

## RESEARCH ARTICLE

# Early-life effects of juvenile Western diet and exercise on adult gut microbiome composition in mice

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## ABSTRACT

Alterations to the gut microbiome caused by changes in diet, consumption of antibiotics, etc., can affect host function. Moreover, perturbation of the microbiome during critical developmental periods potentially has long-lasting impacts on hosts. Using four selectively bred high runner and four non-selected control lines of mice, we examined the effects of early-life diet and exercise manipulations on the adult microbiome by sequencing the hypervariable internal transcribed spacer region of the bacterial gut community. Mice from high runner lines run ~3-fold more on wheels than do controls, and have several other phenotypic differences (e.g. higher food consumption and body temperature) that could alter the microbiome, either acutely or in terms of coevolution. Males from generation 76 were given wheels and/or a Western diet from weaning until sexual maturity at 6 weeks of age, then housed individually without wheels on standard diet until 14 weeks of age, when fecal samples were taken. Juvenile Western diet reduced bacterial richness and diversity after the 8-week washout period (equivalent to ~6 human years). We also found interactive effects of genetic line type, juvenile diet and/or juvenile exercise on microbiome composition and diversity. Microbial community structure clustered significantly in relation to both line type and diet. Western diet also reduced the relative abundance of *Muribaculum intestinale*. These results constitute one of the first reports of juvenile diet having long-lasting effects on the adult microbiome after a substantial washout period. Moreover, we found interactive effects of diet with early-life exercise exposure, and a dependence of these effects on genetic background.

**KEY WORDS:** Early-life, Exercise, Gut microbiome, ITS rRNA, Selection experiment, Western diet

## INTRODUCTION

Animals have evolved in a bacterial world. Coevolution between hosts and symbionts has resulted in complex relationships, wherein the diverse community of species inhabiting the gastrointestinal tract in mammals is essential for breaking down nutrients from ingested food, normal metabolic function and protection through enhanced immunity (Dominguez-Bello et al., 2019; Gilbert et al., 2018; Kohl and Carey, 2016). Many factors have been shown to influence the gut microbial community and diversity, including diet,

exercise, antibiotics and age (Bokulich et al., 2016; Clark and Mach, 2016; Lozupone et al., 2012; Yatsunenkov et al., 2012). Alterations to the community can result in potentially irreversible (Dethlefsen and Relman, 2011; Langdon et al., 2016) changes in the microbiome. Compositional changes in the gut microbiome can, in turn, affect many aspects of host biology, including physiology and behavior.

Diet can rapidly alter the gut microbiome community in as short as 24 h (David et al., 2014). For example, many laboratory studies of adult rodents have shown that a typical Western diet (high in fat and sugar) alters the gut microbiome community and reduces diversity of bacterial species (Becker et al., 2020; Beilharz et al., 2017; Leamy et al., 2014; Pindjakova et al., 2017; Turnbaugh et al., 2008). In multiple strains of inbred, outbred and transgenic mice, a shift in diet can have lasting effects on the community, as repetitive switching from a high-fat, high-sugar diet to a low-fat diet results in altered community membership and composition that does not revert to the original state (Carmody et al., 2015). Rodent studies also indicate that diet can alter microbial function. For example, adult mice fed a high-fat diet for 12 weeks had unique gut microbiome communities, increased body mass, and altered gut bacterial function as measured by metaproteome and metabolome analyses (Daniel et al., 2014). In that study, high-fat diet led to an increase in amino acid metabolism and enzymes involved in the oxidative stress response, possibly in response to the shift in nutrient availability within the gut.

Acute and chronic exercise can also affect the microbiome (Clark and Mach, 2016; Codella et al., 2018; Mach and Fuster-Botella, 2017; Mailing et al., 2019; O'Sullivan et al., 2015; Scheiman et al., 2019). The first paper highlighting the relationship between exercise and the microbiome found that adult rats with wheel access for 5 weeks had an increased amount of cecal n-butyrate, a short-chain fatty acid byproduct of bacterial fermentation (Matsumoto et al., 2008). Butyrate can be transported from the small intestine to muscles, where it can lead to activation of several regulatory pathways linked to ATP production as well as muscle integrity, thus potentially altering athletic ability and/or performance (Ticinesi et al., 2017; Walsh et al., 2015). Approaches for measuring the effect of exercise on the gut microbiome vary widely in the literature, but consistent trends in results are emerging. For example, both rodent and human studies have reported increased butyrate-producing bacteria (Barton et al., 2018; Matsumoto et al., 2008), and also increases in taxa such as *Lactobacillus* (Batacan et al., 2017; Lambert et al., 2014; Petriz et al., 2014; Queipo-Ortuño et al., 2013), *Bifidobacterium* (Bressa et al., 2017; Lambert et al., 2014; Queipo-Ortuño et al., 2013) and *Akkermansia* (Barton et al., 2018; Bressa et al., 2017; Clarke et al., 2014; Liu et al., 2015). In amateur half-marathon runners, the relative abundances of several bacterial taxa and fecal metabolites were significantly different pre- and post-race (Zhao et al., 2018).

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Diet and exercise have also been shown to interactively influence the gut microbiome community and diversity in rodents (Batacan et al., 2017; Denou et al., 2016; Evans et al., 2014). Mice placed on a high-fat diet for 6 weeks followed by 6 weeks of high-intensity interval training had greater bacterial diversity in the feces compared with sedentary mice on standard chow (Denou et al., 2016). Exercise-trained mice on a high-fat diet had significant changes in the relative abundance of the phylum *Bacteroidetes* in the small intestine, cecum and colon compared with mice on a high-fat diet without exercise training. In another study on the interactions between exercise and diet, mice given 12 weeks of voluntary wheel access on a standard or high-fat diet had higher diversity than sedentary controls as well as significant main effects of diet, exercise and their interactions on taxa relative abundance (Evans et al., 2014). More specifically, that study found an increase in the relative abundance of butyrate-producing taxa in the *Clostridiales* order compared with sedentary mice. In rats, high-intensity and light-intensity interval training regimens resulted in unique microbiome communities regardless of whether they were on a high-fat, high-fructose diet or a standard diet (Batacan et al., 2017). The scarcity of studies examining diet–exercise interactions highlights the need for more research in this growing field.

In mammals, the period of development from weaning to sexual maturity is a crucial time during which environmental conditions can have a lasting impact on many traits (Garland et al., 2017), including normal development of the microbiome (Kerr et al., 2015). Immediately after birth, initial colonizers of the gut microbiome in placental mammals are dominated by microbes from the mother, followed by further acquisitions from the early-life environment (Funkhouser and Bordenstein, 2013; Milani et al., 2017). A clear example of developmental effects on the gut microbiome is early-life diet: babies that are breastfed have a unique microbiome compared with those fed formula (Sprockett et al., 2018), and have higher bacterial diversity during the first 12–24 months of age (Bokulich et al., 2016). In mice, early-life antibiotic treatment followed by placement on a high-fat, high-sugar diet as adults results in increased adult adiposity and an increase in the ratio of *Firmicutes* to *Bacteroidetes* as compared with mice on a normal diet (Schulfer et al., 2019). In a recent study, juvenile mice given 3 weeks of high-fat diet or cafeteria diet starting at 4 weeks of age followed by an approximately 7-week-long washout period had altered adult gut microbiome communities (Fülling et al., 2020). More specifically, mice with a juvenile high-fat diet had reduced diversity of the adult gut microbiome at approximately 14 weeks of age. However, only one study has tested whether early-life effects of exercise on the microbiome can persist after a substantial washout period. Mika et al. (2015) found that after a 25-day washout period, rats with 6 weeks of juvenile wheel access tended to have decreased *Firmicutes* abundance as adults.

The first goal of the present study was to test for long-lasting effects of early-life Western diet and exercise on the adult microbiome. To do so, we used a unique animal model: four lines of high runner (HR) mice that have been selectively bred for high voluntary wheel-running behavior and their four non-selected control (C) lines (Swallow et al., 1998). The HR mice differ from C mice in several ways that might affect the microbiome through alterations in the gut environment. HR mice have higher activity levels and food consumption even when housed without wheels, and increased body temperature when active (Copes et al., 2015; Malisch et al., 2009; Swallow et al., 2009; Wallace and Garland, 2016), all of which might affect the gut environment. In the absence of compensatory reductions in other aspects of physical activity,

exercise leads to increased energy expenditure and hence necessitates greater food consumption (Garland et al., 2011), which should directly impact the gut microbiome. Exercise also causes many acute changes in physiology, including increases in body temperature, and changes in hormone levels, intestinal barrier function and digestive transit time that could feedback into the gut environment (Campbell and Wisniewski, 2017; Mach and Fuster-Botella, 2017). HR and C mice also differ in circulating concentrations of hormones (Garland et al., 2016). When housed without wheels, HR and C mice do not differ in small or large intestine mass or length, suggesting that the former might have faster digestive throughput (Kelly et al., 2017). Therefore, our second goal was to test for microbiome differences between the HR and C lines, which could result from acute effects of the noted phenotypic differences. Another possibility is coevolution of the gut microbiome across many tens of generations of selective breeding, but we cannot differentiate that from acute/chronic effects of exercise with the present experimental design. Our analyses also considered the possibility of interactive effects, e.g. that genetic background (Benson et al., 2010; Carmody et al., 2015; Leamy et al., 2014) might influence whether and how early-life Western diet or exercise opportunity affects the adult microbiome.

