Effects of voluntary exercise on plasma and urinary metabolites and gut microbiota in mice fed with high-fat-diet

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Abstract The aim of this study was to examine the effects of voluntary exercise on plasma and urinary metabolites and the changes in gut microbiota in mice fed with a high-fat-diet. Healthy male C57BL/6J mice (four-week-old, n = 27) were fed a normal controlled diet (CD) and a high-fat-diet (HFD) for 10 weeks under two conditions: voluntary wheel running (W) and sedentary controlled condition (C). The metabolites in the collected plasma and urine were detected using ¹H-NMR spectroscopy techniques. Also, 16S-rRNA gene next-generation sequencing was carried out on the collected feces. Wheel running activity in HFDW mice was slightly higher than that in CDW mice (p = 0.075). Exercise and diet significantly altered body weight, fat accumulation, and glucose tolerance tests. In plasma, amino acids such as Leu, Ile, Ala and Tyr, were increased by exercise. Diet influenced the metabolites in both the plasma and urine of mice and showed significant differences; in plasma, Leu, Ile, Glu, 3-HB, lactate and acetate, whereas in urine, citrate, trimethylamine (TMA), trimethylamine-N-oxide (TMAO), taurine, hippurate and allantoin. With regard to beta diversity, unweighted UniFrac analysis (principal coordinate analysis: PCoA) showed the difference between CD and HFD mice could be observed under PC1 (22.61%). Although there was substantial overlap between two conditions (C vs. W), HFD groups were positioned in a slightly different area when compared to the C and W conditions. *Firmicutes/Bacteroidetes* ratio, which might be associated with obesity, in HFD mice was significantly higher than that in CD mice, but not affected by wheel running. Our results suggest that testing in both plasma and urinary metabolites may prove to be a more reliable approach to quantitative metabolite analysis on the effects of exercise-dependent hosts or as an independent alteration of gut microbiota on the hosts.

Keywords : wheel running, cecal microbiota, metabolome analysis

Introduction

Poor health, such as obesity and associated various disorders, is linked to sedentary lifestyle; but the corollary of this is that exercise offers preventive effects¹⁾. The scope of the beneficial effects of exercise extends well beyond energy expenditure, and has been termed “Exercise is Medicine (EIM)” by the American College of Sports Medicine (ACSM), to refer to not only safe and reliable disease prevention, but also treatment²⁾.

It has been well reported that high-fat-diet-induced obesity is associated with changes in gut microbiota³⁻⁶⁾. It is especially known that a balance of phyla *Firmicutes* and *Bacteroidetes* (F/B ratio) is significantly associated with obesity³⁾. Also, recent studies investigating the effects of physical exercise in experimental animal models⁷⁻¹⁰⁾ and human studies^{11,12)} have shown that exercise might induce changes in the gut microbiota, but the influence of physical activity on gut microbiota has largely been understudied. The effect of exercise on metabolites related to changes in gut microbiota is little known, although exercise certainly prevents a high-fat-diet (HFD) and Western

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diet-induced obesity¹³⁻¹⁵).

Recent studies suggest that short chain fatty acids (SCFAs), which are produced by microbial fermentation, regulate appetite and energy expenditure via specific receptors [Reviews]^{16,17}. The major products forming microbial fermentative activity in the gut are SCFAs, in particular, acetate, propionate and butyrate. It was reported in a metabolomics study that both exercise training and diet-induced obesity decreased plasma acetate¹⁵. In contrast, recent studies show that endurance capacity might be associated with gut microbiome-produced acetate¹⁸. However, the impact of gut microbiota on plasma acetate after exercise training has not been established. Furthermore, a high blood concentration of microbiota-dependent metabolite trimethylamine-N-oxide (TMAO) has been linked to an increased risk of atherosclerosis^{19,20}. However, the changes in gut microbiota and plasma TMAO concentration after exercise training remain unclear. In exercise physiology, the study of metabolites is important, and researchers are now able to provide for the simultaneous quantification of numerous metabolites in a small sample volume due to advances in technology. In addition, the effect of exercise and high-fat-diet (HFD) feeding on changes in metabolites via gut microbiota needs to be investigated. In the present study, we investigated the effects of voluntary exercise on plasma and urinary metabolites and changes in gut microbial community structure in mice with high-fat-diet induced-obesity.

