Research article

Microbiome potentiates endurance exercise through intestinal acetate production

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Running title:

Role of microbiome derived acetate in endurance exercise

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Abstract

The intestinal microbiome produces short chain fatty acids (SCFAs) from dietary fiber and has specific effects on other organs. During endurance exercise, fatty acids, glucose, and amino acids are major energy substrates. However, little is known about the role of SCFAs during exercise. To investigate this, mice were administered either multiple antibiotics or a low microbiome-accessible carbohydrate diet (LMC), prior to endurance testing on a treadmill. Two-week antibiotic treatment significantly reduced endurance capacity *versus* the untreated group. In the cecum, acetate, propionate, and butyrate became almost undetectable in the antibiotic-treated group, plasma SCFA concentrations were lower, and the microbiome was disrupted. Similarly, 6-week LMC treatment significantly reduced exercise capacity, and fecal and plasma SCFA concentrations. Continuous acetate, but not saline, infusion in antibiotic-treated mice restored their exercise capacity ($p<0.05$), suggesting that plasma acetate may be an important energy substrate during endurance exercise. In addition, running time was significantly improved in LMC-fed mice by fecal microbiome transplantation from others fed a high microbiome-accessible carbohydrate diet and administered a single portion of fermentable fiber ($p<0.05$). In conclusion, the microbiome can contribute to endurance exercise by producing SCFAs. Our findings provide new insight into the effects of the microbiome on systemic metabolism.

Keywords microbiome, skeletal muscle, short-chain fatty acid, acetate

45 **Introduction**

46

47 Skeletal muscle constitutes ~30%–50% of total body mass, making it the largest organ in
48 the body. Muscle mass is one of the major determinants of basal metabolic rate (30) and is a
49 substantial consumer of ATP during physical activity. Its metabolism is also differentially
50 regulated under fasting and fed conditions. In the fasting state, approximately 80% of blood
51 glucose is metabolized in an insulin-independent manner by the brain, gut, and red blood
52 cells, whereas skeletal muscle needs only small quantities of glucose. However, in the
53 postprandial state, skeletal muscle accounts for 75% of glucose utilization, a shift that is
54 insulin-dependent (6).

55 Maximal exercise can increase ATP turnover more than 100-fold over the resting
56 level (9). Contracting skeletal muscle is able to use a number of intra- and extramuscular
57 substrates to generate ATP during exercise. These include creatine phosphate, muscle
58 glycogen, plasma glucose, and free fatty acids (FFA), derived from either adipose tissue or
59 intramuscular triglyceride stores. The relative importance of these substrates for
60 metabolism during exercise is primarily determined by the intensity and duration of the
61 exercise (27).

62 Acetate is included in Ringer's solution (an isotonic solution of electrolytes for
63 infusion) for the clinical correction of dehydration, because HCO_3^- is generated by the
64 oxidation of acetate in the liver and skeletal muscles (24). Acetate is converted to acetyl-
65 CoA by acetyl-CoA synthetase 2 (AceCS2), and can be used as a metabolic substrate, such
66 as for fatty acid synthesis or in the tricarboxylic acid cycle. In the gut, microbes metabolize

dietary fiber by fermentation, producing short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, in the cecum and colon (13-15). In addition, acetate is generated in the liver by fatty acid oxidation during more extreme fasting conditions (23). AceCS2 knockout mice, which cannot use acetate as a substrate for acetyl-CoA generation, show low endurance exercise tolerance and hypothermia during fasting (23), implying that acetate supply is important under fasting conditions. However, the roles of the intestinal microbiome and SCFAs during exercise have not been established. In this study we have determined the effects of antibiotic treatment, a high-fiber diet, and a low-fiber diet in mice, to explore the importance of SCFAs derived from the intestine microbiome during exercise.

Methods

Animals and diet groups

Male C57BL/6J mice were housed under a 12-h light-dark cycle at 24°C, with free access to food and water. At 10 weeks of age, mice were randomly assigned to receive one of two types of dietary fiber or antibiotic treatment. The mice in the diet groups were fed either a low microbiome-accessible carbohydrate diet (LMC) or a high microbiome-accessible carbohydrate diet (HMC). In addition, other mice were allocated to either an antibiotic treatment group (Abx) or an antibiotic-free group (Abx-free). Daily food intake was estimated by measuring weekly food intake and calculating a mean amount per mouse per day. Mice were fed with the study diets from 10 to 16 weeks of age. All surgery was performed under anesthesia with 40 mg/kg BW of pentobarbital injection

following 4% sevoflurane inhalation, and efforts were made to minimize suffering throughout the study. All experimental protocols were approved by the Animal Care and Use Committee of Shiga University of Medical Science (Identification codes: 2015-11-3, 2016-7-19, 2017-6-9, and 2018-7-5. Approval dates: 2015-11-24, 2016-08-08, 2017-07-03, and 2018-07-30). Animals were treated in accordance with the guidelines of the United States National Institutes of Health.

Blood glucose measurement

Blood glucose concentrations were measured using glucose dehydrogenase-pyrroloquinoline quinone glucose test strips (Glutest Sensor; Sanwa Kagaku Kenkyusho, Nagoya, Japan). Blood samples were collected from a tail vein into heparinized tubes and centrifuged at 700 × *g* for 15 min. The derived plasma was stored at −80°C until further analysis.

Measurement of cecal, skeletal muscle, and adipose tissue mass

The adipose tissue, skeletal muscle, and cecal masses were measured in each mouse and normalized to body mass.

SCFA analysis

SCFAs in the feces and plasma were quantified using liquid chromatography (LC)-mass spectrometry (MS)/MS. To extract SCFAs (acetate, propionate, and n-butyrate) from samples, ethanol:water (3:7, v/v) was added at room temperature. After extraction, internal standard solutions, 2-nitrophenyl hydrazine, and condensation reagent were added to the tube for derivatization, which was performed in iced water for 60 min. An alkaline solution was then added to stop the reaction and the tubes were left in iced water for a further 30 min. After the reaction had been stopped, an acidic solution and hexane were then added for liquid-liquid extraction. The hexane layer was removed, and then ether was added. Afterwards, the ether layer was transferred to another test tube and dried under a nitrogen stream. An ammonium formate/methanol solution was added to dissolve the residues in the tubes and aliquots were injected into the LC-MS/MS system. LC was performed using an Acquity UPLC system (Waters, Milford, MA, USA) and an analytical column (Acquity HSS T3 2.1 × 150 mm, 1.8 μm; Waters). For mass detection, an API4000 tandem mass spectrometer (AB Sciex, Foster City, CA, USA) was used.

Subcutaneous infusion of acetate or butyrate using an osmotic pump

We used an osmotic pump for acetate or butyrate delivery, as reported previously, with minor modifications (21). Alzet Mini-Osmotic Pumps (Model 2001; Durect Corp, Cupertino, CA, USA), releasing 1 μL/h for 7 days, were filled with 4 M sodium acetate or butyrate solution, or saline, following the manufacturer's instructions. A saline, acetate, or butyrate-dispensing pump was then inserted subcutaneously between the

scapulae of the mice and connected to a fitted cannula. The procedure was performed under anesthesia (intraperitoneal administration of 10% pentobarbital, followed by maintenance using sevoflurane inhalation). All mice were housed individually during the post-surgical observation period. Mice that died within a few days of the operation were excluded from the statistical analysis. The chemicals and reagents used were sodium acetate from Sigma (S5636), sodium butyrate from Sigma (B5887), streptomycin, and penicillin from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan) and vancomycin hydrochloride (V0252), metronidazole (M1977), ciprofloxacin (C3262), ceftazidime hydrate (C1635), gentamycin sulfate (G1658), and neomycin sulfate (N1755) from LKT laboratories, Inc. (St Paul, MN, USA).

