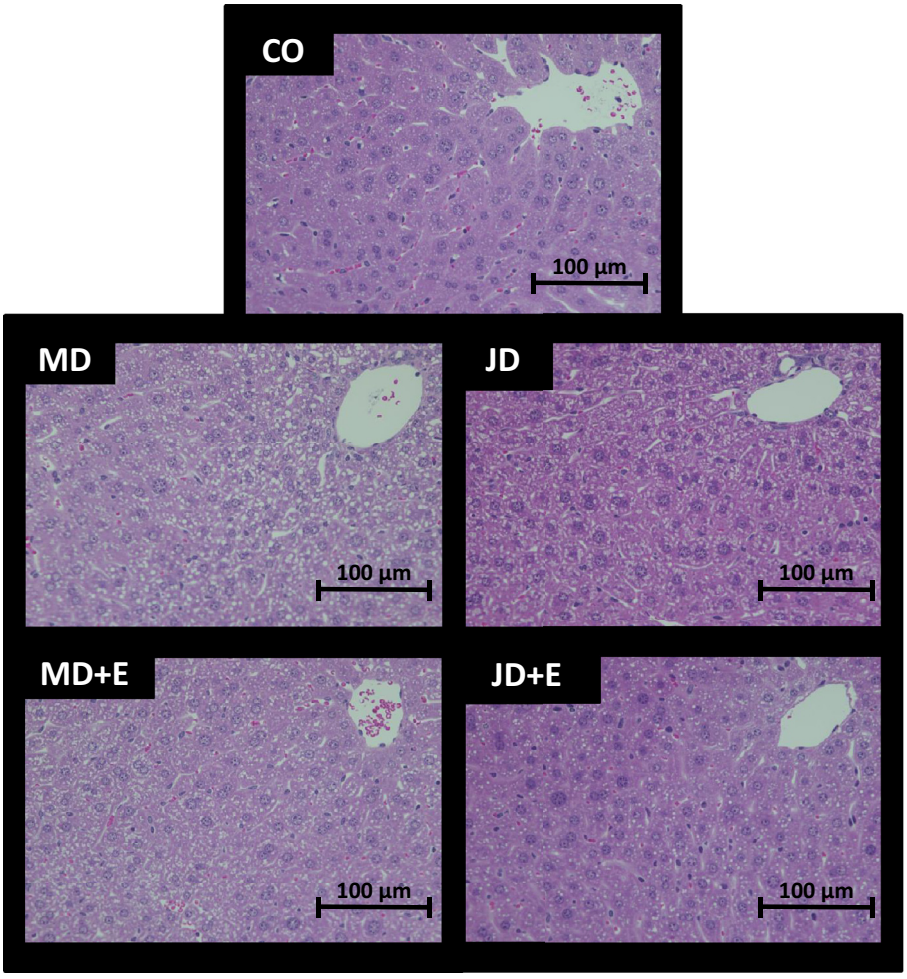

Fig 2. Effects of test diet and exercise on the liver in mice. Liver sections from representative mice in each group (hematoxylin and eosin, scale bar = 100 μm).

Epididymal adipose tissue weight decreased in mice after the ingestion of the JD and exercise, and the interaction between diet and exercise shows that visceral fat was synergistically decreased by the combination of JD and exercise. The decreased size of adipocytes and changes in mRNA levels for lipid metabolism-related genes in epididymal adipose tissue were also consistent with reduced lipid accumulation after ingestion of the JD and exercise.