Materials and Methods

Animals and housing conditions. Male C57BL/6J mice (four-week-old, $n = 27$) were used in this experiment (Clea Japan, Tokyo, Japan). They were housed individually in cages, under a controlled environment ($22 \pm 1^\circ\text{C}$, 12:12-h light-dark cycle) in conventional conditions with free access to food and water. The experiments involving mice, including the procedures performed, were approved by the Institutional Animal Care and Use Committee of Kawasaki University of Medical Welfare (#17-004).

HFD diet. The mice were fed a normal controlled diet (CD; MF, Oriental Yeast, Tokyo, Japan) and a high-fat-diet (HFD; D12492, Research Diets, New Brunswick, NJ) that contained 60% fat, 20% protein and 20% carbohydrates (of total calories) for 10 weeks.

Voluntary wheel running. The mice were randomly assigned to two conditions after an acclimation period of one week: voluntary wheel running (W) and sedentary control (C). The W mice ran on a wheel (10 x 23 x 10 cm cage with wide 5.5cm x ϕ 22 cm wheel, Natsume Seisakusho, Tokyo, Japan) in their cages freely for 10 weeks. Wheel running activity and body weight were measured weekly. Accordingly, in this experiment, mice were divided into four groups: CDC ($n = 6$), CDW ($n = 7$), HFDC (n

$= 7$) and HFDW ($n = 7$) groups.

Fecal sample collection and bacterial taxonomic analysis.

Feces were collected after the voluntary exercise period. The mice were placed in clean cages with a minimum of bedding for 24 hours. Feces was collected from each cage, frozen in liquid nitrogen, and stored at -80°C ¹⁰. The extraction of DNA from the frozen fecal samples using a QIAamp DNA mini kit (Qiagen, Valencia, CA). The 16S ribosomal RNA (16S rRNA) gene amplicon sequencing libraries were prepared following the “16S Metagenomic Sequencing Library Preparation Protocol” (Illumina, San Diego, CA, USA). Sequencing was performed using an Illumina MiSeq sequencer (Illumina) with 2 x 300 cycle MiSeq Reagent Kit v3. Reads were trimmed, combined and aligned to 16S rDNA database (Greengenes version 13_8).

Glucose tolerance test (GTT). Each mouse was lightly anesthetized by inhalation of Isoflurane prior to glucose administration (2 g/kg, i.p.) two days after the voluntary exercise period. Blood samples were collected from the eye vessel under Isoflurane anesthesia, and blood glucose levels were measured using the glucose monitoring device Accu-Chek, (Roche, Basel, Switzerland) immediately prior to, and at 15, 30, 60 and 120 minutes after glucose administration¹³.

Blood, urine and tissue collection. Whole blood and urine samples were directly collected from the inferior vena cava and the bladder under Isoflurane anesthesia, when the mice have at least 48 hours after the end of the voluntary running period. The whole blood sample was centrifugated at $800 \times g$ for 30 minutes at 4°C to isolate plasma. Collected urine was snap-frozen immediately in liquid nitrogen. Plasma and urine samples were stored at -80°C for metabolomics analysis. Epididymal adipose tissue was isolated and fixed by a 4% formaldehyde solution in PBS at 4°C , and then was treated with paraffin embedding according to standard methods. The specimen was stained by H and E, and was observed by microscope (BZ-X, Keyence, Osaka, Japan). The cross-sectional area of adipocytes was measured using ImageJ⁵.

Metabolomics analysis using nuclear magnetic resonance (NMR) spectroscopy. Quantitative analysis of metabolites in urine was performed by ^1H nuclear magnetic resonance (NMR) analysis as described by Heath and Claus (2011)²¹. On the other hand, plasma samples were measured after drying 40 μL solution in a vacuum centrifugal dryer²².