Fecal microbial transplantation and oral inulin administration

The fecal microbial transplantation (FMT) experiment was performed as previously described (29), with minor modifications. Briefly, mice were first fed an LMC diet for 6 weeks, then divided into two groups. Group 1 mice underwent oral FMT from HMC-fed mice (donor) to LMC-fed mice (receiver) daily for 3 days, then inulin was administered by gavage, 6 h prior to treadmill testing. Group 2 mice were administered distilled water by gavage instead of FMT. One hundred milligrams of fresh feces were collected from each mouse fed for 6 weeks with the HMC immediately following defecation, and this was resuspended in 1 ml normal saline, vortexed for 3 min, and allowed to settle for 2

min. Two hundred microliters of the supernatant were then transplanted into the recipient mice by gavage once daily for 3 days.

Exercise training and tolerance testing

The exercise protocol used was as previously reported (18). During the experimental period, all animals were trained on a motorized treadmill (TMW-4 #TW4160601 Melquest Ltd. Toyama, Japan) three times a week. Training was performed for 15 min (5 min at 10 m/min, then an increase of 1 m/min every minute for 10 min) on a 10° incline, with a shock grid (0.97 mA, 1 Hz) placed below to encourage the mice to run. Exercise tolerance was determined at the end of each treatment by placing the animals on an individual treadmill at room temperature. The regimen commenced with the shock grid turned on, a 5° incline, and a speed of 10 m/min for 10 min. The speed was then increased by 0.07 m/min, up to 20 m/min (a total of 140 min of increasing speed), and was then held at 20 m/min until exhaustion. The mice were deemed to be exhausted if they were willing to sustain shocks five times for more than 3 sec, or remain on the shock grid for 5 consecutive seconds. At this time, the mouse was removed from the treadmill. The total running time until exhaustion was recorded and used as an index of exercise capacity.

Fecal specimen collection, culture, and DNA extraction

169 In all experiments, fecal samples were collected from mice under fed conditions, and
170 were immediately frozen at −80°C, except for when used for FMT. The samples were
171 freeze-dried and lyophilized using VD-250R (Taitec), then powdered using a Shake
172 Master Neo (BMS). DNA was extracted from the pulverized samples using an Mpure
173 Bacterial DNA Extraction Kit (MPBio). The concentrations of the extracted DNA
174 solutions were measured using Synergy H1 (BioTek) and the QuantiFluords DNA
175 System (Promega).

176

177 ***Quantitative PCR***

178 Sets of qPCR primers (Table 1) and universal bacterial 16S rRNA sequences were used
179 to quantify the sizes of bacterial populations (16).

180

181 ***Library preparation for high-throughput sequencing***

182 The concentration of the prepared library was measured using Synergy H1 and the
183 QuantiFluords DNA System. The quality of the prepared library was confirmed using a
184 Fragment Analyzer and a dsDNA915 Reagent Kit (Advanced Analytical Technologies).
185 Sequencing analysis was performed using MiSeq, requiring 2 × 300 bp sequences. The
186 library was prepared using a 2-step tailed PCR method. The first PCR reaction was
187 performed in 1.0 µl 10 × Ex buffer, 0.8 µl dNTPs (2.5 mM each), 0.5 µl 10 µM forward
188 primer, 0.5 µl 10 µM reverse primer, 2.0 µl template DNA (0.5 ng/µl), 0.1 µl ExTaq

189 [TaKaRa] (5 U/µl), and 5.1 µl deionized water. The reaction conditions were 94°C for 2
190 min, then 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 30 cycles, followed
191 by a final extension of 5 min at 72°C (16). We used the following forward primer: 1st-
192 341f_MIX #1
193 ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNCCTACGGGNGGCWGC
194 AG and the following reverse primer: 1st-805r_MIX #2
195 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGACTACHVGGGTAT
196 CTAATCC. The second PCR reaction mixture was composed of 1.0 µl 10 × Ex buffer,
197 0.8 µl dNTPs (2.5 mM each), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer,
198 2.0 µl template DNA (0.5 ng/µl), 0.1 µl ExTaq (Takara) (5 U/µl) and 5.1 µl deionized
199 water. The reaction conditions were 94°C for 2 min, then 94°C for 30 sec, 60°C for 30
200 sec, and 72°C for 30 sec for 10 cycles, followed by a final extension for 5 min at 72°C
201 (16). We used the following forward: 2nd-F
202 AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGC and
203 reverse: 2nd-R
204 CAAGCAGAAGACGGCATACGAGATTGACTGGAGTTCAGACGTGTG primers.
205
206 ***Taxonomic and ecological analyses***
207 For reading quality filtering we used Fastx toolkit's Fastq barcode splitter (part of the
208 FASTX-toolkit from http://hannonlab.cshl.edu/fastx_toolkit/index.html) to extract only
209 the sequences that had a start sequence exactly matching that of the primer used. The

primer sequence of the extracted sequence was deleted. After that, we removed the sequences with quality values < 20 using Sickle tools and discarded sequences of ≤ 150 bases and their paired sequences. For merge reads we merged arrays that passed the quality filtering criteria using the paired-end merge script FLASH. The condition of merging was set to a fragment length of 420 bases after merging, a fragment length of read of 280 bases, and a minimum overlap length of 10 bases. For chimera checking we used all the sequences that had passed filtering and Usearch's Uchime algorithm. The database contained 97% of the operational taxonomic unit from Greengenes attached to the pipeline Qiime for flora analysis, and all the sequences that were not judged to be chimeric were extracted and used for subsequent analyses. Microbial diversity was measured using the Shannon index, which takes into account both overall richness and evenness.

Statistical analyses

All quantitative data except for running time are expressed as median and interquartile range, and running time data are expressed using Kaplan-Meier estimates. Cecal mass is expressed as mean and standard deviation. Student's *t*-test or the Mann-Whitney U-test were used to evaluate differences between two groups, and one-way ANOVA and subsequent *post hoc* Tukey tests were used to determine the significance of differences where multiple comparisons were required. $p<0.05$ was considered to represent statistical significance.

Results

Oral administration of antibiotics reduces treadmill running time

Antibiotic treatment is known to largely eliminate the intestinal microbiome. To explore the role of the microbiome in endurance exercise, multiple antibiotics were administered orally to mice (Abx mice) consuming a regular chow diet (Fig. 1A). Antibiotic treatment for 2 weeks significantly reduced treadmill running time compared with the untreated group (Fig. 1B). Although dietary intake was significantly higher in the Abx group, body mass gain and blood glucose were not different between the two groups (Fig. 1C, 1D, 1G). This can be explained by differences in body composition, because Abx mice had lower muscle and white adipose tissue masses than the control group. These mice also had enlarged ceca, suggesting that dietary fiber was accumulating there, due to disruption of the microbiome (0.53 ± 0.08g in the control mice versus 4.2 ± 0.14g in the Abx mice, $p<0.001$ by two-tailed Student's *t*-test.). Gut microbes generate SCFAs from fermentable fiber in the intestine. Therefore, we measured the acetate, propionate, and butyrate content of the feces of both groups. We found that fecal SCFA content was significantly lower in the Abx than the untreated group (Fig. 1H). Most of the SCFAs produced are known to be used locally in the intestine, but some are absorbed into the circulation. As a result, acetate is present at significant concentrations in the plasma (Fig. 1I). Similar to the fecal content, plasma SCFA concentrations were also significantly lower in the Abx group (Fig. 1I).