The mRNA level for *Pparγ*, which controls the differentiation and proliferation of adipocytes, increased after the ingestion of the JD and further increased with the addition of exercise. *Pparγ* plays an important role in the suppression of excessive hypertrophy and maintenance of the normal size of adipocytes [22]. The mRNA level for *Hsl*, which is related to lipolysis, increased in mice after JD intake and exercise. *Hsl* is a major fat lipase [23] and exercise increases the protein

Table 7
Messenger RNA expression level in liver

	CO	MD	JD	MD + E	JD + E	Interaction	Gene function
Fas	1.00 ± 0.16	1.30 ± 0.13	1.49 ± 0.19	0.90 ± 0.12	1.10 ± 0.09	E	Fatty-acid synthesis
Me	1.00 ± 0.13*	1.01 ± 0.06*	0.83 ± 0.03*†	0.65 ± 0.04†	0.57 ± 0.04†	D, E	
G6 pdx	1.00 ± 0.09	0.83 ± 0.04	0.84 ± 0.07	0.79 ± 0.05	0.71 ± 0.04		
Srepb1 c	1.00 ± 0.14	1.28 ± 0.17	1.15 ± 0.16	1.07 ± 0.21	1.40 ± 0.24		
Aco	1.00 ± 0.08*†	1.02 ± 0.04*	0.76 ± 0.07†‡	0.75 ± 0.05†‡	0.74 ± 0.04†‡	D, E, D × E	Fatty-acid β-oxidation
Cpt1	1.00 ± 0.09†	2.13 ± 0.19*	1.25 ± 0.15†	1.38 ± 0.18†	1.09 ± 0.21†	D, E	
Cpt2	1.00 ± 0.05*†	1.10 ± 0.03*	0.99 ± 0.03*†	0.89 ± 0.02†	0.99 ± 0.04*†	E, D × E	
Pparα	1.00 ± 0.08	1.09 ± 0.05	0.82 ± 0.10	0.89 ± 0.08	1.00 ± 0.06	D × E	

CO, CE-2 without exercise; D, diet; E, exercise; *Fas*, fatty-acid synthase; *G6 pdx*, glucose-6-phosphate dehydrogenase X-linked; *Hsl*, lipase, hormone sensitive; JD, 1975 Japanese diet; MD, modern Japanese diet; *Me*, malic enzyme; *Srebp1 c*, sterol regulatory element binding transcription factor 1; *Pparγ*, peroxisome proliferator activated receptor gamma.
Values are mean ± standard error; n = 8 to 10. The results of two-way analysis of variance were expressed by the effect of D, the effect of E, and the interaction between the two.
*†‡Significantly different means at $P < 0.05$.