## MATERIALS AND METHODS

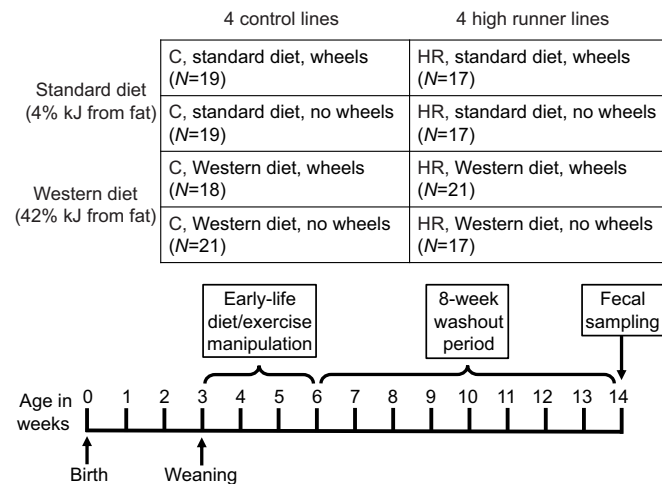
All experiments and methods were approved by the Institutional Animal Use and Care Committee of the University of California, Riverside.

### Experimental animals

Mice were sampled from generation 76 of an ongoing selection experiment selecting for high voluntary wheel-running behavior. Four replicate HR lines were bred for high levels of voluntary wheel running and were compared with four non-selected C lines. The base population was 224 outbred Hsd:ICR laboratory house mice (Swallow et al., 1998). Mice were weaned at 21 days of age and housed four per cage separated by line and sex until ~6–8 weeks of age. Mice were then placed into individual cages attached to a 1.12 m circumference wheel (Lafayette Instruments, Lafayette, IN, USA) with a sensor to record the total number of revolutions per day (e.g. see Swallow et al., 1998). For HR mice, the highest running male and female from each family based on the average revolutions on days 5 and 6 of a 6-day period of wheel access were chosen as breeders for the next generation. Breeders in the C lines were chosen without regard to how much they run. Each generation had ~10 breeding pairs per line, and sibling pairings were not allowed.

### Early-life diet and exercise treatment

A total of 165 male mice, sampled approximately equally from the four replicate HR and four non-selected C lines, were weaned at 21 days of age and placed into one of four treatment groups for 3 weeks: (1) standard diet, no wheels; (2) Western diet, no wheels; (3) standard diet, wheels; and (4) Western diet, wheels (see Fig. 1). Mice were provided with *ad libitum* food and water for the duration of the experiment. Standard Laboratory Rodent Diet (SD) from Harlan Teklad (W-8604) contained 4% kJ from fat and the Western diet (WD) from Harlan Teklad (TD.88137) contained 42% kJ from fat. After the 3 weeks of juvenile exposure, which allowed them to reach sexual maturity, all mice were housed individually without wheel access on standard diet for an 8-week washout period (equivalent to approximately 6 human years: Dutta and Sengupta, 2016). Mice were maintained in rooms with lights on at 07:00 Pacific Standard Time for a 12 h:12 h light:dark photoperiod, and at approximately 22°C.



**Fig. 1. Early-life experimental design and treatment groups (N=149 mice).** Fecal sampling occurred as adults (14 weeks of age) after the 8-week washout period on standard diet with no wheel access.

### Juvenile wheel running

Juvenile wheel running was measured during weeks 3–6 of the early-life diet and/or exercise manipulation. Mice were housed individually in home cages with attached wheels, as used during the routine selective breeding protocol (Swallow et al., 1998). Sensors attached to the wheel record the number of revolutions in each 1-min interval during a 23 h measurement period. We measured wheel freeness by recording the number of revolutions per wheel until it reached a stop after accelerating each wheel to a constant speed (Copes et al., 2015).

### Juvenile food consumption

Juvenile food consumption was measured during weeks 3–6 of the early-life diet and/or exercise manipulation. Food hoppers were weighed at the start and end of each week to measure apparent food consumption after accounting for food wasting (Koteja et al., 2003). Food consumption was converted to caloric intake as the diets differed in energy content (Meek et al., 2010).

### Fecal sampling

At 14 weeks of age, individual mice were placed into a clean, empty cage and watched until defecation. We obtained fecal samples from 149 individuals. The samples were placed into a sterile tube and held on dry ice prior to storage at  $-80^{\circ}\text{C}$ , where they remained until DNA extraction.

### Bacterial rRNA ITS analysis

Illumina bacterial rRNA internal transcribed spacer (ITS) libraries were constructed as follows. PCRs were performed using a DNA Engine thermal cycler (Bio-Rad Inc., Hercules, CA, USA) as 25- $\mu\text{l}$  reactions containing: 50  $\text{mmol l}^{-1}$  Tris (pH 8.3), bovine serum albumin (BSA) at 500  $\mu\text{g ml}^{-1}$ , 2.5  $\text{mmol l}^{-1}$   $\text{MgCl}_2$ , 250  $\mu\text{mol l}^{-1}$  of each deoxynucleotide triphosphate (dNTP), 400  $\text{nmol l}^{-1}$  of the forward PCR primer, 200  $\text{nmol l}^{-1}$  of each reverse PCR primer, 2.5- $\mu\text{l}$  of DNA template, and 0.625 units JumpStart Taq DNA polymerase (Sigma-Aldrich, St Louis, MO, USA). PCR primers targeted a portion of the small-subunit (ITS-1507F, GGTGAAGTCGTAACAAGGTA) and large-subunit (ITS-23SR, GGGTTBCCCCATTCRG) rRNA genes and the hypervariable ITS region (Ruegger et al., 2014), with the reverse primers including a 12-bp barcode and both primers including the sequences needed for

Illumina cluster formation; primer binding sites are the reverse and complement of the commonly used small-subunit rRNA gene primer 1492R (Frank et al., 2008) and the large-subunit rRNA gene primer 129F (Hunt et al., 2006). PCR primers were only frozen and thawed once. Thermal cycling parameters were as follows:  $94^{\circ}\text{C}$  for 5 min; 35 cycles of  $94^{\circ}\text{C}$  for 20 s,  $56^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 40 s; followed by  $72^{\circ}\text{C}$  for 10 min. PCR products were purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA sequencing (single-end 250 base) was performed using an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). Clusters were created using template concentrations  $2.5 \text{ pmol l}^{-1}$  and phi X at  $107,000 \text{ mm}^{-2}$ .

Data processing was performed with USEARCH v10.0 (Edgar, 2010). We used the UPARSE pipeline for de-multiplexing, length trimming, quality filtering and operational taxonomic unit (OTU) picking using default parameters or recommended guidelines that were initially described in Edgar (2013) and which have been updated at [https://www.drive5.com/usearch/manual10/uparse\\_pipeline.html](https://www.drive5.com/usearch/manual10/uparse_pipeline.html). Briefly, after demultiplexing and using the recommended 1.0 expected error threshold, sequences were trimmed to a uniform length of 248 bp and then dereplicated. Dereplicated sequences were subjected to error correction (denoised) and chimera filtering to generate zero-radius operational taxonomic units (ZOTUs) using UNOISE3 (Edgar, 2016b preprint). An OTU table was then generated using the otutab command. ZOTUs with non-bacterial DNA were identified and enumerated by performing a local BLAST search (Altschul et al., 1990) of their seed sequences against the nucleotide database. ZOTUs were removed if any of their highest scoring BLAST hits contained taxonomic IDs within the rodent family, Fungi, Viridiplantae or phi X. Taxonomic assignments to bacterial ZOTUs were made with the SINTAX taxonomy prediction algorithm (Edgar, 2016a preprint) on an updated SSU-ITS database (Ruegger et al., 2014). This resulted in 2730 OTUs with an average of 47,851 sequences per sample. Data were normalized within each sample by dividing the number of reads in each OTU by the total number of reads in that sample.

The bacterial rRNA ITS sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under SRA BioProject Accession PRJNA624662.

### Statistical analyses

#### Juvenile wheel running and food consumption

As used in numerous previous studies of these lines of mice, we used linear mixed models in SAS 9.4 Proc Mixed (SAS Institute, Cary, NC, USA). The effect of line type is tested against the variance among replicate lines, which are a nested random effect within line type. Wheel access $\times$ line(line type), diet $\times$ line(line type) and wheel access $\times$ diet $\times$ line(line type) were also nested random effects. In these full models, the effects of wheel access, diet, line type and their interactions were tested with 1 and 6 degrees of freedom. If the covariance parameter estimate for higher-order random effects was zero, we removed them in a stepwise fashion. In other words, if the covariance parameter estimate for the three-way interaction was 0, we removed the wheel access $\times$ diet $\times$ line(line type) random effect. Then, if one of the two-way random interaction effects was also zero, we removed it. However, we always retained the line(line type) random effect, given the nature of the experimental design (e.g. see Castro and Garland, 2018; Castro et al., 2020; Swallow et al., 1998). For juvenile wheel running, we



included wheel freeness as a covariate in the model. For caloric intake, we included body mass as a covariate.

In these statistical models, we also tested for effects of the mini-muscle phenotype (present in two of the HR lines) on juvenile wheel running, juvenile caloric intake, adult gut microbiome richness and relative abundance. The mini-muscle phenotype is caused by an autosomal recessive allele, a single base pair change in a myosin heavy chain gene (Kelly et al., 2013). Homozygotes for this naturally occurring mutation are characterized by a 50% reduction in hindlimb muscle mass, larger internal organs and various other differences as compared with unaffected individuals (Garland et al., 2002; Swallow et al., 2009; Wallace and Garland, 2016). In the present study, the number of mini-muscle individuals varied among analysis. For example, of the 88 mice for which we obtained wheel-running data during week 1 of juvenile exposures, 12 had the mini-muscle phenotype (all nine in line 3 and three of 11 in line 6). Of the 165 mice for which we obtained week 1 food consumption data, 43 had the mini-muscle phenotype (all 21 in line 3 and five of 22 in line 6). Of the 149 mice for which we obtained microbiome data, 25 had the mini-muscle phenotype (all 20 in line 3 and five of 20 in line 6).