253 ***Antibiotic treatment alters the composition of the microbiome***

254 Next, we determined the effects of antibiotic treatment on the microbiome by analyzing
255 fecal bacterial composition in the Abx and untreated groups using 16S/rRNA analysis.
256 Antibiotic treatment for 2 weeks was associated with a larger population of Firmicutes and
257 a smaller population of Bacteroidetes than in the control group (Fig. 2A, 2B). Furthermore,
258 antibiotic treatment significantly reduced bacterial DNA concentration in the feces (Fig.
259 2C) and fecal bacterial diversity, assessed using the Shannon index (Fig. 2D).

260

261 ***Reduction of the fermentable fiber content of the diet is associated with lower white***
262 ***adipose tissue mass, tibialis anterior mass, and treadmill running time***

263 The effect of dietary fiber level on skeletal muscle performance was also assessed. Mice
264 were randomly assigned to consume either an HMC or an LMC (Fig.3A). The HMC
265 contained hemi-cellulose and lignin, which are microbiome-accessible, and cellulose,
266 which is microbiome-resistant (Table 2). In contrast, the LMC contained only cellulose as a
267 fiber source (Table 2). After 6 weeks of dietary treatment, treadmill running time was
268 significantly lower in the LMC than the HMC diet group (Fig. 3B). Muscle mass was also
269 significantly lower and white adipose tissue mass was significantly higher in the LMC diet
270 group (Fig. 3E, 3F), although body mass gain and dietary intake were similar in the two
271 groups (Fig. 3C, 3D). Similar to the effect of antibiotic treatment, we found that fecal
272 SCFA content was significantly lower in the LMC than the HMC diet group (Fig. 3G). In
273 addition, plasma acetate and propionate concentrations were significantly lower, and
274 plasma butyrate concentration tended to be lower, in the LMC diet group (Fig. 3H).

Low dietary fermentable fiber content affects fecal microbial composition

To explore the effect of the level of fermentable fiber in the diet on microbiome composition, we analyzed the composition of the fecal bacterial population in mice fed an HMC or an LMC diet using 16S/rRNA analysis. LMC feeding for 6 weeks was associated with larger numbers of Firmicutes and Actinobacteria and smaller numbers of Bacteroidetes than in the HMC diet group (Fig. 4A, 4B). Furthermore, the ratio of Firmicutes:Bacteroidetes was higher in the LMC diet group (Fig. 4C). There was no difference in bacterial DNA concentration, suggesting that the total number of microbes was similar (Fig. 4D). The LMC-fed group also demonstrated lower fecal bacterial diversity, estimated using the Shannon index (Fig. 4E). *Prevotella* and S24-7 were present in lower numbers in the LMC-fed group than in the HMC group. In contrast, the numbers of *Lactococcus* and *Allobaculum* were higher in LMC group (Fig. 4F). Thus, low dietary fermentable fiber content alters the composition of the microbiome in favor of bacteria that produce less SCFA.

Continuous acetate infusion ameliorates the impairment in treadmill running time in antibiotic-treated mice

We speculated that acetate would be a key metabolite determining exercise tolerance in mice, because it is the dominant SCFA in plasma and is generated in the largest quantity by intestinal microbes (Fig. 1I, 3I). Continuous acetate, but not saline, infusion significantly increased endurance running time in antibiotic-treated mice (Fig. 5A, 5B). However, 1

297 week of infusion did not affect body or muscle mass, suggesting that low muscle mass was
298 not the cause of the lower exercise tolerance observed in the antibiotic-treated mice (Fig.
299 5C, 5D). To explore the effect of SCFAs other than acetate, we also performed a continuous
300 butyrate infusion experiment (Fig. 5E), but found no improvement in running time
301 following continuous butyrate infusion at a similar rate to that of acetate (Fig. 5F).
302
303 ***Fecal microbial transplantation and a single administration of fermentable fiber in mice***
304 ***fed an LMC diet normalizes exercise tolerance***
305 To further explore the mechanism underlying the LMC-induced loss of exercise tolerance
306 in mice, we determined the impact of FMT and a single oral administration of inulin, a
307 fermentable form of fiber, to LMC-fed mice (Fig. 6A). This did not affect body mass or
308 tibialis anterior mass (Fig. 6C, 6D), but there was a significant improvement in treadmill
309 running time in mice that had received a FMT and inulin compared with those that had not
310 (Fig. 6B). We found that fecal SCFA content was significantly improved in the mice that
311 had received FMT and inulin than the LMC diet group (Fig. 6E).
312
313

Discussion

There were three important findings of this study. First, plasma acetate is an important energy source for skeletal muscle, at least during endurance exercise. Second, plasma acetate is derived mainly from the intestine and generated from fermentable dietary fiber by microbes. Third, of the SCFAs, acetate is likely to be the most important energy source in skeletal muscle.

Plasma acetate is an important energy source in skeletal muscle, at least during endurance exercise. Our study has demonstrated that acetate infusion normalizes endurance exercise performance, which is impaired by antibiotic treatment. This is consistent with the findings of a previous study in which AceCS2 knockout mice were shown to have low exercise tolerance, assessed using a similar protocol (23). AceCS2 is the only enzyme that can convert acetate to acetyl-CoA (8), while the liver can generate acetate during starvation from the beta-oxidation of fatty acids, using AceCS1 (11). In our study, food was withdrawn from the mice for only 6 h prior to treadmill running, so that acetate would not be released from the liver. The turnover of acetate is quite rapid, having been estimated to be ~20 nmol/g.min by the infusion of isotope-labeled acetate in mice (19). In horses, acetate infusion during exercise reduces FFA and glycerol concentrations, which may be due to a reduction in lipolysis during endurance exercise (22), and oral administration of acetate rapidly restores muscle glycogen (28). In humans, acetate infusion increases acetyl-CoA concentrations in skeletal muscle (7). Taken together, these findings indicate that plasma acetate is an energy source for skeletal muscle.

Plasma acetate is mainly derived from the intestine and generated by microbes using fermentable dietary fiber. Oral administration of multiple antibiotics has been shown to substantially reduce microbial mass in the intestine (4, 26), and indeed fecal microbial mass was dramatically lower in mice administered an antibiotic cocktail in this study, as shown by the measurement of DNA concentration (Fig. 2). Moreover, severe dysbiosis was observed in the Abx group (Fig. 2D), and the resulting accumulation of undigested dietary fiber probably explains the observed enlargement of the cecum and the lower fecal SCFA concentration (Fig. 1H). Similarly, LMC treatment for 6 weeks was associated with lower fecal SCFA concentration (Fig. 3H) and microbial diversity (Fig. 4E), which are consistent with the findings of a previous study (25).

The difference in the effects of LMC and HMC consumption may be due to the content of hemi-cellulose, which has been reported to be a suitable substrate for SCFA generation by the microbiome in rats (17). LMC consumption was associated with a smaller population of *Prevotella* (Fig. 4F), which generate SCFA, and this is also consistent with the findings of a previous study that a microbiome containing substantial numbers of *Prevotella* produces a great deal of SCFA, whereas one in which *Bacteroides* predominates does not (3). Both antibiotic and LMC administration reduced exercise endurance in the mice, probably because of reductions in acetate influx from the intestine. Consistent with this, acetate infusion in Abx mice and FMT plus inulin administration significantly improved exercise endurance. These findings suggest that intestinally produced acetate is an important energy source during prolonged exercise. However, it is likely that acetate is not the sole mediator of the observed improvement,

because we have also identified effects of antibiotic treatment on propionate and butyrate concentrations during antibiotic treatment (Fig. 5E). However, butyrate may not be important for the improvement in endurance running capacity, based on the results of the continuous butyrate infusion experiment (Fig. 5F).