Briefly, the sample was prepared by mixing 30 μL solution and 30 μL 0.2 M phosphate buffer pH 7.4, in D_2O containing 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The mixture 50 μL was transferred into a

1.7 mm NMR capillary tube (NE-262-1.7; New Era Enterprises, Vineland, NJ, USA), which was placed into a Micro NMR sample tube (NE-H5/3; New Era Enterprises). NMR spectra were recorded on a NMR spectrometer (JNM-ECX400P; JEOL, Tokyo, Japan).

Statistical analysis. Principal coordinate analysis (PCoA) which is a method to explore and visualize similarities in data, was performed with unweighted UniFrac distance using Origin 2019 software (OriginLab, Northampton, MA, USA)²³. Data were expressed as the mean $\pm$ SEM. Statistical analyses were performed using IBM SPSS Statistics 24.0 for Windows software program. The data were analyzed using two-way analysis of variance (ANOVA).

A two-way repeated-measures ANOVA was used to analyze the changes in the wheel-running activity and body weight over 10 weeks. Bonferroni's test was used for the post hoc analyses. *P* values of <0.05 were considered to indicate statistical significance.

Results

The wheel running in both CDW and HFDW groups gradually increased to a peak at 4–5 weeks, and then running activity was constantly maintained. In the HFDW group, running activity was slightly, but not significantly, higher than that in CDW mice ($p = 0.075$, Fig. 1a). Exercise and diet significantly altered body weight (Fig. 1b).

Fig. 1 Changes in wheel-running activity and body weight in mice during experimental period.

(a) Wheel-running activity in exercised mice. Data were expressed as the mean $\pm$ SEM. (b) Changes in body weight. ●: control diet mice (CDW) and ●: high-fat-diet mice (HFDW). ** $p < 0.01$ vs. data at 1st week in each time.

Table 1. Body, heart, fat pad and skeletal muscle weight and cecal content.

Organ	CDC (6)	CDW (7)	HFDC (7)	HFDW (7)	Ex	Diet	Ex x Diet
Body weight (g)	28.33 $\pm$ 0.68	26.98 $\pm$ 0.26	36.85 $\pm$ 1.45	30.9 $\pm$ 0.30	**	**	*
Heart (mg)	140 $\pm$ 5.8	151.4 $\pm$ 9.9	132.3 $\pm$ 1.40	164.3 $\pm$ 12.10	*	N.S.	N.S.
(%)	0.49 $\pm$ 0.01	0.56 $\pm$ 0.03	0.36 $\pm$ 0.01	0.53 $\pm$ 0.04	**	*	N.S.
Cecal content (mg)	590 $\pm$ 11.5	627 $\pm$ 30.8	211.4 $\pm$ 7	217.1 $\pm$ 18.7	N.S.	**	N.S.
(%)	2.09 $\pm$ 0.06	2.32 $\pm$ 0.11	0.58 $\pm$ 0.03	0.70 $\pm$ 0.06	*	**	N.S.
Epidymal fat (mg)	413 $\pm$ 41.4	366.6 $\pm$ 98.4	1226 $\pm$ 287.6	624.3 $\pm$ 88.1	N.S.	**	N.S.
(%)	1.47 $\pm$ 0.16	1.36 $\pm$ 0.37	3.33 $\pm$ 0.78	2.01 $\pm$ 0.27	N.S.	*	N.S.
Visceral fat (mg)	303.3 $\pm$ 43.9	92.4 $\pm$ 15	605.7 $\pm$ 144.4	311.4 $\pm$ 40.4	**	**	N.S.
(%)	1.07 $\pm$ 0.15	0.34 $\pm$ 0.05	1.65 $\pm$ 0.40	1.00 $\pm$ 0.12	**	*	N.S.
Subcutaneous fat (mg)	506.7 $\pm$ 108.2	337.1 $\pm$ 48.5	3277.1 $\pm$ 336.2	724.6 $\pm$ 123.6	**	**	**
(%)	1.77 $\pm$ 0.36	1.25 $\pm$ 0.18	8.81 $\pm$ 0.61	2.33 $\pm$ 0.39	**	**	**
Total fat (mg)	1223.3 $\pm$ 158.2	796.2 $\pm$ 121.8	5109.5 $\pm$ 514.2	1660 $\pm$ 237.6	**	**	**
(%)	4.31 $\pm$ 0.53	2.95 $\pm$ 0.46	13.79 $\pm$ 1.12	5.35 $\pm$ 0.73	**	**	**
Tibialis anterior (mg)	53 $\pm$ 2	49.8 $\pm$ 2	45.2 $\pm$ 6.1	57.4 $\pm$ 1.6	N.S.	N.S.	*
(%)	0.19 $\pm$ 0.01	0.18 $\pm$ 0.01	0.13 $\pm$ 0.02	0.19 $\pm$ 0.01	*	*	*
Gastrocnemius (mg)	135.4 $\pm$ 5	163.7 $\pm$ 37.8	132.9 $\pm$ 5.3	154.9 $\pm$ 4	N.S.	N.S.	N.S.
(%)	0.48 $\pm$ 0.02	0.60 $\pm$ 0.13	0.36 $\pm$ 0.01	0.50 $\pm$ 0.02	p=0.076	N.S.	N.S.
Soleus (mg)	9.5 $\pm$ 0.2	10 $\pm$ 0.6	15.4 $\pm$ 0.6	13.4 $\pm$ 0.5	N.S.	N.S.	N.S.
(%)	0.03 $\pm$ 0.00	0.04 $\pm$ 0.00	0.04 $\pm$ 0.01	0.04 $\pm$ 0.00	N.S.	N.S.	N.S.