#### Beta diversity of the adult gut microbiome

Gut microbiome membership and community structure were compared by calculating unweighted UniFrac and Hellinger distance matrices in QIIME version 1.9.1. Unweighted UniFrac distance utilizes the presence and absence of bacterial species while accounting for the phylogenetic relationship between bacterial species. For statistical and graphical representation, we used an OTU table rarified to an even sequencing depth of 14,000 reads per sample. We used a principal coordinates analysis (PCoA) to visualize the communities in a 3D space. For beta diversity, we used a PERMANOVA test in QIIME to determine statistical significance (Anderson, 2001). For these tests we did not treat replicate line as a nested random effect because the software to do this is not currently available.

#### Alpha diversity of the adult gut microbiome

To determine the effects of diet, exercise, line type and their interactions on alpha diversity of the adult gut microbiome, we used the Chao1 index and Shannon index calculated in QIIME Version 1.9.1 from an OTU table rarified to the lowest common sequencing depth of 14,000 reads. We also totaled the number of non-zero OTUs identified in each mouse using the rarified OTU table. We used the statistical procedures described above in 'Juvenile wheel running and food consumption'. Because ANOVAs have relatively low power to detect interactions (Wahlsten, 1990), and following our laboratory's previous analyses of these mice (e.g. Belter et al., 2004; Houle-Leroy et al., 2000), we considered interactions significant if  $P < 0.10$ .

#### Lower-level taxa summary comparisons

We compared the relative abundance data of identified phylum, class, order, family, genus and species groups produced by the summarize\_taxa.py script in QIIME. Based on the simulations reported by Aschard et al. (2019), we only analyzed taxa found in >85% of the mice [phylum ( $N=6$ ), class ( $N=9$ ), order ( $N=8$ ), family ( $N=16$ ), genus ( $N=17$ ), species ( $N=26$ ) and OTUs ( $N=140$ , of the total 2730 identified OTUs)], which totaled 221 tests and 1761  $P$ -values. We used the statistical procedures described above in 'Juvenile wheel running and food consumption'. Bacterial relative abundance data were log or arcsine square-root transformed to normalize residuals (Brown et al., 2020; Kohl et al., 2016).  $P$ -values

were corrected for multiple comparisons using the false discovery rate (FDR; Benjamini and Hochberg, 1995). For these analyses, we accepted statistical significance at  $P < 0.05$  after adjustment for FDR.

## RESULTS

### Line type, diet and exercise affect juvenile wheel running and food consumption

Diet had an interactive effect on wheel running across the 3 weeks of early-life exposure (full statistical results are in Table S1). During the first week, Western diet increased wheel running, but the effect was greater in HR mice (interaction  $F_{1,76}=7.62$ ,  $P=0.0072$ ; Fig. 2A), and mini-muscle mice ran more than normal-muscle mice ( $F_{1,76}=6.12$ ,  $P=0.0156$ ). During the second week, mice with a Western diet continued to run significantly more than those with standard diet, and HR mice ran 2.6-fold more revolutions per day than C mice, with no interaction between diet and line type (interaction  $F_{1,76}=0.51$ ,  $P=0.4765$ ; Fig. 2A). By the third week of juvenile wheel access, HR mice ran 3.4-fold more than C mice and diet no longer significantly affected wheel running.

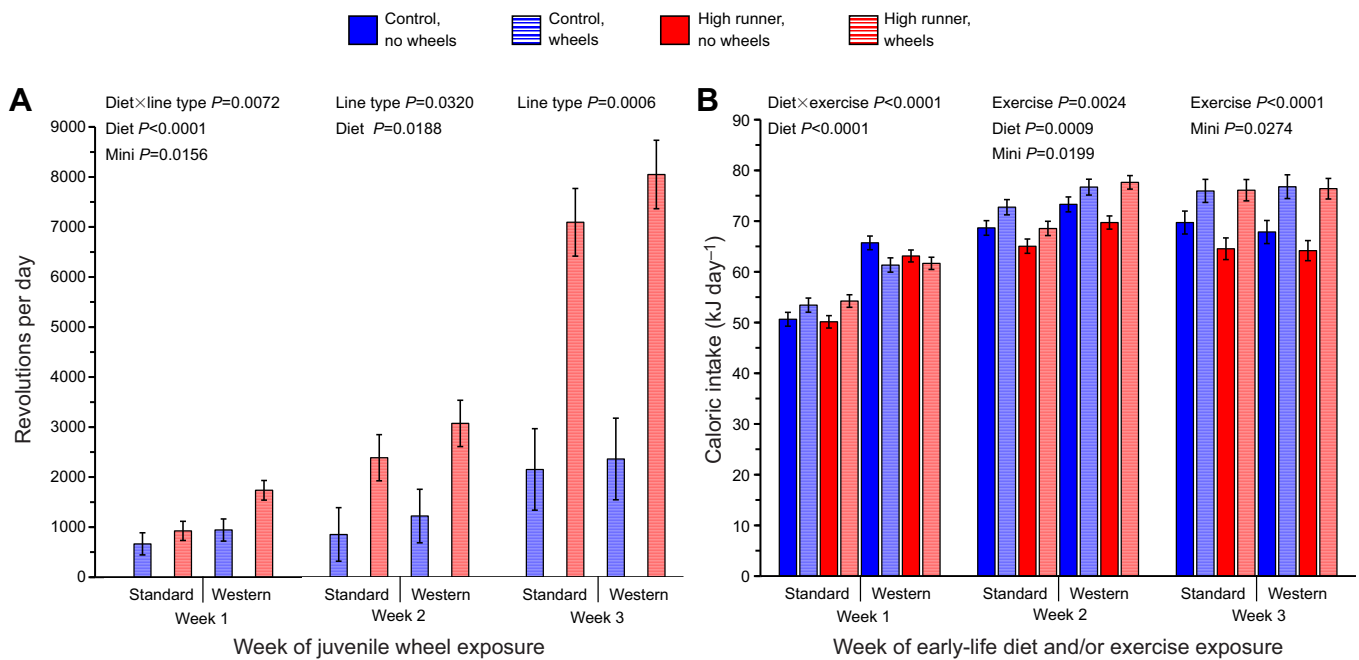
During the first week of early-life exposure, diet and wheel access had an interactive effect on caloric intake (interaction  $F_{1,143}=26.62$ ,  $P < 0.0001$ ; Fig. 2B). Western diet increased caloric intake in all groups, by ~21% on average ( $F_{1,143}=313.25$ ,  $P < 0.0001$ ; Fig. 2B). However, wheel access increased intake in mice on a standard diet but decreased it in those on a Western diet. During the second week, mice on the Western diet had increased caloric intake ( $F_{1,6}=37.71$ ,  $P=0.0009$ ; Fig. 2B) and those with wheels consumed more than mice without wheels ( $F_{1,6}=25.18$ ,  $P=0.0024$ ; Fig. 2B). In the third week, mice with wheels again consumed more calories than those without wheels ( $F_{1,6}=84.23$ ,  $P < 0.0001$ ; Fig. 2B), but the effect of diet was no longer significant. Mini-muscle mice consumed significantly more food than normal-muscle mice during both weeks 2 ( $F_{1,137}=5.55$ ,  $P=0.0199$ ) and 3 ( $F_{1,136}=4.97$ ,  $P=0.0274$ ).

### Dominant phyla of the adult gut microbiome

The 2730 identified OTUs were classified into seven phyla, 22 classes, 36 orders, 58 families, 79 genera and 112 species. Community composition for the entire set of experimental mice ( $N=149$ ) was dominated by the phyla Bacteroidetes (68.1±17.4%) (mean±s.d.) and Firmicutes (27.9±16.7%), with additional phyla being much less abundant: Proteobacteria (1.2±2.1%), *Candidatus* Melanobacteria (0.3±0.6%), Tenericutes (0.2±0.3%) and Actinobacteria (0.05±0.04%) (Fig. 3).

### Juvenile diet and line type affect adult community membership (Beta diversity)

Community membership measured by unweighted UniFrac distance and by Hellinger distance plotted in a PCoA plot (Figs 4 and 5, respectively; corresponding statistical results in Tables 1 and 2, respectively) showed clustering of mice by line type and by juvenile diet exposure. HR and C mice significantly clustered independent of one another (PERMANOVA,  $F_{1,147}=1.56$ ,  $P=0.009$ , Fig. 4A; PERMANOVA,  $F_{1,147}=2.31$ ,  $P=0.001$ , Fig. 5A). Mice fed a juvenile Western diet resulted in significant clustering of samples compared with mice fed a juvenile standard diet (PERMANOVA,  $F_{1,147}=2.72$ ,  $P=0.001$ , Fig. 4B; PERMANOVA,  $F_{1,147}=2.85$ ,  $P=0.001$ , Fig. 5B). Within both HR and C line types, mice clustered together by diet (C,  $F_{1,75}=1.64$ ,  $P=0.007$ ; HR  $F_{1,70}=0.001$ ,  $P=0.001$ : Fig. S1F). Wheel access did not result in significant clustering within line types (PERMANOVA,  $F_{1,70}=1.30$ ,  $P=0.072$ , Fig. S1G). HR mice also clustered independently by diet (PERMANOVA,  $F_{1,70}=3.783$ ,  $P=0.001$ , Fig. S2F).



**Fig. 2. Weekly revolutions per day and caloric intake in response to juvenile diet and/or exercise treatment.** Data are presented as untransformed least squares means  $\pm$  s.e.m. (values for mini-muscle versus normal-muscle mice are not shown). Shown above each week are the significant main effects and interactions (two-tailed ANCOVAs  $P < 0.05$ , not adjusted for multiple comparisons). Full statistical results are in Table S1. (A) Weekly juvenile wheel running for half of the mice during the 3 weeks of early-life exposure ( $N = 88$ ). (B) Weekly mass-adjusted juvenile caloric intake during the 3 weeks of early-life exposure ( $N = 165$ ).