Of the SCFAs, acetate is likely to be the most important energy source in muscle. We have shown that acetate is the dominant SCFA in plasma (Fig. 1I, 3I) (13), and a previous study in mammalian models showed that SCFAs are an important energy source for intestinal epithelial cells (1). Moreover, SCFAs entering the portal vein from the intestine are metabolized in the liver (5). Recently, a randomized trial of colonic infusion of the various SCFAs was performed in obese subjects (2). This showed that acetate, propionate, and butyrate increase energy expenditure and fat oxidation, but reduce carbohydrate oxidation, even though only a slight increase in the plasma concentration of each SCFA was achieved, probably because the turnover of these compounds is very rapid. In humans, acetate is the principal anion in the intestine, but there is greater uptake of butyrate by the colonic epithelium and propionate by liver (5). Nevertheless, the evidence suggests that acetate is the most important energy source among the SCFAs.

In the present study, we have demonstrated the importance of the microbiome for endurance exercise (Fig. 7). Consistent with this, a recent study showed that specific microbes affect the performance of professional cyclists (20). Here, the beneficial effect of FMT plus inulin suggests that preloading with fermentable dietary fiber prior to endurance exercise may be beneficial for skeletal muscle performance in endurance athletes, because acetate is generated. One small randomized study of six women who

were exercised to exhaustion on a cycle ergometer showed that prior ingestion of whole oat flour with a high fiber content was associated with better performance than ingestion of whole oat flour with a lower fiber content (12), a finding that was attributed to the lower glycemic index. However, we speculate that adequate fiber intake can enhance exercise tolerance because of greater SCFA production by gut microbes. Therefore, we propose that intestinal fiber and the microbiome represents the fourth energy storage organ, in addition to muscle (glycogen, amino acids, and lipids), liver (glycogen), and adipose tissue (lipids). Further studies, including randomized controlled trials, are necessary to further elucidate this concept by evaluating the effects of supplementing dietary fiber and optimizing the microbiome prior to endurance exercise.

This study had several limitations. First, the use of multiple antibiotics to disrupt the intestinal microbiome may be associated with systemic side effects, although the antibiotics were chosen on the basis of their use in a previous study (4). To reduce the significance of this possibility further, we chose to compare the effects of LMC-feeding on plasma and fecal SCFAs, a comparison that is independent of the microbial population size (Fig. 3). Second, plasma acetate concentrations following acetate or saline infusion were similar (data not shown), probably because of the rapid turnover of acetate (10, 19). Finally, this study was performed in mice. Because the microbiome and its relationship with metabolism varies among mammalian species, any generalization to humans must be made with caution.

In conclusion, our study has revealed that acetate generated in the intestine by microbes is an important energy substrate during endurance exercise. This study

provides new insights into the effect of the microbiome on systemic metabolism, and especially on the capacity for physical activity involving skeletal muscle.

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Author contributions

T.O. performed the experiments, designed the project, and wrote and edited the manuscript. K.M. interpreted the results, designed the project, and wrote and edited the manuscript. F.N., M.L., N.O., S.I., D.S., and Y.F. discussed the results and commented on the manuscript. S.U. and H.M. reviewed the manuscript and directed all the work.

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Disclosures

The authors have declared that no conflict of interest exists.

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525 **Figure Legends**

526

527 **Figure 1. Effects of antibiotic treatment for 2 weeks**

528 (*A*) Experimental protocol. C57BL/6 mice were allocated to two groups and administered
529 antibiotics (100 μg/ml neomycin, 50 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml
530 vancomycin, 100 μg/ml metronidazole, 125 μg/ml ciprofloxacin, 100 μg/ml ceftazidime,
531 and 170 μg/ml gentamicin in their drinking water) or not for 2 weeks (n=6 per group). (*B*)
532 Running time on the treadmill. Time until exhaustion on the treadmill after 6 h of fasting.
533 (*C*) Body mass, measured before and after treatment. (*D*) Dietary intake. (*E*) Mass of
534 tibialis anterior muscle. (*F*) Epididymal and inguinal adipose tissue depot masses. (*G*)
535 Casual blood glucose. (*H*) Fecal short-chain fatty acid (SCFA) concentrations. (*I*) Plasma
536 SCFA concentration. The boxes indicate the interquartile ranges and the lines within the
537 boxes indicate the medians. *p<0.05 and **p<0.01, analyzed using the Log-Rank test (*B*)
538 or Student's *t*-test (*D, E, F, H, I*). Abx: antibiotics treated, WAT: white adipose tissue, Epi:
539 epididymal adipose tissue, Ing: inguinal adipose tissue, SCFA; short chain fatty acid.

540

541 **Figure 2. Analysis of the fecal microbiome in control and antibiotic-treated groups**

542 n=4 per group. (*A*) Composition of the fecal microbiome at the phylum level. (*B*)
543 Comparison of Firmicutes and Bacteroidetes populations (percentages of the total). (*C*)
544 Concentration of fecal bacterial DNA. (*D*) Fecal bacterial diversity, assessed using the
545 Shannon index. The boxes indicate the interquartile ranges and the lines within the boxes
546 indicate the medians. * p<0.05, **p<0.01, and ***p<0.001, analyzed using the Mann-

Whitney U test (*A, B*) or Student's *t*-test (*C, D*). Abx: antibiotics treated.

Figure 3. Metabolic phenotype of mice fed a high microbiome-accessible carbohydrate diet (HMC) or a low microbiome-accessible carbohydrate diet (LMC)

(*A*) Experimental protocol: C57BL/6 mice were allocated to two groups and fed either an LMC or HMC for 6 weeks. n = 6 per group. The LMC contains cellulose, while the HMC contains cellulose, hemi-cellulose, and lignin. (*B*) Running time on a treadmill. Time until exhaustion after 6 h of fasting. (*C*) Body mass before and after dietary treatment. (*D*) Dietary intake during the experiment. (*E*) Mass of tibialis anterior muscle. (*F*) Masses of epididymal and inguinal adipose depots (WAT). (*G*) Casual blood glucose. (*H*) Fecal short-chain fatty acid (SCFA) concentration. (*I*) Plasma SCFA concentration. The boxes indicate the interquartile ranges and the lines within the boxes indicate the medians. *$p< 0.05$ and **$p<0.01$, analyzed using the Log-Rank-test (*B*) or Student's *t*-test (*C, D, E, F, H, I*). LMC: low microbiome-accessible carbohydrate diet, HMC: high microbiome-accessible carbohydrate diet, WAT: white adipose tissue, Epi: epididymal adipose tissue, Ing: inguinal adipose tissue, SCFA; short chain fatty acid.

Figure 4. Analysis of the fecal microbiome in high microbiome-accessible carbohydrate diet (HMC) and low microbiome-accessible carbohydrate diet (LMC)-fed groups

n=4 per group. (*A*) Composition of the fecal microbiome at the phylum level. (*B, C*) Comparison of the percentages (*B*) and ratio (*C*) of the Firmicutes and Bacteroidetes in the

entire microbiome. (*D*) Concentration of fecal bacterial DNA. (*E*) Fecal bacterial diversity, assessed using the Shannon index. The boxes indicate the interquartile ranges and the lines within the boxes indicate the medians. (*F*) Heat-map and list of bacterial taxa showing significant differences in the percentage composition of the entire microbiome, analyzed using the Mann-Whitney U test. Values are medians and interquartile ranges. *p<0.05, **p<0.01, and ***p<0.001, analyzed using the Mann-Whitney U test (*B, C*) or Student's *t*-test (*E*). LMC: low microbiome-accessible carbohydrate diet, HMC: high microbiome-accessible carbohydrate diet.