*: $p < 0.05$ and **: $p < 0.01$

Fig. 2 Effects of wheel running on adipocyte cells in HFD mice.

(a) H&E sections of white adipose tissue from CD and HFD mice after treatment with W and C (Scale bar = 50 μm) and (b) Effects of wheel running on adipocyte cell surface area from CD & HFD mice after treatment with W and C.

** $p < 0.01$

Fig. 3 Effects of wheel running on glucose metabolism in HFD mice.

(a) Intraperitoneal glucose tolerance test (GTT) and (b) its blood glucose area under the curve (BGAUC). ** $p < 0.01$

Wheel running induced hypertrophy of the heart ($p < 0.01$, Table 1) and skeletal muscles, such as tibialis anterior (TA, $p < 0.05$) and gastrocnemius ($p = 0.07$). In addition to higher fat mass (epididymal adipose tissue, visceral adipose tissue, subcutaneous adipose tissue and total fat mass), the adipocyte area in HFDC was significantly larger than that in CDC mice ($p < 0.01$), but was attenuated by wheel running despite HFD feeding ($p < 0.01$, Fig. 2a and b). To evaluate the effects of wheel running on HFD-induced insulin resistance, GTT were performed in the four groups (Fig. 3a). Glucose tolerance was assessed by

calculating the incremental area under the curve in blood glucose (BGAUC). As for the results of GTT, high levels of BGAUC in the HFDC group ($p < 0.01$, vs. CDC) were significantly inhibited by wheel running ($p < 0.01$, Fig. 3b).

PCoA score plots derived from the $^1\text{H-NMR}$ spectra of plasma and urine are shown in Fig. 4. Each group was treated with sedentary (C) and wheel running (W) conditions for 10 weeks. In both plasma and urine samples, CD and HFD groups were clearly separated by PC1s (31.3% and 60.0%, respectively). However, PC2s (19.6%

Fig. 4 $^1\text{H-NMR}$ -based metabonomic analysis of plasma and urine samples.

PCoA score plots derived from the $^1\text{H-NMR}$ spectra of (a) plasma and (b) urine extracts obtained from control diet (CD) and high-fat diet (HFD) mice. Each group was treated with sedentary (C) and wheel running (W) conditions for 10 weeks.