### Early-life exposures, line type and their interactions affect adult gut microbiome richness (alpha diversity)

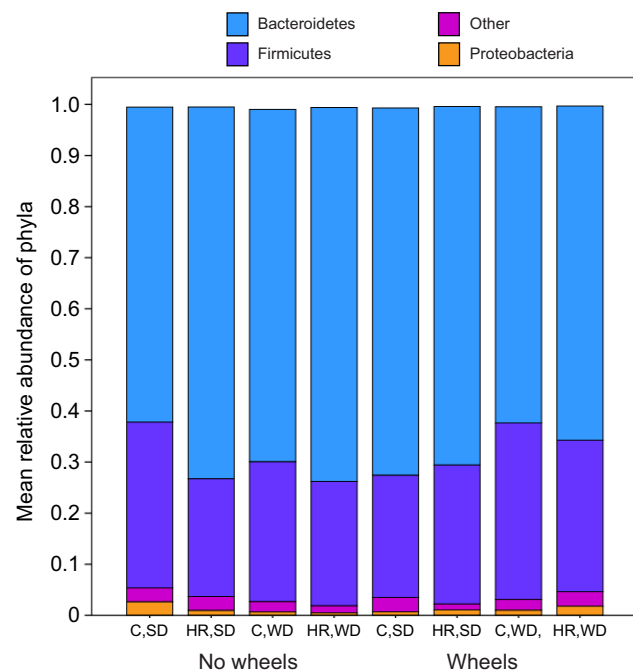
For the total number of OTUs, early-life diet and exercise exposures altered the adult gut microbial richness in a line-type-dependent

manner: the three-way interaction of juvenile diet, wheel access and line type was significant (interaction  $F_{1,128} = 2.83$ ,  $P = 0.095$ ; Fig. 6A). Early-life Western diet tended to have a lasting impact on gut microbiome diversity by reducing the total OTUs (ANOVA,  $F_{1,6} = 5.67$ ,  $P = 0.055$ ; Fig. 6A).

The three-way interaction of juvenile diet, exercise and line type was significant for the Chao1 index, a corrected index of gut microbial richness that accounts for rarer taxa (interaction  $F_{1,128} = 2.83$ ,  $P = 0.013$ ; Fig. 6B). Early-life exposure to Western diet tended to have a lasting impact on the gut microbiome by reducing adult gut community richness (ANOVA,  $F_{1,6} = 5.68$ ,  $P = 0.054$ ; Fig. 6B). The Shannon index, another measure of gut microbial richness that accounts for the abundance of taxa in a sample, was not statistically different among groups (Fig. 6C).

### Juvenile Western diet affects adult gut microbiome community

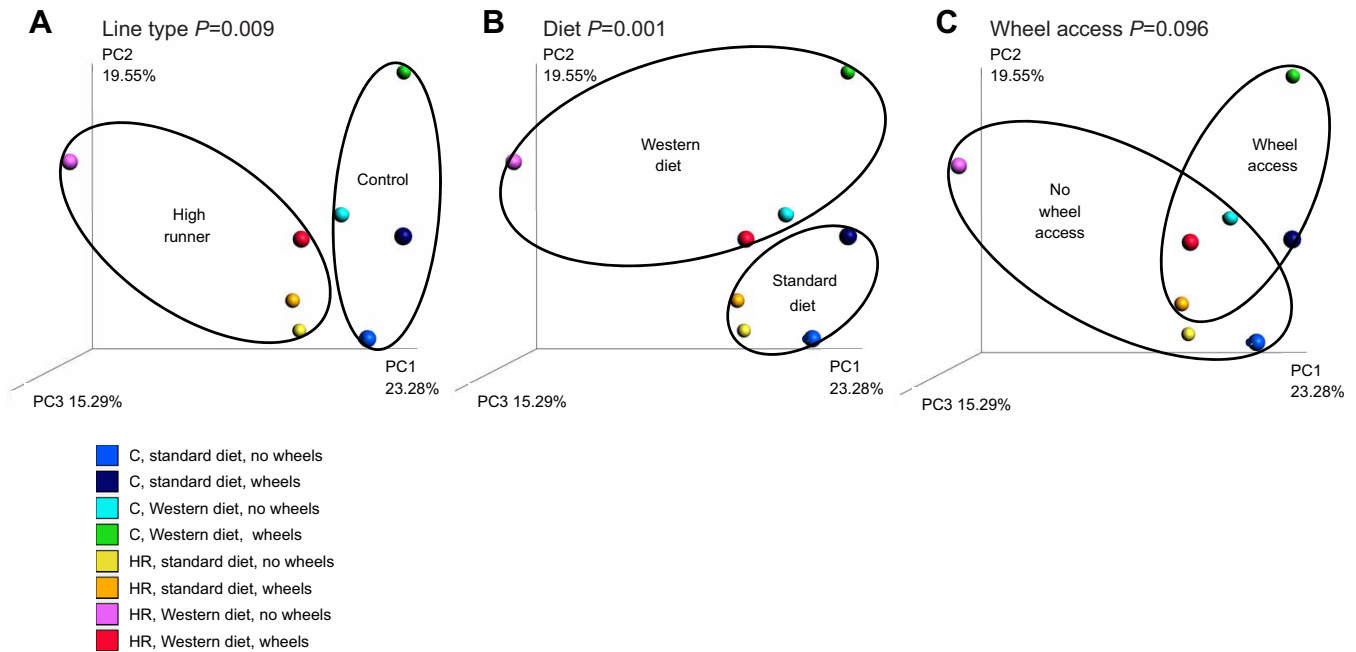
Of the 1760  $P$ -values tested, only two remained significant at  $P < 0.05$  after correcting for multiple comparisons using a Benjamini and Hochberg FDR (see Table S2 for phylum through genus  $P$ -values before FDR). Western diet significantly reduced the relative abundance of the family *Muribaculaceae*, which is commonly found in the mouse gut microbiome (ANOVA,  $F_{1,128} = 19.2$ ,  $P = 0.021$ ). This decrease is explained by the gut bacterial species *Muribaculum intestinale*, which was found in all mice from our study (ANOVA,  $F_{1,128} = 19.2$ ,  $P = 0.021$ ; Fig. 7). *Muribaculum intestinale* made up 0.38% of the identified OTUs. Mini-muscle mice did not significantly differ in the relative abundance of any of the tested taxa.



**Fig. 3. Community composition of the adult gut microbiome for all experimental mice ( $N = 149$ ).** Bars represent the mean relative abundance of the three main phyla found in greater than 1% of the population, separated by treatment group. C, control; HR, high running; SD, standard diet; WD, Western diet.

### DISCUSSION

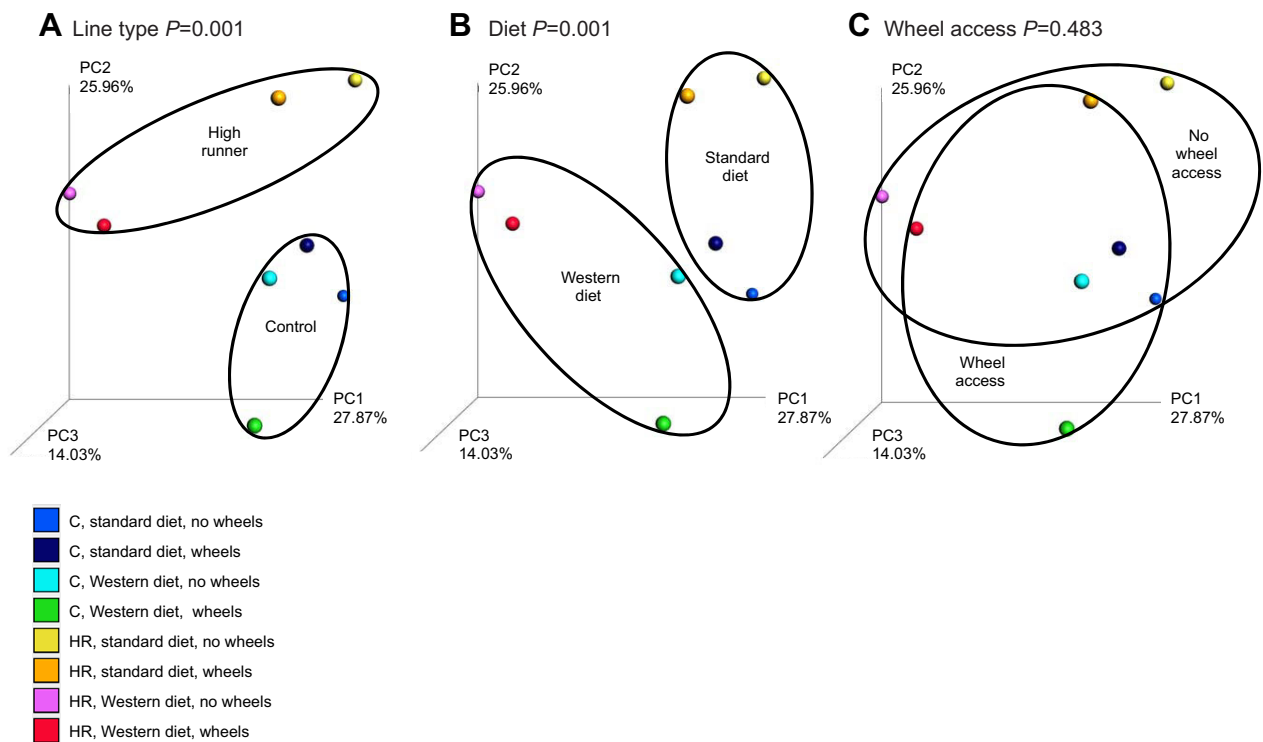
Our results constitute one of the first reports of juvenile diet having long-lasting effects on the adult microbiome after a substantial washout period (equivalent to  $\sim 6$  human years). Moreover, we