Figure 5. Effect of acetate infusion on running time

(*A*) The experimental protocol. An osmotic pump containing either saline or 4 M acetate was inserted dorsally, between the scapulae of mice, 2 weeks after antibiotic treatment, and left for 1 week. (n=6 per group) (*B*) Comparison of running time. Time until exhaustion after 6 h of fasting. (*C*) Body mass after treatment. (*D*) Mass of tibialis anterior muscle. (E) The experimental protocol. An osmotic pump containing either saline or 0.2ml of 4 M butyrate was inserted dorsally, between the scapulae of mice, 2 weeks after antibiotic treatment, and left for 1 week. (n=4 per group) (F) Comparison of running time. Time until exhaustion after 6 h of fasting. The boxes indicate the interquartile ranges and the lines within the boxes indicate the medians. * p<0.05 analyzed using the Log-Rank test (*B*) or ANOVA (*D*). Abx: antibiotics treated.

Figure 6. Effect of fecal microbial transplantation (FMT) and oral inulin

administration on running time

(*A*) The experimental protocol. FMT from high microbiome-accessible carbohydrate (HMC)- to low microbiome-accessible carbohydrate diet (LMC)-fed mice was performed after 6 weeks of LMC consumption. Inulin was orally administered 6 h prior to treadmill running. Black arrows indicate the timing of FMT (once daily for three days), and the open arrow indicates the time of inulin administration (once). (n=4 per group) (*B*) Comparison of running time. Time until exhaustion after 6 h of fasting. (*C*) Body mass after treatment. (*D*) Mass of tibialis anterior muscle. (*E*) Fecal Short chain fatty acid (SCFA) concentration. The boxes indicate the interquartile ranges and the lines within the boxes indicate the medians. *p<0.05 and **p<0.01, analyzed using the Log-Rank test (*B*) or Student's t-test (*E*). LMC: low microbiome-accessible carbohydrate diet, FMT: fecal microbial transplantation.

Figure 7. Energy source during endurance exercise

(*A*) Fermentable fiber with normal microbial flora in the intestine. (*B*) Low fermentable fiber and /or dysbiosis in the intestine. During endurance exercise, skeletal muscles require 100 times more energy than during the resting state. Plasma FFAs derived from adipose tissue and glucose from the liver are the two major energy sources. SCFAs derived from the intestinal microbiome may represent an additional energy substrate for skeletal muscle during endurance exercise. FFA: free fatty acid, SCFAs: short-chain fatty acids.

Table 1. Sets of qPCR primers and universal bacterial 16S rRNA sequences

Sample	Concentration of DNA solution		Sequence for sample identification		Concentration of the library		Library quality check	Sequencing results			Number of leads used in Qiime analysis
	Concentration (ng/μl)	Liquid volume (μl)	Index 1	Index 2				Raw number of reads	Q20 (%) × 2	Q30 (%) × 2	Non-chimera
Abx-1	21.7	50	ACGAGTCC	TCGACTAC	25.9	20	Passed	49,602	94.2	87.3	23.160
Abx-2	23.5	50	ACCAGTCC	TTCTACCT	24.5	20	Passed	49,131	94.3	87.6	22.330
Abx-3	20.2	50	AGGAGTCC	CCTACACT	26.4	20	Passed	44,445	94.3	87.5	20.320
Abx-4	16.0	50	ACCACTCC	GCGTAAGA	27.2	20	Passed	41.503	94.0	86.9	19.902
Abx+1	0.555	50	AGCAGTCC	CTATTAAG	25.7	20	Passed	46.451	95.1	88.5	32.987
Abx+2	2.20	50	ACGAGTCC	AACGCTAT	26.2	20	Passed	56.687	95.4	89.1	50.066
Abx+3	2.64	50	AGCACTCC	CACCCTTA	25.0	20	Passed	40,916	95.4	88.9	36.622
Abx+4	1.68	50	AGGAGTCC	TTATCCCA	25.2	20	Passed	40,290	95.6	89.3	35.756
LMC1	10.9	50	CATGCCTA	TCGACTAC	26.1	20	Passed	46,326	94.4	87.3	25.943
LMC2	21.5	50	CATGCCTA	TTCTAGCT	26.3	20	Passed	46.705	93.9	86.4	28.621
LMC3	21.2	50	CATCCCTA	CCTACACT	25.5	20	Passed	40,486	93.5	85.9	23.995
LMC4	22.3	50	CATCCCTA	GCGTAACA	25.7	20	Passed	44,838	93.5	85.7	25.981
HMC1	22.3	50	CATGCCTA	CTATTAAC	24.2	20	Passed	48,365	94.3	87.2	23.740
HMC2	22.5	50	CATGCCTA	AACGCTAT	25.7	20	Passed	45.730	93.5	86.2	21.252
HMC3	22.0	50	CATGCCTA	GAGCCTTA	23.4	20	Passed	45,531	93.5	86.3	20.470
HMC4	21.8	50	CATCCCTA	TTATCCCA	23.1	20	Passed	54,486	93.4	86.0	23.925

Abx: antibiotics treated, LMC: low microbiome-accessible carbohydrate diet, HMC: high microbiome-accessible carbohydrate diet

Table 2. Nutritional information for the mouse diets

Diet	Supplier/ Product Name	Energy (kcal/g)	Protein (% by mass)	Fat (% by mass)	Carbohydrates (% by mass)	Crude Fiber (% by mass)	Neutral Detergent Fiber (% by mass)	Fiber Content
Regular chow	Clea CE-2	3.4	25.5	4.6	54.1	5.1	ND	ND
HMC	Lab Diet 5L65	3.1[A]	24.3	5	50	4.6	14.6	Cellulose Hemi-cellulose Lignin
LMC	Envigo TD.86489	3.7	18.3	5.2	61.7	5	5[B]	Cellulose

[A] Metabolizable energy.

[B] Exclusively from cellulose.

HMC: high microbiome-accessible carbohydrate diet; LMC: low microbiome-accessible carbohydrate diet, ND: not determined.