Table 2. Effect of voluntary exercise and a high-fat-diet fed on plasma and urine metabolite in mice.

		CDC	CDW	HDC	HDW	Ex	Diet	Ex x Diet
Plasma (mM)	Leu	0.050 $\pm$ 0.002	0.094 $\pm$ 0.011	0.052 $\pm$ 0.004	0.066 $\pm$ 0.003	**	*	*
	Ile	0.174 $\pm$ 0.014	0.106 $\pm$ 0.010	0.059 $\pm$ 0.004	0.061 $\pm$ 0.015	**	**	**
	Val	0.205 $\pm$ 0.017	0.206 $\pm$ 0.029	0.163 $\pm$ 0.010	0.227 $\pm$ 0.010	N.S.	N.S.	N.S.
	3-HB	0.171 $\pm$ 0.016	0.162 $\pm$ 0.023	0.133 $\pm$ 0.017	0.125 $\pm$ 0.018	N.S.	*	N.S.
	Lactate	9.525 $\pm$ 0.596	10.989 $\pm$ 1.142	6.978 $\pm$ 0.463	7.931 $\pm$ 0.583	N.S.	**	N.S.
	Ala	0.681 $\pm$ 0.103	0.852 $\pm$ 0.053	0.614 $\pm$ 0.067	0.798 $\pm$ 0.095	*	N.S.	N.S.
	Acetate	0.309 $\pm$ 0.034	0.495 $\pm$ 0.026	0.086 $\pm$ 0.006	0.139 $\pm$ 0.016	**	**	**
	Tyr	0.102 $\pm$ 0.015	0.101 $\pm$ 0.017	0.076 $\pm$ 0.014	0.130 $\pm$ 0.011	p=0.07	N.S.	p=0.06
	Formate	0.096 $\pm$ 0.012	0.075 $\pm$ 0.011	0.073 $\pm$ 0.008	0.065 $\pm$ 0.016	N.S.	N.S.	N.S.
Urine (mM)	Glu	16.380 $\pm$ 1.005	13.261 $\pm$ 0.629	11.398 $\pm$ 1.014	13.385 $\pm$ 1.391	N.S.	*	*
	Succinate	1.483 $\pm$ 0.166	1.196 $\pm$ 0.240	0.948 $\pm$ 0.780	0.309 $\pm$ 0.066	N.S.	N.S.	N.S.
	OG	3.239 $\pm$ 0.455	2.980 $\pm$ 0.506	3.175 $\pm$ 2.409	0.845 $\pm$ 0.092	N.S.	N.S.	N.S.
	Citrate	22.672 $\pm$ 2.820	21.120 $\pm$ 1.351	2.459 $\pm$ 0.860	1.197 $\pm$ 0.102	N.S.	**	N.S.
	TMA	4.525 $\pm$ 0.313	3.794 $\pm$ 0.597	1.433 $\pm$ 0.813	0.924 $\pm$ 0.300	N.S.	**	N.S.
	TMAO	1.427 $\pm$ 0.129	1.329 $\pm$ 0.161	0.513 $\pm$ 0.107	0.443 $\pm$ 0.097	N.S.	**	N.S.
	Taurine	18.588 $\pm$ 3.232	29.508 $\pm$ 5.798	14.565 $\pm$ 2.130	13.559 $\pm$ 2.041	N.S.	*	N.S.
	Creatine	1.685 $\pm$ 0.417	1.540 $\pm$ 0.405	1.077 $\pm$ 0.273	0.866 $\pm$ 0.180	N.S.	p=0.06	N.S.
	Hippurate	3.495 $\pm$ 0.284	3.001 $\pm$ 0.423	1.136 $\pm$ 0.136	1.217 $\pm$ 0.260	N.S.	**	N.S.
	Allantoin	22.391 $\pm$ 3.672	25.363 $\pm$ 4.228	13.046 $\pm$ 0.966	13.947 $\pm$ 2.904	N.S.	**	N.S.
	Creatinine	4.275 $\pm$ 0.439	4.311 $\pm$ 0.753	5.692 $\pm$ 0.477	6.054 $\pm$ 1.257	N.S.	N.S.	N.S.