**Fig. 4. Community membership of the adult gut microbiome principal coordinate analysis (PCoA) using unweighted UniFrac distances.** (A) Clustering of mice by high runner ( $N=72$ ) and control ( $N=77$ ) lines of mice (PERMANOVA,  $F_{1,147}=1.56$ ,  $R^2=0.010$ ,  $P=0.009$ ). (B) Clustering of mice by Western diet ( $N=77$ ) and standard diet ( $N=72$ ) (PERMANOVA,  $F_{1,147}=2.72$ ,  $R^2=0.018$ ,  $P=0.001$ ). (C) Clustering of mice by wheel access ( $N=75$ ) and no wheel access ( $N=74$ ) (PERMANOVA,  $F_{1,147}=1.24$ ,  $R^2=0.008$ ,  $P=0.096$ ). Results of statistical analyses are shown in Table 1.

found interactive effects of diet with early-life exercise exposure, and a dependence of these effects on genetic background. The overall bacterial community composition that we found (Fig. 3) is

similar to that reported in many other studies of adult laboratory house mice (e.g. Benson et al., 2010; Lamoureux et al., 2017). However, beta diversity metrics indicated that community



**Fig. 5. Community membership of the adult gut microbiome PCoA using a Hellinger distance matrix.** (A) Clustering of mice by high runner ( $N=72$ ) and control ( $N=77$ ) lines of mice (PERMANOVA,  $F_{1,147}=2.31$ ,  $R^2=0.015$ ,  $P=0.001$ ). (B) Clustering of mice by Western diet ( $N=77$ ) and standard diet ( $N=72$ ) (PERMANOVA,  $F_{1,147}=2.85$ ,  $R^2=0.019$ ,  $P=0.001$ ). (C) Clustering of mice by wheel access ( $N=75$ ) and no wheel access ( $N=74$ ) (PERMANOVA,  $F_{1,147}=0.99$ ,  $R^2=0.007$ ,  $P=0.483$ ). Results of statistical analyses are shown in Table 2.

**Table 1. Community membership of the adult gut microbiome assessed by PERMANOVA statistical tests using unweighted UniFrac distances**

	SS	d.f.	F	R <sup>2</sup>	P	Figure
Line type	0.213	1, 147	1.560	0.010	0.009	4A
Diet	0.369	1, 147	2.719	0.018	0.001	4B
Wheel access	0.170	1, 147	1.243	0.008	0.096	4C
C:Diet	0.225	1, 75	1.644	0.021	0.007	4B, S1F
HR:Diet	0.328	1, 70	2.462	0.034	0.001	4B, S1F
C:Wheel access	0.116	1, 75	0.838	0.011	0.832	4C, S1G
HR:Wheel access	0.176	1, 70	1.304	0.018	0.072	4C, S1G

HR, high runner; C, control. Statistical analyses corresponding to Fig. 4.

membership was unequal between the two genetic line types we studied (replicate, selectively bred HR and C lines of mice), and was also affected by early-life Western diet (Figs 4, 5). Bacterial richness and alpha diversity were also affected by an interaction of juvenile diet, exercise and line type (Fig. 6). Finally, juvenile Western diet significantly decreased the relative abundance of the *Muribaculaceae* family driven by the species *M. intestinale* (Fig. 7).

Selective breeding for high voluntary wheel running resulted in unique clustering of gut microbiomes by line type (Figs 4, 5). These results are consistent with the fact that selection for wheel-running behavior has caused many exercise-associated biological changes that could influence the gut environment, including higher food consumption even when housed without wheels, higher body temperatures when active, and differences in circulating concentrations of multiple hormones, including corticosterone, a classic 'stress hormone' (Copes et al., 2015; Garland et al., 2016; Malisch et al., 2009; Swallow et al., 2009; Wallace and Garland, 2016). Our results and those of other recent studies also demonstrate the utility of selectively bred rodent models for understanding possible coevolutionary changes in the microbiome (e.g. see Kohl et al., 2016; Liu et al., 2015; van der Eijk et al., 2020; Zhang et al., 2020).

A Western diet can negatively impact the host's normal gut barrier function by increasing intestinal permeability (Martinez-Medina et al., 2014) and by increasing inflammation of the gut environment (Agus et al., 2016). Several studies have demonstrated effects of a Western diet on the gut microbiome in adult rodents. For example, Western diet results in unique clustering of microbiome communities (Carmody et al., 2015; Pindjakova et al., 2017). We also found significant clustering of microbiome communities by diet (Figs 4, 5). Previous studies of adult mice have reported that a high-fat or high-sugar diet can decrease bacterial diversity (Pindjakova et al., 2017; Sonnenburg et al., 2016; Turnbaugh et al., 2008). Adult rats on standard chow supplemented with 10% sucrose solution and a selection of cakes, biscuits and high-protein foods continuously for 25 days had a significantly reduced alpha diversity, evidenced by a reduction in the total number of OTUs compared with control rats (Beilharz et al., 2017). In our study,

**Table 2. Community membership of the adult gut microbiome assessed by PERMANOVA statistical tests using a Hellinger distance matrix**

	SS	d.f.	F	R <sup>2</sup>	P	Figure
Line type	1.150	1, 147	2.310	0.015	0.001	5A
Diet	1.414	1, 147	2.851	0.019	0.001	5B
Wheel access	0.497	1, 147	0.989	0.007	0.483	5C
C:Diet	0.534	1, 75	1.043	0.014	0.384	5B, S2F
HR:Diet	1.753	1, 70	3.783	0.051	0.001	5B, S2F
C:Wheel access	0.385	1, 75	0.749	0.010	0.843	5C, S2G
HR:Wheel access	0.458	1, 70	0.951	0.013	0.518	5C, S2G

Statistical analyses corresponding to Fig. 5.

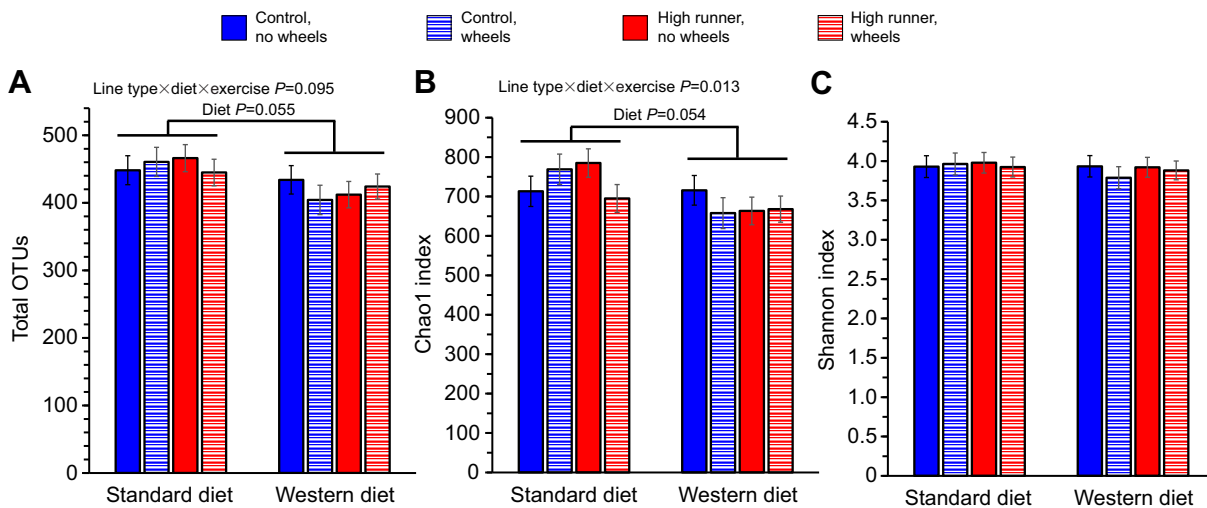
Western diet during the juvenile period increased wheel-running behavior and food consumption in both selectively bred HR mice and non-selected C mice (Fig. 2). Both altered diet and increased food consumption can affect the gut environment and thus alter the bacterial community. In principle, early-life Western diet could have altered the gut microbiome in a way that persists into adulthood, an effect that we did indeed find (Figs 4–7).

Only one other publication has examined the long-lasting effects of juvenile diet on the adult gut microbiome after a significant washout period in mice. Mice with 3 weeks of juvenile high-fat diet followed by a 7-week washout period had decreased alpha diversity as measured by the Shannon index as adults (Fülling et al., 2020). In our study, perturbation of the juvenile gut microbiome with Western diet also had long-lasting effects on species community indicators of adult gut microbial richness by reducing the total number of OTUs and the Chao1 index, though no differences in Shannon diversity were found (Fig. 6). Similarly to Carmody et al. (2015), who demonstrated that a high-fat, high-sugar diet in multiple inbred, outbred and transgenic strains of mice resulted in clustering of mice by both diet and genotype within diet treatment, we found significant clustering of genetic lines within diet treatment (Fig. S1), showing the response to diet can be genotype-dependent.