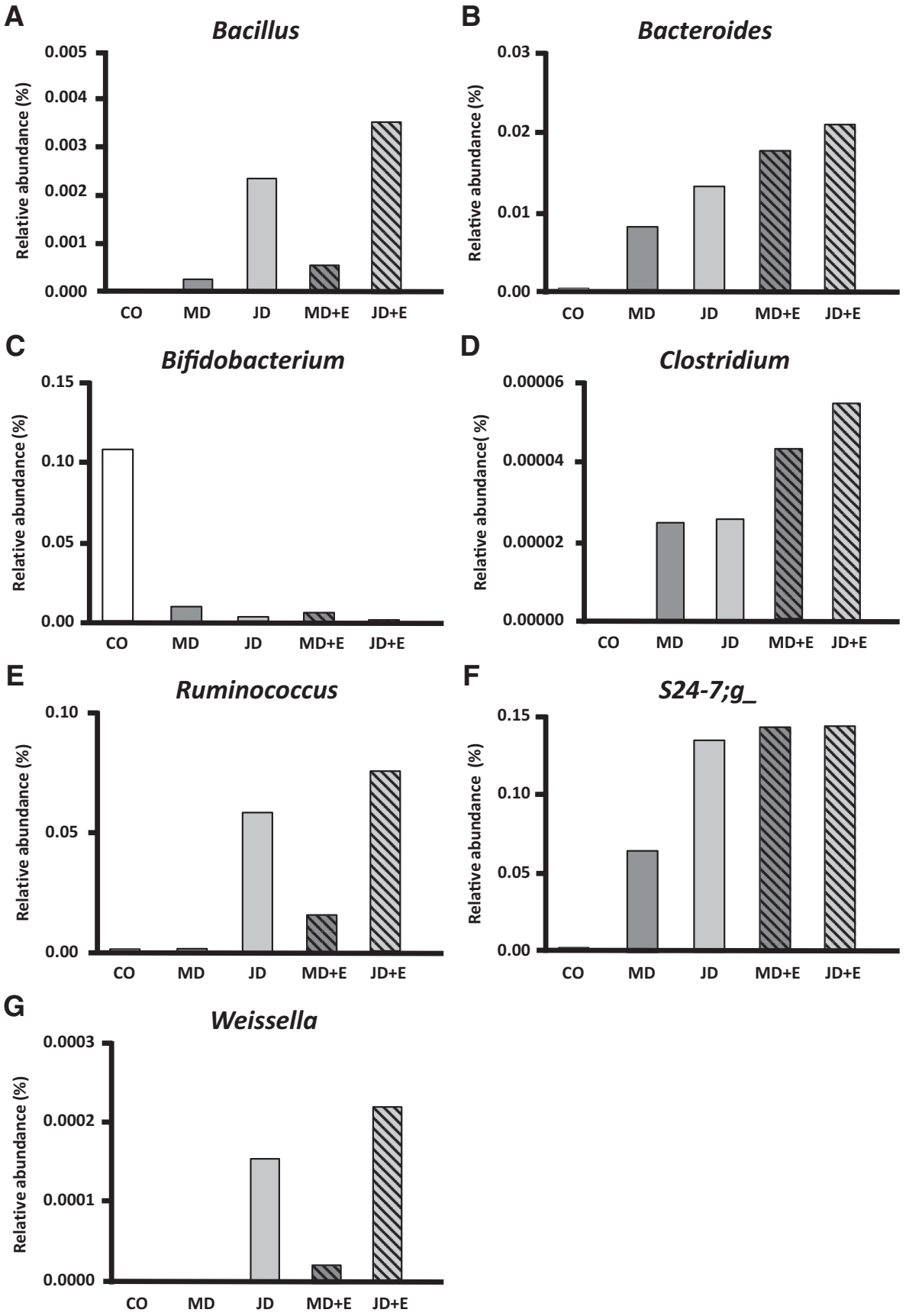

Fig. 3. Effects of test diet and exercise on gut microbiota in mice. Relative abundance of (A) *Bacillus*, (B) *Bacteroides*, (C) *Bifidobacterium*, (D) *Clostridium*, (E) *Ruminococcus*, (F) *S24 to 7; g*, and (G) *Weissella*.

level of *Hsl* [24]. In our previous study, *Hsl* mRNA showed an increasing trend with JD intake [25]. Therefore, the increase in mRNA for *Hsl* appears due to both exercise and diet, and the increases in mRNA levels for *Pparγ* and *Hsl* both suppressed adipocyte hypertrophy.

The mRNA level for *Fas*, which promotes fatty-acid synthesis [26], increased in mice after the ingestion of the JD and exercise. Fatty-acid biosynthesis is activated in rats that are subjected to calorie restriction even though the weight of white adipose tissue decreased [27] and the JD had a calorie restriction-like effect in our previous study [25]. Therefore, the calorie restriction-like effect of the JD was further enhanced by exercise, which resulted in visceral fat strongly decreasing, and the fatty-acid synthesis pathway was activated to compensate for this effect.

Liver TG and TC were the lowest in mice after the ingestion of the JD and exercise, which shows that these actions suppressed lipid accumulation in the liver. To confirm this finding, liver tissue was stained with H&E. The results showed that the number of lipid droplets in hepatocytes were reduced by the JD and exercise. To clarify this mechanism, the mRNA levels of lipid metabolism-related genes in the liver were measured. The mRNA levels for *Me* and *G6 pdx*, which are related to fatty-acid synthesis [28,29], and those for *Cpt1* and *Aco*, which are related to fatty-acid β-oxidation [30,31], were the lowest after ingestion of the JD and exercise. Therefore, fatty-acid synthesis was inhibited, lipid accumulation in the liver was decreased, and genes related to β-oxidation were decreased because of the reduction in liver lipid accumulation after the combination of JD intake and exercise.