Leu: Leucine, Ile: Isoleucine, Val: Valine, 3-HB: 3-hydroxybutyrate, Ala: Alanine, Tyr: Tyrosine, Glu: Glucose, OG: 2-oxoglutarate, TMA: Trimethylamine and TMAO: Trimethylamine *N*-oxide. *: $p < 0.05$ and **: $p < 0.01$.

and 14.5%, respectively) were substantially overlapped between the two conditions (C vs. W). In plasma, amino acids such as Leu ($p < 0.01$), Ile ($p < 0.01$) and Ala ($p < 0.05$), were significantly increased by exercise; whereas in urine, exercise did not affect the significant changes in the detected metabolites (Table 2).

Plasma acetate, which is one of the SCFAs, was observed to significantly increase by exercise ($p < 0.01$), as well as have an effect on diet ($p < 0.01$) and the interaction between exercise and diet ($p < 0.01$).

In addition, diet also influenced certain metabolites in both the plasma and urine of mice, and showed significant differences: in plasma, Leu ($p < 0.05$), Ile ($p < 0.01$), 3-HB ($p < 0.05$), lactate ($p < 0.01$) and Glu ($p < 0.05$), and in urine, citrate ($p < 0.01$), TMA ($p < 0.01$), TMAO ($p < 0.01$), taurine ($p < 0.05$), hippurate ($p < 0.01$) and allantoin ($p < 0.01$).

In the alpha diversity of gut microbiota, the observed species index, but not the phylogenetic diversity of the whole tree and Chao1, was significantly lower in HFD mice than in CD mice ($p < 0.05$, Fig. 5a). With regard to beta diversity, unweighted UniFrac analysis PCoA showed the difference between CD and HFD mice could be observed under PC1 (22.61%) (Fig. 5b). Although there was substantial overlap between two of the conditions (C vs. W), HFD groups were positioned in slightly different areas than those in the C and W conditions. Fig. 5c shows the results of clustering of gut microbiota, and stacked bar plots indicate the proportion of microbial abundances at the order level in each sample.

Discussion

In this study, we investigated the impact of voluntary exercise via changes of gut microbiota on plasma and urine metabolites in high-fat-diet (HFD) fed mice. Results showed an impact of gut microbiota especially on plasma acetate and urine TMAO after voluntary exercise training.

Although, in this experiment, HFD-induced metabolic disorders such as obesity, high fat accumulation and low levels of glucose clearance were observed, voluntary physical activity greatly attenuated these disorders. These results are in agreement with previous studies that voluntary exercise inhibits obesity and improves GTT¹³. The F/B ratio in gut microbiota is a well-known parameter of obesity^{3,4}. In this study, there was a difference between the F/B ratio in CD and HFD mice (Fig. 6a). However, wheel running didn't induce attenuation of the F/B ratio in either CD or HFD mice. Moreover, there wasn't any significant correlation between the F/B ratio and body weight ($r = 0.383$, N.S.) or between F/B ratio and locomotor activity ($r = 0.113$, N.S.). In addition to this study, other research has reported that wheel running did not affect^{8,10} or increase the F/B ratio^{7,24}. Therefore, the suppression of weight gain simply by exercise habits may not necessarily improve gut microbiota, such

as the F/B ratio. In this study, however, we just showed the F/B ratio in mice after voluntary wheel running for 10 weeks. In fact, human studies have shown a relationship between $\dot{V}O_2\text{max}$ and F/B ratio²⁵, and an animal experiment showed that juvenile onset exercise increased *Bacteroidetes* and decreased *Firmicutes*⁸. Hence, further research for elucidation of the effect of exercise on the F/B ratio is needed.