After correction for multiple comparisons of 1760 *P*-values comparing taxa at the level of phylum, class, order, family, genus, species and OTU, we found one species (and its family) whose relative abundance was significantly decreased by juvenile Western diet, *Muribaculum intestinale* (Fig. 7, Table S2). The *Muribaculaceae* family is commonly found in mouse (but not human) gut microbiomes (previously referred to as S24-7; Lagkouravdos et al., 2016; Seedorf et al., 2014). *Muribaculaceae* has been linked with propionate production, a short-chain fatty acid, in a mouse longevity study (Smith et al., 2019). This family was also seen to increase in abundance in mice given voluntary wheel access while on a high-fat or standard diet, and decrease in relative abundance in mice on a high-fat diet with or without exercise (Evans et al., 2014). This finding is similar to our study in which the relative abundance of *M. intestinale*, a species of the *Muribaculaceae* family, was unaffected by exercise but decreased in abundance with juvenile Western diet (Fig. 7). *Muribaculaceae* belongs to the phylum Bacteroidetes, one of the two most abundant phyla in the gut microbiome. A Western diet has been shown to usually decrease the relative abundance of Bacteroidetes, a primarily acetate- and propionate-producing phylum, while increasing the relative abundance of Firmicutes, a primarily butyrate-producing phylum (Carmody et al., 2015; den Besten et al., 2013; Ley et al., 2006). If species in the *Muribaculaceae* family could potentially influence the energy substrate availability to the host, this could lead to a differential effect of diet and exercise treatments on normal host function. As *M. intestinale* is a newly cultured species, it remains to be seen what other functions it might have (Lagkouravdos et al., 2019). In a small sample of adult wild-type and AC5KO mice (known for their exercise-associated traits of longevity and increased mitochondrial metabolism in skeletal muscle; Ho et al., 2015), a taxon with high sequence similarity to *M. intestinale* was enriched in adult AC5KO mice after 5 weeks of treadmill training, suggesting that *M. intestinale* is a potentially exercise-associated species (Dowden et al., 2020).

To our knowledge, only one previous study of rodents has tested for long-lasting effects of juvenile exercise on the adult microbiome. Mika et al. (2015) found that juvenile rats given 6 weeks of wheel access, followed by a 25-day washout period, tended (not statistically significant) to have a decreased abundance of the





**Fig. 6. Alpha diversity metrics of the adult gut microbiome (N=149 mice).** Data are presented as untransformed least squares means $\pm$ s.e.m. (A) Total operational taxonomic units (OTUs) when the OTU table was rarified to an even number of reads per sample. The three-way interaction between juvenile diet, exercise and line type on fecal bacterial richness was significant (two-tailed ANOVA interaction,  $F_{1,128}=2.83$ ,  $P=0.095$ , not adjusted for multiple comparisons). Early-life exposure to Western diet tended to have a lasting impact on gut microbiome diversity by reducing the total OTUs (two-tailed ANOVA,  $F_{1,6}=5.67$ ,  $P=0.055$ , not adjusted for multiple comparisons). (B) Chao1 index. The three-way interaction between Western diet, exercise and line type was statistically significant (two-tailed ANOVA interaction,  $F_{1,128}=6.39$ ,  $P=0.013$ , not adjusted for multiple comparisons). Early-life exposure to Western diet tended to have a lasting impact on the gut microbiome by reducing adult gut community richness (two-tailed ANOVA,  $F_{1,6}=5.68$ ,  $P=0.054$ , not adjusted for multiple comparisons). (C) The Shannon index was not significantly affected by any experimental factor.

*Firmicutes* phylum compared with sedentary juveniles. We found that early-life exercise significantly interacted with diet and line type to influence gut microbial diversity (Fig. 6). Given that we have shown long-lasting effects of relatively mild and natural early-life changes (diet, exercise), more severe treatments, such as antibiotics, might have even stronger long-lasting effects (Ma et al., 2020).

#### Limitations and future directions

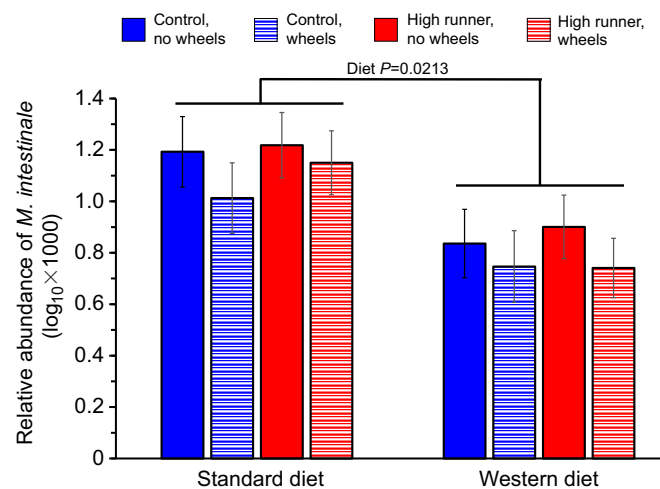
When examining the gut microbiome, variation in sequencing methods can lead to different results under similar experimental conditions. Much of the literature consists of 16S rRNA analysis. Instead, we sequenced the ITS rRNA gene for finer resolution of the

gut microbial community (Ruegger et al., 2014). This poses a challenge when comparing ITS data with 16S data. Nevertheless, by examining broad patterns in diversity and community structure (Figs 4–6), we were able to find similar patterns between our data and the literature (see above). For example, a Western diet tends to decrease gut microbiome diversity (Fig. 6) and alters the gut microbiome community measured by beta diversity (Figs 4, 5).

We were only able to sample feces and obtain microbial sequence data for one time point. Logistical constraints precluded our obtaining fecal samples at the beginning of the study. In future studies, repeating this experiment with a baseline sample at weaning and immediately after the juvenile exposure to diet and/or exercise would increase the power to detect longitudinal changes. As we had only the microbiome data after the washout period, we cannot know when the effects of the experimental treatments first appeared. They might have appeared during the 3-week treatment period, which seems likely, or they might have appeared later, at any time prior to when we took fecal samples. Regardless of when the effects first appeared, they were detectable when we analyzed the adult fecal samples. This is an important result, even in the absence of information regarding the longitudinal trajectory of the effects. Future studies should examine the time course of early-life effects. In addition, study of the cecum would allow a more *in situ* view of the microbiome.

We did not separate or sterilize cages, bedding, food or water, thus giving the mice constant exposure to environmental bacteria. This exposure should have tended to homogenize the gut microbiome, thus possibly erasing any early-life effects of diet or exercise. Nevertheless, we were able to detect such effects after a substantial washout period, supporting the idea that the early-life developmental period of the microbiome is sensitive and responsive to change, and can be impacted in ways that resist subsequent environmental perturbations.

Future experiments involving antibiotic reduction and transplantation of the microbiome will be required to determine



**Fig. 7. Relative abundance of the species *Muribaculum intestinale* (N=149 mice).** Data are presented as transformed least squares means $\pm$ s.e.m. Mice with juvenile exposure to Western diet had a significantly lower relative abundance of the species *M. intestinale* (two-tailed ANOVA,  $F_{1,128}=19.2$ ; FDR-adjusted  $P=0.0213$ ).



whether the unique microbial community of HR mice (Figs 4, 5), which has potentially co-evolved during the selection experiment, contributes to their high motivation and/or ability for sustained, aerobically supported exercise (Hsu et al., 2015; Nay et al., 2019; Okamoto et al., 2019; Scheiman et al., 2019). More specifically, one could administer antibiotics to eliminate the existing gut microbiome, monitor changes in wheel running, and then transplant the HR microbiome into C mice and vice versa. Additional groups would receive their own line-type-specific microbiome in the reseeded phase of the experiment (i.e. HR to HR and C to C). If a unique microbiome is partly responsible for the HR phenotype, then we would predict that (1) antibiotics would reduce their wheel running and (2) reseeded with HR (but not C) microbiome would recover the normal wheel-running behavior for HR mice. It is also possible that transplanting the HR microbiome to C mice would increase their wheel running, at least if some other inherent factor does not limit their running motivation or ability.

Overall, we found that an early-life Western diet had more long-lasting effects on the microbiome than did early-life exercise. Future studies will be required to determine whether this is a general result. In particular, we need dose–response studies of how much exercise, and what type of exercise, is needed to elicit a permanent, potentially beneficial, change in the gut microbiome. The field also needs more studies of how voluntary exercise can acutely change the gut microbiome (e.g. by short-term or alternate-day wheel access), combined with longitudinal sampling. Finally, milder diet alterations should be examined, in addition to effects of probiotics (Sanders et al., 2019).

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: M.P.M., J.M.S., M.D.C., J.B., T.G.; Methodology: M.P.M., J.M.S., M.D.C., J.B., T.G.; Software: J.B.; Validation: J.B., T.G.; Formal analysis: M.P.M., M.D.C., P.M.R., J.B., T.G.; Investigation: M.P.M., J.M.S., M.D.C., P.M.R., J.B., T.G.; Resources: J.B., T.G.; Data curation: M.P.M., M.D.C., P.M.R., J.B., T.G.; Writing - original draft: M.P.M., P.M.R., J.B., T.G.; Writing - review & editing: M.P.M., J.M.S., M.D.C., P.M.R., J.B., T.G.; Visualization: M.P.M., P.M.R., J.B., T.G.; Supervision: J.B., T.G.; Project administration: T.G.; Funding acquisition: J.B., T.G.

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#### Data availability

Microbiome data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under SRA BioProject Accession PRJNA624662.

#### Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.239699.supplemental>

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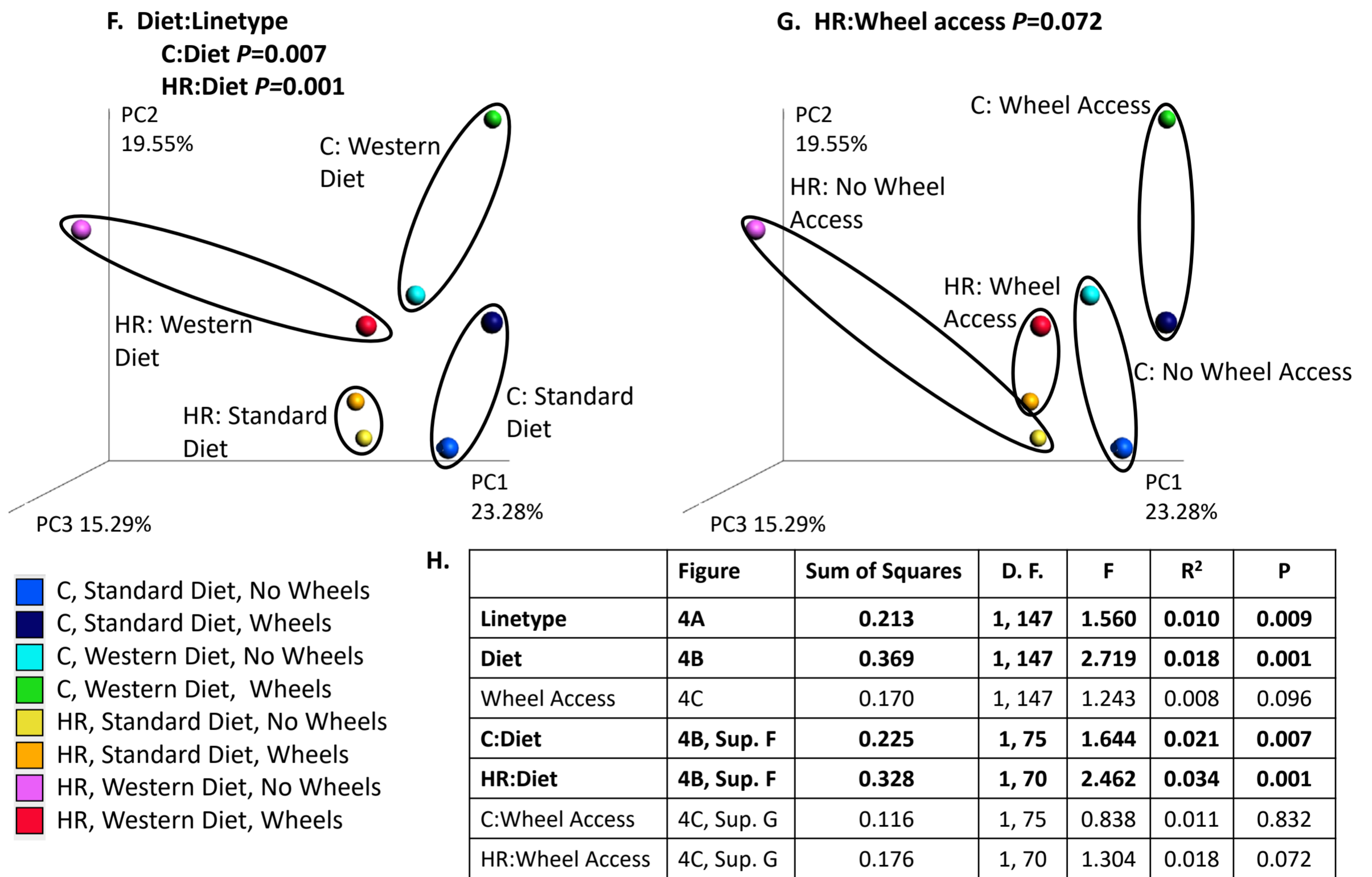
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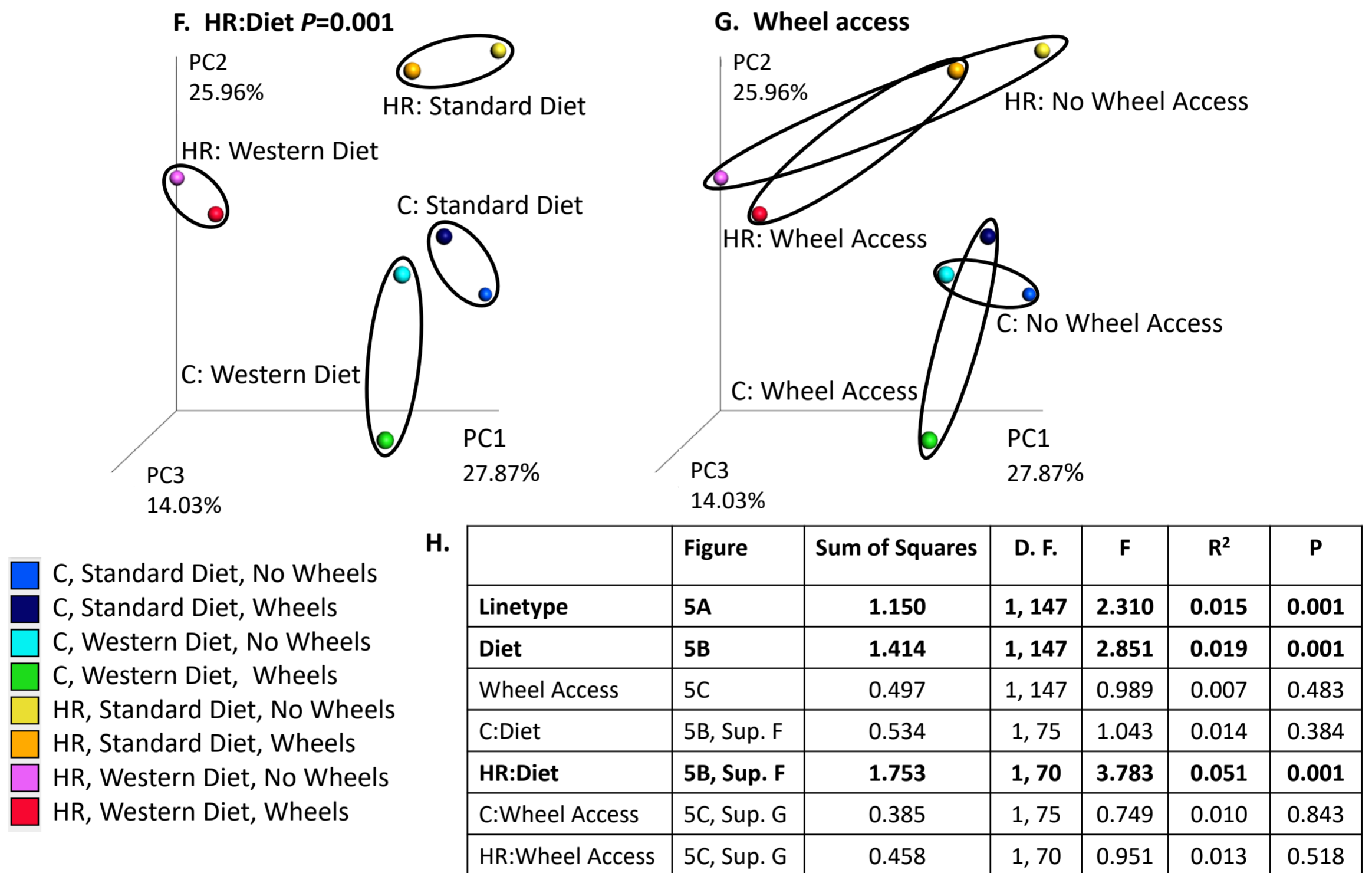
Figure S1.



**Figure S1.** **F.** Community membership of the adult gut microbiome Principal Coordinate Analysis using unweighted UniFrac distances. Clustering of mice by HR:WD ( $N=38$ ), HR:SD ( $N=34$ ), C:WD ( $N=39$ ), and C:SD ( $N=38$ ). **G.** Clustering of mice by HR:Wheel access ( $N=38$ ), HR:No wheel access ( $N=34$ ), C:Wheel access ( $N=37$ ), H:No wheel access ( $N=40$ ). **H.** Statistical results.



Figure S2.



**Figure S2. F.** Community membership of the adult gut microbiome Principal Coordinate Analysis using a Hellinger distance matrix. Clustering of mice by HR:WD ( $N=38$ ), HR:SD ( $N=34$ ), C:WD ( $N=39$ ), and C:SD ( $N=38$ ). **G.** Clustering of mice by HR:Wheel access ( $N=38$ ), HR:No wheel access ( $N=34$ ), C:Wheel access ( $N=37$ ), C:No wheel access ( $N=40$ ). **H.** Statistical results.

**Table S1.** *P* values from analyses of juvenile wheel running and caloric intake. Tests for main and interactive effects on juvenile wheel running and caloric intake. For wheel running, a measure of wheel freeness was included but was not significant (results not shown); for caloric intake, body mass was included as a covariate. Significance levels (*P* values; bold indicates  $P < 0.05$ , two-tailed, unadjusted for multiple comparisons).

Early-life Trait	N	D.F.	$P_{\text{linetype}}$	$P_{\text{exercise}}$	$P_{\text{diet}}$	$P_{\text{diet} \times \text{linetype}}$	$P_{\text{diet} \times \text{exercise}}$	$P_{\text{exercise} \times \text{linetype}}$	$P_{\text{diet} \times \text{linetype} \times \text{exercise}}$	$P_{\text{body mass}}$	$P_{\text{mini-muscle}}$
Week 1 Revolutions/day	88	6, 76	0.0854	NA	<b>&lt;0.0001</b>	<b>0.0072</b>	NA	NA	NA	NA	<b>0.0156</b>
Week 2 Revolutions/day	88	6, 76	<b>0.0320</b>	NA	<b>0.0188</b>	0.4765	NA	NA	NA	NA	0.9060
Week 3 Revolutions/day	88	6, 80	<b>0.0006</b>	NA	0.2848	0.4950	NA	NA	NA	NA	<b>0.3800</b>
Week 1 Caloric Intake	165	6, 143	0.7158	0.7553	<b>&lt;0.0001</b>	0.2983	<b>&lt;0.0001</b>	0.2399	0.5121	<b>&lt;0.0001</b>	0.9206
Week 2 Caloric Intake	165	6, 137	0.0658	<b>0.0024</b>	<b>0.0009</b>	0.2030	0.2941	0.3391	0.1514	<b>&lt;0.0001</b>	<b>0.0199</b>
Week 3 Caloric Intake	164	6, 136	0.3158	<b>&lt;0.0001</b>	0.8676	0.8806	0.3842	0.0881	0.5950	<b>&lt;0.0001</b>	<b>0.0274</b>

## Table S2. Significance levels for Phylum, etc.

Excel file for table of *p* values for phylum through genus. Taxafound in >85% of the mice were analyzed [phyla (*N*=6), class (*N*=9),order (*N*=8), family (*N*=16), genus (*N*=17), species (*N*=26), andOTUs (*N*=140, of the total 2,730 identified OTUs) totaling 221 testsand 1,761 *P* values. 448 *P* values shown in the table. *P* values

before FDR ≤ 0.1 are highlighted in red.