Figure 1

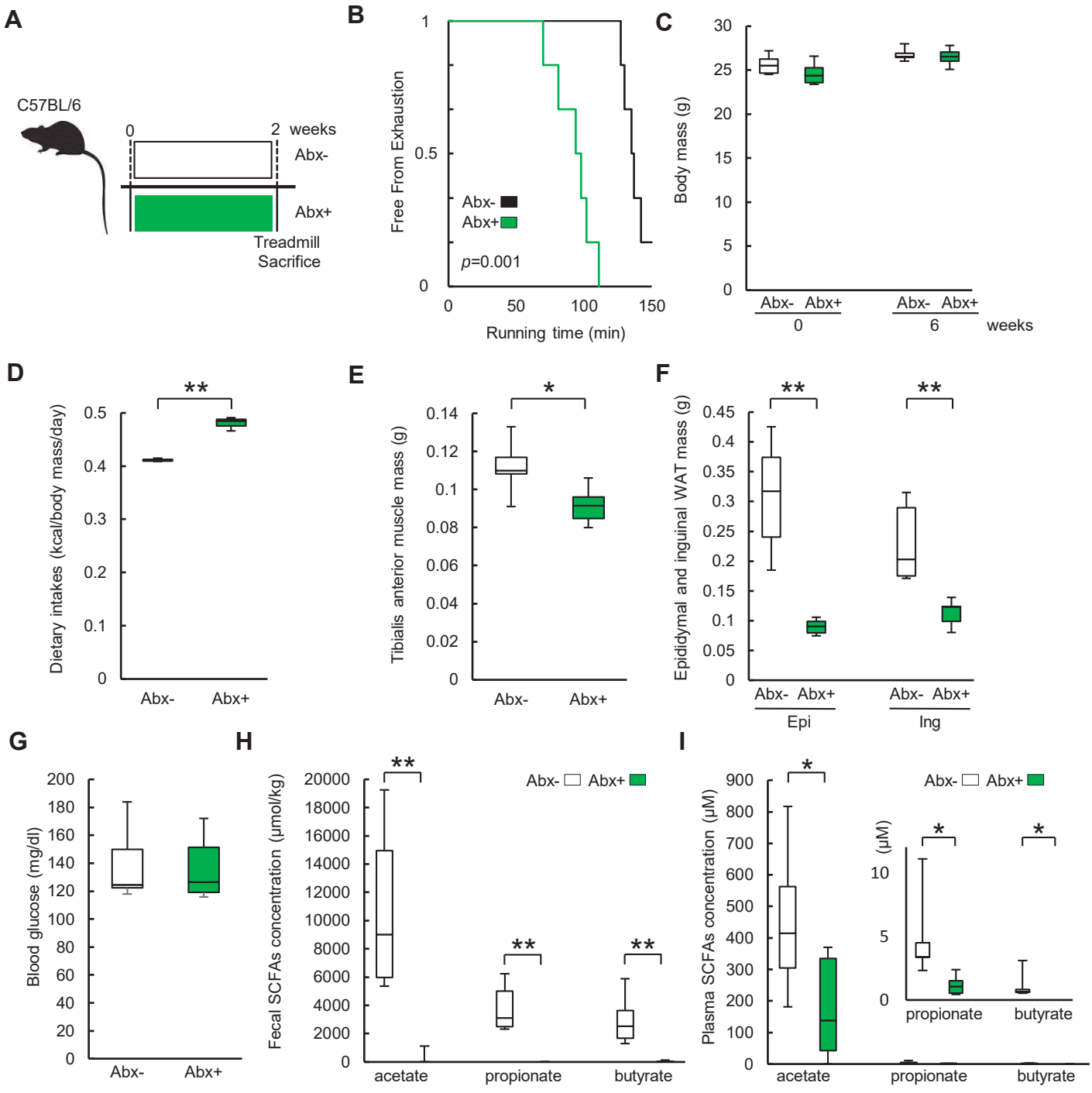


Figure 2

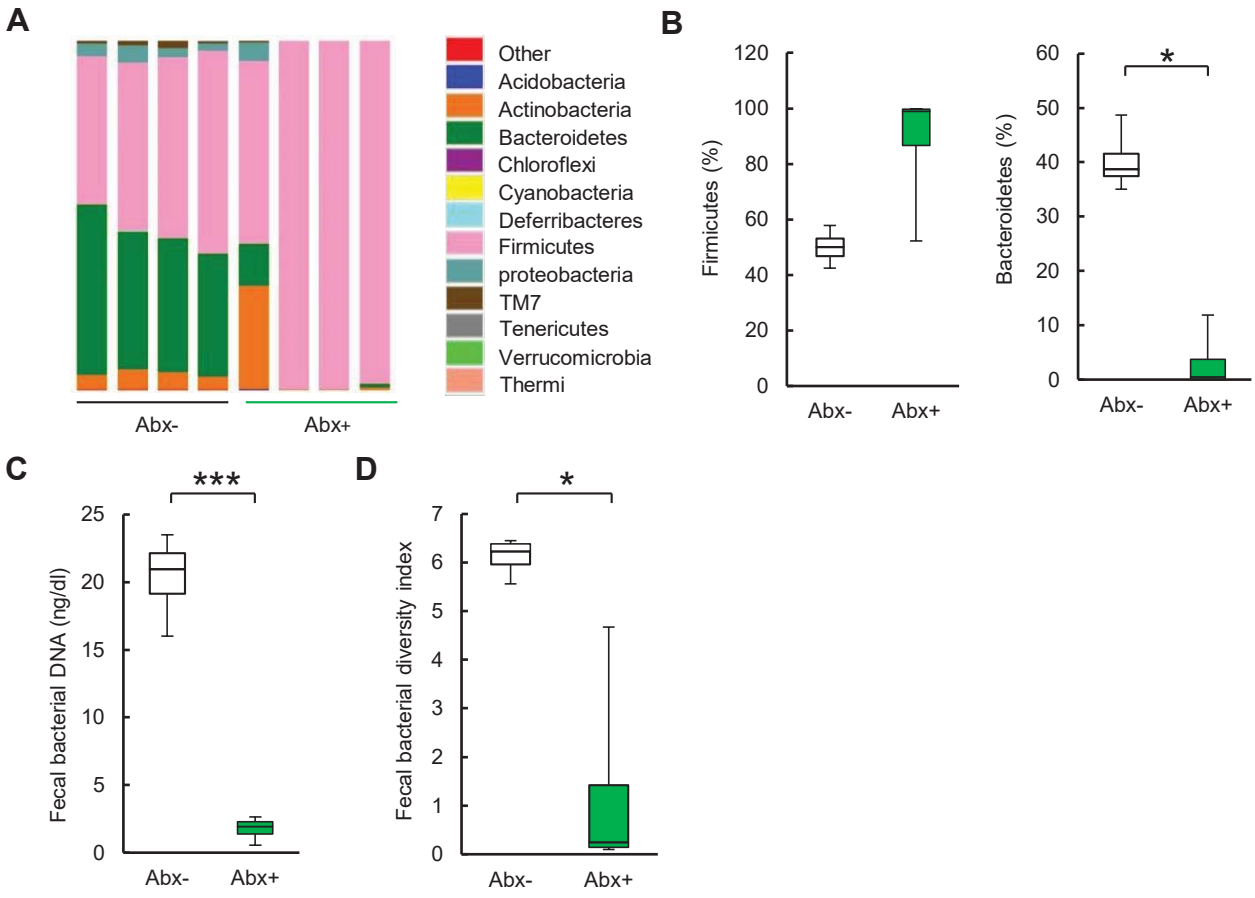


Figure 3

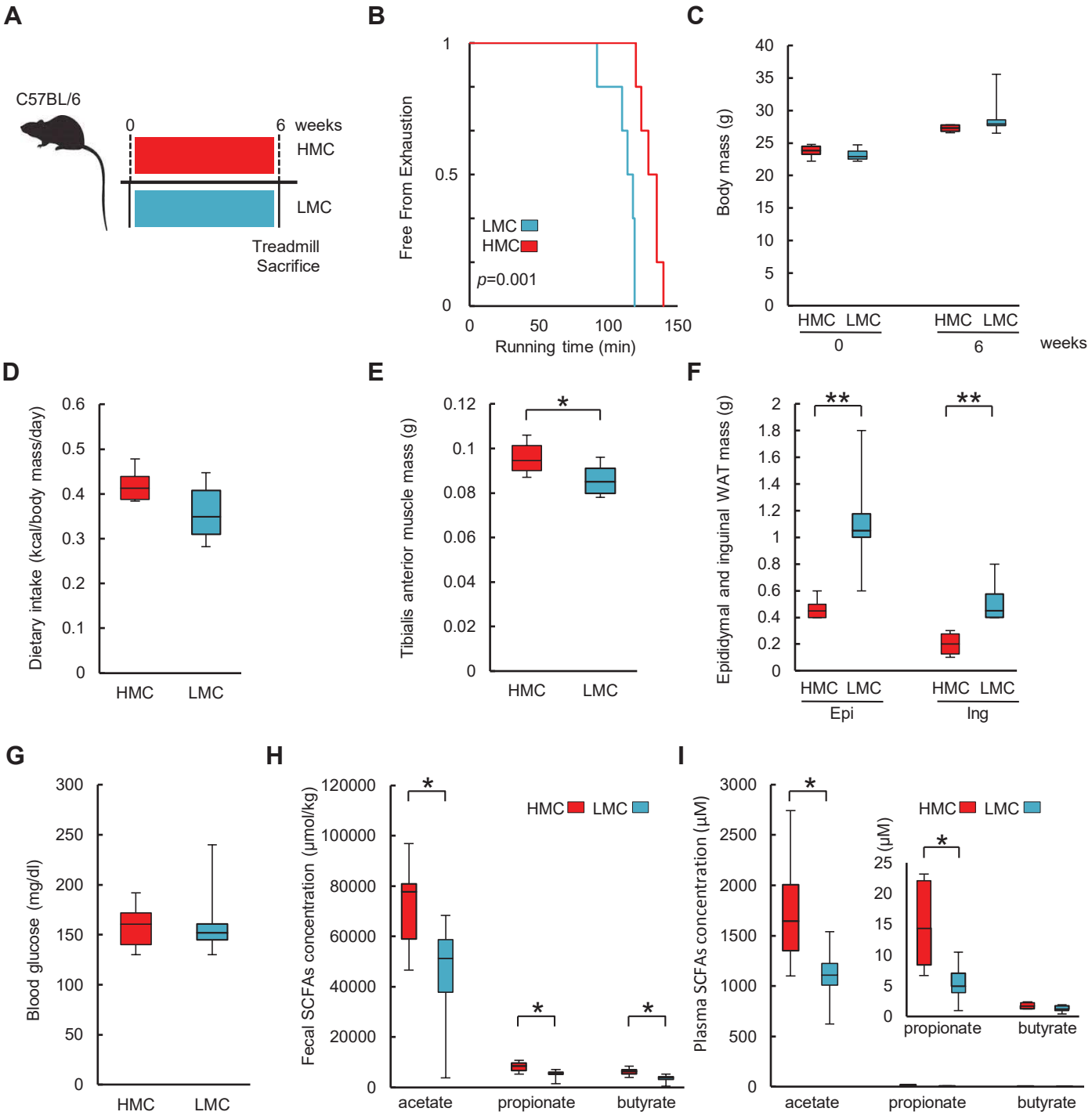
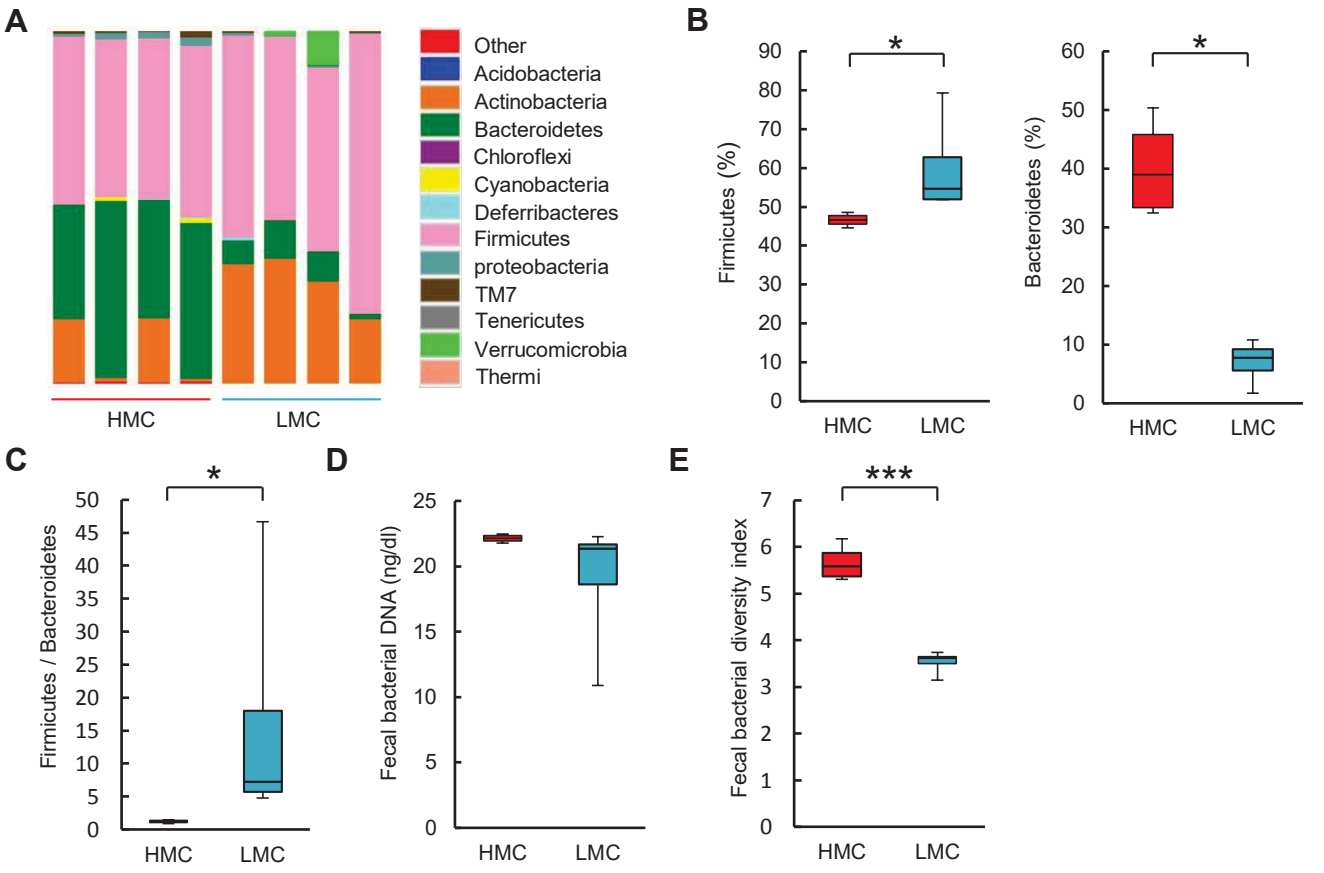


Figure 4



	HMC	LMC
f__Coriobacteriaceae;g__	0.125 (0.125-0.1)	3.65 (5.5-1.4)
f__Prevotellaceae;g__	0.275 (0.3-0.275)	0
f__Prevotellaceae;g__*Prevotella*	3.825 (5.05-2.475)	0
f__S24-7;g__	27.625 (28.075-24.8)	4.925 (6.075-4.45)
f__Paraprevotellaceae;g__*Prevotella*	7.075 (7.875-5.35)	0.525 (0.8-0.075)
f__Streptococcaceae;g__*Lactococcus*	0	4.7 (5.625-4.275)
f__Turicibacteraceae;g__*Turicibacter*	0.75 (1.025-0.525)	0.025 (0.025-0)
f__Lachnospiraceae;g__*Coprococcus*	0.375 (0.425-0.25)	0.025 (0.025-0)
f__Lachnospiraceae;g__*Ruminococcus*	2.3 (2.825-1.175)	0.45 (0.55-0.35)
f__Erysipelotrichaceae;g__*Allobaculum*	9.875 (19.1-0.075)	40.15 (41.275-37.775)
f__Alcaligenaceae;g__*Sutterella*	0.675 (0.775-0.45)	0