On the other hand, combined data between metabolites and gut microbiota has provided much useful information about exercise and microbiology. First, we confirmed the effect of HFD and wheel running on plasma acetate which might be induced by gut microbiota fermentation. Namely, acetate concentration in plasma was increased by exercise and decreased by HFD. Frost et al.²⁶ reported that acetate derived from colonic fermentation acts to direct brain function such as central hypothalamic mechanisms. Therefore, plasma acetate might contribute to supporting the central-mediated anorectic role of gut microbiota-induced SCFAs. Acetate might induce not only suppression of body fat accumulation^{27,28} and inflammation²⁹ in obese and diabetic animals, but also promote endurance capacity by increasing fatty acid oxidation in muscles³⁰. Indeed, Okamoto et al.¹⁸ recently reported that the microbiome was disrupted by the antibiotic treatment-induced reduction of endurance capacity; and the infusion of acetate in those antibiotic-treated mice restored their exercise capacity. Therefore, it was suggested that the microbiome might contribute to endurance exercise by producing acetate. In our study, plasma acetate concentration in both control diet and high-fat-diet fed mice was increased by voluntary exercise (1.6 fold compared with each sedentary condition in both diet groups). Since it was already reported that HFD decreased concentrations of acetate in cecal content compared to a control diet (similar to our results⁵), absolute concentrations in HFD mice were lower than those in CD mice. Carvalho et al.²⁹ suggests that modulation of gut microbiota in obesity can improve insulin signaling and glucose tolerance by reducing circulating lipopolysaccharide (LPS) levels and inflammatory signaling. Modulation of gut microbiota appears to increase levels of circulating acetate, which activates AMP-activated protein kinase (AMPK) and finally leads to reduced macrophage infiltration. We already showed that in high-fat-diet-induced obese mice, exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages, suggesting that exercise training attenuates obese induced low-grade chronic inflammation¹⁴. The suppression of macrophage infiltration is consistent with reductions in adipocyte size, resulting in attenuated systemic levels of gut-derived endotoxin^{29,31} and increased in portal levels of circulating acetate²⁹. Murphy et al.³² indicated that SCFA concentrations were unrelated to changes in proportions of *Firmicutes* and *Bacteroidetes*, as well as changes in

Fig. 5 Microbial diversities and differences in bacterial order within fecal microbiomes of controlled and exercised high-fat-diet mice.

(a) Rarefaction curves of α -diversity (Whole tree and Chao-1). (b) Visualized PCoA of unweighted UniFrac distances among the groups. (c) Bacterial classification at the genus level. Stacked bar plots indicating the proportion of microbial abundances in each sample. $\square$: CD, $\blacksquare$: CDW, $\square$: HFDC and $\blacksquare$: HFDW.

Fig. 6 Effect of wheel running and high-fat-diet feeding on fecal F/B ratio, *Bifidobacterium* and *Clostridia* in mice.

actinobacteria abundance. Therefore, the relationship between microbial composition and microbiota-related metabolites might be more complex than previously thought. Antibiotic treatment greatly modified the gut microbiota, reducing levels of circulating LPS levels and increasing circulating acetate²⁹. In this study, exercise modified the gut microbiota and increased circulating acetate. Although there is not a clear understanding of what kind of bacteria affect acetate production, there is at least a suggestion of the relationship between plasma metabolites and gut microbiota through exercise. This is because we already showed that voluntary wheel running increased the amount of acetate in cecum contents²³. Moreover, acetate enhances endurance capacity of exercise-trained mice by increasing skeletal muscle AMPK-mediated fatty acid oxidation³⁰. In contrast, it was shown that plasma acetate decreased in obese diabetic mice³³ as well as HFD mice in this study. Also, acetate attenuates obese and diabetic function in animal models via AMPK-induced phosphorylated carbohydrate-responsive element-binding protein (ChREBP)²⁷. It is well known that acetate is recognized by G protein-coupled receptor 43 (GPR43) which is associated with a lot of host functions in several tissues¹⁶. SCFAs can directly activate GPRs, and then the processes induce signaling molecules as well as role of energy sources. Thus bacterial metabolites affect various physiological processes. These findings may also provide new and exciting possibilities for modulation of host physiology. In addition, exercise may be a predominant factor influencing the alteration of gut microbiota composition and its function.