Serum glucose levels increased significantly in mice that ingested the JD, but this was due to the high carbohydrate content of this diet. However, there was no significant difference in the Homeostatic Model Assessment of Insulin Resistance (index of insulin resistance), which suggests that the change in glucose level did not have an adverse effect on glucose metabolism. Exercise improves glucose tolerance [6], and glucose levels decreased in mice after the intake of the JD and exercise. These results show that the combination of the JD and exercise had no significant effect on glucose metabolism.

The Bacteroidetes to Firmicutes ratio is increased in the gut microbiota of mice after long voluntary exercise [32,33]. The gut microbiota varies depending on activities such as eating habits, antibiotics, pathogens, and lifestyle, and is thought to have various effects on the host [11]. Recently, the relationship between the gut microbiota and the cause of obesity and related metabolic diseases has become apparent [7]. The gut microbiota is dominated by Bacteroidetes and Firmicutes and in obese people, the relative proportion of Bacteroidetes decreases and that of Firmicutes increases [34]. In this study, the Bacteroidetes to Firmicutes ratio was increased by exercise and inversely related to white adipose tissue weight. Therefore, the change in balance of the gut microbiota may be a factor in the decrease in total white adipose tissue weight.

Among the gut microbiota at the genus level that showed the same or an opposite relationship to white adipose tissue weight, the bacteria with the highest occupancy belonged to the S24 to 7 family, and the proportion was increased by exercise and JD intake. The S24 to 7 family are butyrate-producing bacteria and are significantly increased by exercise, which shows an association between voluntary exercise and diet-induced obesity [32]. Bacteria in the *Clostridiales* order are also butyrate-producing [35], and bacteria of the genus *Clostridium* were increased by exercise and the JD in this study. Therefore, the production of butyric acid was increased by exercise and the JD.

In addition, almost all species in the genus *Bacteroides* produce acetate [33]. Therefore, acetic acid, which is a short-chain fatty acid, was increased because the *Bacteroides* genus was increased by exercise and the JD. Short-chain fatty acids have an antiinflammatory effect through the suppression of production of proinflammatory molecules by inhibiting inflammatory pathways [36], and also promote energy metabolism [37]. Thus, short-chain fatty acids both prevent inflammation in the intestine and control obesity by regulating metabolism. These results suggest that the functions of the gut microbiota are related to the suppression of visceral fat accumulation by the JD and exercise.

The genera *Clostridium, Bacteroides*, and *Ruminococcus* increase in humans who ingest diets that are rich in resistant starch [38]. This starch is contained in foods such as beans and potato and not digested in the large intestine [39]. In this study, these bacteria may have increased because the amount of cooked soybeans was higher in the JD than in the MD. The *Bifidobacterium* genus decreased in the other four groups compared with the CO group and was decreased by exercise and JD intake.

The composition of the gut microbiota is changed by stress that may occur due to changes in diet and exercise [40] and may explain the decrease in *Bifidobacterium* in this study. *Bacillus* is a probiotic that improves intestinal gut microbiota and the immune function and lowers cholesterol levels [41], and *Bacillus subtilis (natto)* is in the *Bacillus* genus. There is more *natto* in the JD, which may have increased *Bacillus* after ingestion of this diet. This increase was also enhanced by exercise. *Weissella* is found in pickles and has an anti-obesity effect [42]. The JD consists of more pickles than the MD, which may have increased *Weissella*. Therefore, changes in the gut microbiota may have been related to the anti-obesity effect of the JD and exercise that were observed in this study.

Conclusions

This study showed that a combination of the JD and exercise strongly suppresses increases in white adipose tissue weight, adipocyte hypertrophy, and lipid accumulation in the liver. Exercise in this study was moderate and equivalent to 50% to 70% of the maximum oxygen consumption [43], which is similar to light running in humans. Therefore, well-balanced light running and the intake of a diet with ingredients such as those in the JD had a synergistic positive influence on the maintenance of health.

Supplementary data

Supplementary data related to this article can be found, at http://doi.org/10.1016/j.nut.2018.05.023.

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