**Table S2. Table with Phylum through Genus *p* values for taxa found in ≥85% of the population. 448 *p* values.** *P* values ≤ 0.1

(not adjusting for multiple comparisons) are highlighted in red.

Taxon	N	Model	Classification	ANOVA					3-way		
				Diet	Exercise	Linetype	Diet*Exercise	Exercise*Linetype	Diet*Linetype	action	Mini
Actinobacteria	149 reduced		phylum	0.8360	0.3004	0.3197	0.4088	0.8155	0.9529	0.7841	0.8522
Bacteroidetes	149 reduced		phylum	0.4663	0.6103	0.3558	0.0585	0.2107	0.8462	0.2428	0.8854
Firmicutes	149 reduced		phylum	0.4052	0.4555	0.4739	0.1604	0.2914	0.9176	0.1440	0.9672
Proteobacteria	149 reduced		phylum	0.9310	0.0726	0.6975	0.0096	0.1802	0.8713	0.9489	0.9402
Tenericutes	135 kept DIET*LINE(LINETYPE)		phylum	0.4340	0.3670	0.2437	0.0126	0.4680	0.5586	0.8328	0.8520
Candidatus Melainabacteria	127 kept WHLACC*LINE(LINETY)		phylum	0.2623	0.3663	0.6868	0.3120	0.6160	0.7507	0.4311	0.8290
Mollicutes	132 full		class	0.3551	0.1318	0.7030	0.0350	0.5210	0.2572	0.7379	0.8583
Coriobacteria	149 reduced		class	0.9976	0.2449	0.2038	0.4585	0.6891	0.9959	0.6782	0.7607
Bacteroidia	149 reduced		class	0.0564	0.6764	0.2399	0.6080	0.3958	0.7162	0.3812	0.8636
Flavobacteriia	147 reduced		class	0.3993	0.1110	0.1607	0.2445	0.4951	0.4104	0.1723	0.3715
Bacilli	149 full		class	0.2314	0.4298	0.8555	0.7741	0.7698	0.8553	0.9322	0.4307
Clostridia	149 kept whlacc*line(linetype)		class	0.1194	0.4476	0.6615	0.2097	0.9554	0.4021	0.5150	0.8490
Erysipelotrichia	149 full		class	0.6853	0.9820	0.2273	0.5235	0.8904	0.7396	0.1582	0.5717
Deltaproteobacteria	149 reduced		class	0.6092	0.1772	0.8848	0.0219	0.5498	0.8843	0.4342	0.9367
Epsilonproteobacteria	148 reduced		class	0.7460	0.4524	0.3640	0.0067	0.0499	0.0284	0.4897	0.7552
Eggerthellales	149 reduced		order	0.9892	0.3061	0.2376	0.7083	0.9535	0.7721	0.7326	0.8387
Bacteroidales	149 reduced		order	0.0408	0.6974	0.2289	0.7505	0.4609	0.8790	0.4542	0.8376
Flavobacteriales	132 reduced		order	0.3907	0.1417	0.1368	0.2050	0.5186	0.4722	0.2296	0.4601
Lactobacillales	149 full		order	0.2293	0.4451	0.8519	0.8139	0.7896	0.8688	0.9463	0.3737
Clostridiales	149 full		order	0.0966	0.4501	0.7298	0.3393	0.8976	0.4949	0.4539	0.8275
Erysipelotrichales	149 full		order	0.6853	0.9820	0.2273	0.5235	0.8904	0.7396	0.1582	0.5717
Desulfovibrionales	149 reduced		order	0.6092	0.1772	0.8848	0.0219	0.5498	0.8843	0.4342	0.9367
Campylobacteriales	148 reduced		order	0.7460	0.4524	0.3640	0.0067	0.0499	0.0284	0.4897	0.7552
Eggerthellaceae	149 reduced		family	0.9892	0.3061	0.2376	0.7083	0.9535	0.7721	0.7326	0.8387
Bacteroidaceae	149 kept DIET*LINE(LINETYPE)		family	0.3250	0.0566	0.3814	0.2480	0.8602	0.0511	0.5977	0.4949
Muribaculaceae	149 kept WHLACC*LINE(LINETY)		family	0.1570	0.00024	0.6391	0.9986	0.8970	0.7370	0.5565	0.5123
Porphyromonadaceae	149 kept DIET*LINE(LINETYPE)		family	0.1457	0.9684	0.1626	0.0637	0.3570	0.8354	0.7085	0.2915
Prevotellaceae	148 kept DIET*LINE(LINETYPE)		family	0.7223	0.9836	0.5898	0.8044	0.2458	0.1490	0.6041	0.3624
Rikenellaceae	149 reduced		family	0.3591	0.2649	0.9491	0.5458	0.2663	0.8497	0.5249	0.4978
Tannerellaceae	149 kept WHLACC*LINE(LINETY)		family	0.5771	0.4489	0.7839	0.8406	0.1861	0.1165	0.0326	0.9592
Flavobacteriaceae	132 reduced		family	0.3907	0.1417	0.1368	0.2050	0.5186	0.4722	0.2296	0.4601
Lactobacillaceae	149 full		family	0.2288	0.4450	0.8515	0.8137	0.7906	0.8684	0.9457	0.3733
Clostridiaceae	143 reduced		family	0.0829	0.5881	0.0750	0.1883	0.4206	0.0884	0.8345	0.0157
Eubacteriaceae	138 full		family	0.7357	0.6149	0.3578	0.4696	0.9538	0.9395	0.2115	0.9677
Lachnospiraceae	149 full		family	0.0614	0.4646	0.4434	0.1929	0.6238	0.5032	0.2879	0.2585
Peptococcaceae	149 kept WHLACC*LINE(LINETY)		family	0.7550	0.6666	0.7603	0.7551	0.8199	0.0761	0.9172	0.9868
Erysipelotrichaceae	149 full		family	0.6853	0.9820	0.2273	0.5235	0.8904	0.7396	0.1582	0.5717
Desulfovibrionaceae	149 reduced		family	0.6092	0.1772	0.8848	0.0219	0.5498	0.8843	0.4342	0.9367
Helicobacteraceae	149 reduced		family	0.7460	0.4524	0.3640	0.0067	0.0499	0.0284	0.4897	0.7552
Adlercreutzia	149 reduced		genus	0.8959	0.3729	0.2462	0.6079	0.9669	0.6887	0.5569	0.9432
Bacteroides	149 kept DIET*LINE(LINETYPE)		genus	0.3250	0.0566	0.3814	0.2480	0.8602	0.0511	0.5977	0.4949
Muribaculum	149 removed 3-way		genus	0.1570	0.0047	0.6391	0.9986	0.8970	0.7479	0.5566	0.5123
Prevotella	148 kept Diet*Line(Linetype)		genus	0.7223	0.9836	0.5898	0.8044	0.2458	0.1490	0.6041	0.3624
Alistipes	149 reduced		genus	0.3591	0.2649	0.9491	0.5458	0.2663	0.8497	0.5249	0.4978
Parabacteroides	149 kept WHLACC*LINE(LINETY)		genus	0.6049	0.5481	0.7654	0.8892	0.1848	0.1506	0.0367	0.8385
Lactobacillus	149 full		genus	0.2288	0.4450	0.8515	0.8137	0.7906	0.8684	0.9457	0.3733
Eubacterium	138 full		genus	0.7357	0.6149	0.3578	0.4696	0.9538	0.9395	0.2115	0.9677
Anaerostipes	137 full		genus	0.5146	0.9474	0.6404	0.4269	0.5480	0.7750	0.5547	0.4490
Lachnoclostridium	149 reduced		genus	0.0384	0.3485	0.4264	0.0786	0.7446	0.5565	0.1710	0.1976
Flavonifractor	148 full		genus	0.2441	0.7499	0.3556	0.1516	0.3188	0.2826	0.3473	0.4316
Intestinimonas	134 reduced		genus	0.7629	0.3761	0.5139	0.0640	0.4040	0.5833	0.1096	0.1745
Erysipelatoclostridium	146 Kept WHLACC*LINE(LINETY)		genus	0.5310	0.3067	0.4209	0.4674	0.3496	0.3693	0.5582	0.6724
Faecalibaculum	147 reduced		genus	0.3132	0.9674	0.6340	0.6402	0.4461	0.3035	0.2706	0.9681
Turicibacter	145 full		genus	0.7131	0.8948	0.4867	0.7333	0.5042	0.9056	0.1967	0.8524
Desulfovibrio	149 reduced		genus	0.6020	0.1748	0.8789	0.0222	0.5478	0.8793	0.4371	0.9398
Helicobacter	148 reduced		genus	0.7460	0.4524	0.3640	0.0067	0.0499	0.0284	0.4897	0.7552