Figure 5

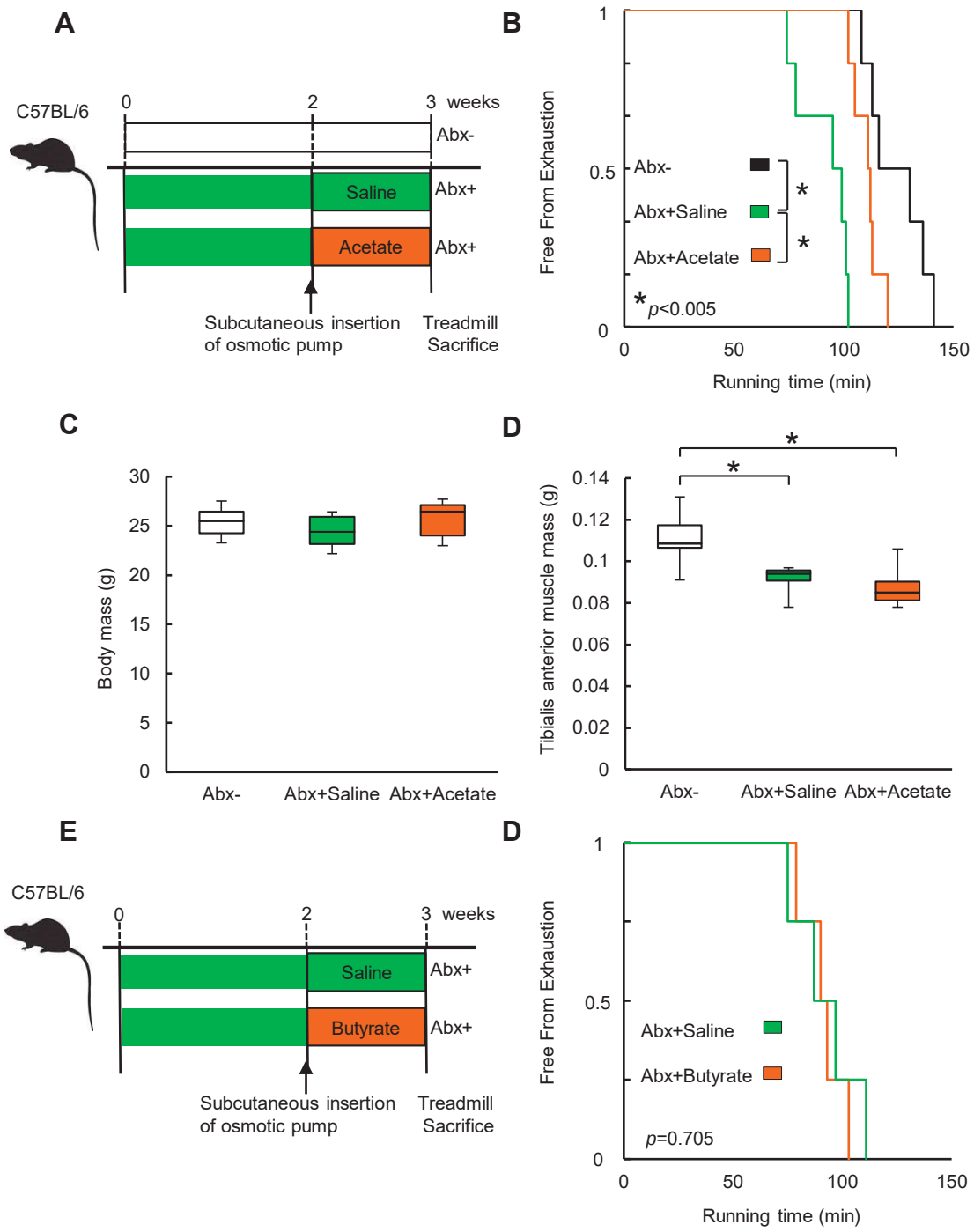


Figure 6

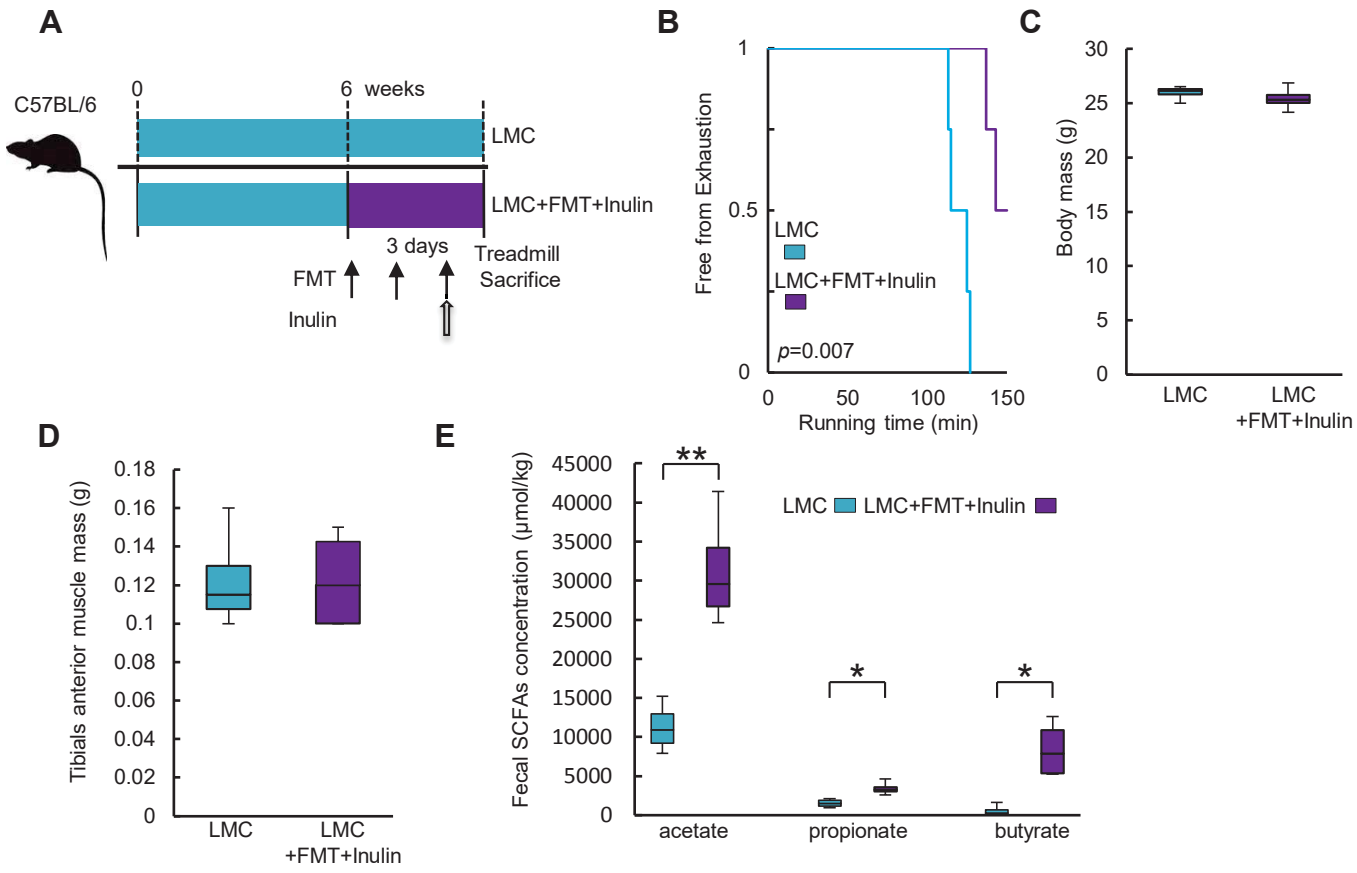


Figure 7