Secondly, recent evidence suggests that gut microbiota-

induced-TMAO-generating [TMA/flavin monooxygenase 3 (FMO3)/TMAO] pathway is linked to atherosclerotic cardiovascular disease, obesity and diabetes^{19,20}. It is known that circulating TMAO levels are associated with metabolic disease³⁴. Indeed, TMAO is associated with survival of patients with chronic heart failure³⁵. In this study, TMA and TMAO were detected in urine, but not in plasma. Furthermore, although HFD decreased active filtration of TMA and TMAO, exercise did not significantly affect it. The *Clostridiales*, *Ruminococcus*, and *Lachnospiraceae* are positive, but *S24-7*, an abundant family from *Bacteroidetes*, are inversely associated with TMA and TMAO levels in plasma of *Apoe*^{-/-} mice³⁶. Indeed, we also observed that *S24-7* in CD mice was higher than that in HFD mice. Since *S24-7* is a major bacteria in the gut microbiota of mice, it might be possible that significant changes in the abundance of *S24-7* have a great influence on the physiological function of the host.

Thirdly, it was reported that branched-chain amino acids (BCAA) and allantoin in serum and urine were increased by probiotics, such as *Bifidobacterium*³⁷. Thus, gut microbiota also have regulating amino acid utilization and nitrogen cycling. In our study, both urine allantoin and fecal abundance of *Bifidobacterium* in HFD mice were lower than in CD mice. In addition, wheel running had a negative effect on *Bifidobacterium* abundance, but not on urine allantoin concentration. However, mice, unlike humans, have an allantoin metabolic pathway via uricase, and it may be difficult to simply compare urine allantoin with the function of gut microbiota (Fig. 6b).

Finally, a reduction of hippurate in urine was observed in HFD mice, suggesting that HFD induced a reduction in

gut microbial metabolites. This observation was possible because metabolites related to stress, including reduced gut microbial metabolites such as hippurate, were already known³⁸). Although altered host metabolic pathways cannot be completely disregarded, differences in urinary co-metabolites may reflect systemic consequences of altered gut microbial metabolism. In fact, hippurate is known to be produced by *Clostridia* spp., and it has been suggested that reduced hippurate excretion in Crohn's disease patients was due to alterations in the gut microbiota³⁹). However, we observed that *Clostridia*, at least, was inhibited by HFD (Fig. 6c). Hence, the pathway of hippurate synthesis by the intestinal microbiota between diet and pathogenesis might be different, so further experiments in models of HFD are warranted.

The limitations of this study include the measurement of metabolites by NMR. The NMR spectroscopy is a relatively insensitive technique, and can only detect metabolites in high concentrations. However, it is mainly useful in determining metabolic fingerprints leading to the identification and quantification of compounds in non-targeted large scale in a non-destructive way and with high reproducibility⁴⁰⁻⁴²). Although we suggest that some microbes are affected by plasma and urine metabolites, neither expression nor activity of enzyme-related bacterial fermentation was measured in this study. Metatranscriptome and metabolome analysis needs to be carried out in future studies.

Taken together, our results suggest that testing in both plasma and urinary metabolites can reveal the effects of exercise on the gut microbiota when it comes to quantitative metabolite analysis and its effect on exercise-dependent or independent alteration of gut microbiota on the host.

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Author contributions

EO, CW, MJK and HY conceptualized the study design and protocol. CW, NK, TA, EO and TN performed the experiment and carried out the analysis. CW, MJK, TN, HY and EO interpreted the data and wrote the manuscript. HY, TA, MJK, TN and EO revised the reviewed manuscript. All authors approved the final version of the manuscript.

Conflict of Interests

The authors declare no conflict of interests associated with the present study.

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