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RESEARCH ARTICLE | *Role of Gut Microbiota, Gut-Brain and Gut Liver Axes in Physiological Regulation of Inflammation, Energy Balance, and Metabolism*

Fecal microbiota transplantation from high caloric-fed donors alters glucose metabolism in recipient mice, independently of adiposity or exercise status

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Zoll J, Read MN, Heywood SE, Estevez E, Marshall JP, Kammoun HL, Allen TL, Holmes AJ, Febbraio MA, Henstridge DC. Fecal microbiota transplantation from high caloric-fed donors alters glucose metabolism in recipient mice, independently of adiposity or exercise status. *Am J Physiol Endocrinol Metab* 319: E203–E216, 2020. First published June 9, 2020; doi:10.1152/ajpendo.00037.2020.—Studies suggest the gut microbiota contributes to the development of obesity and metabolic syndrome. Exercise alters microbiota composition and diversity and is protective of these maladies. We tested whether the protective metabolic effects of exercise are mediated through fecal components through assessment of body composition and metabolism in recipients of fecal microbiota transplantation (FMT) from exercise-trained (ET) mice fed normal or high-energy diets. Donor C57BL/6J mice were fed a chow or high-fat, high-sucrose diet (HFHS) for 4 wk to induce obesity and glucose intolerance. Mice were divided into sedentary (Sed) or ET groups (6 wk treadmill-based ET) while maintaining their diets, resulting in four donor groups: chow sedentary (NC-Sed) or ET (NC-ET) and HFHS sedentary (HFHS-Sed) or ET (HFHS-ET). Chow-fed recipient mice were gavaged with feces from the respective donor groups weekly, creating four groups (NC-Sed-R, NC-ET-R, HFHS-Sed-R, HFHS-ET-R), and body composition and metabolism were assessed. The HFHS diet led to glucose intolerance and obesity in the donors, whereas exercise training (ET) restrained adiposity and improved glucose tolerance. No donor group FMT altered recipient body composition. Despite unaltered adiposity, glucose levels were disrupted when challenged in mice receiving feces from HFHS-fed donors, irrespective of donor-ET status, with a decrease in insulin-stimulated glucose clearance into white adipose tissue and large intestine and specific changes in the recipient's microbiota composition observed. FMT can transmit HFHS-induced disrupted glucose metabolism to recipient mice independently of any change in adiposity. However, the protective metabolic effect of ET on glucose metabolism is not mediated through fecal factors.

exercise; fecal microbiota transplantation; FMT; glucose; metabolism

INTRODUCTION

The gut microbiome is a large and sophisticated community of bacteria, fungi, and archaea that reside within the gastrointestinal tract. It has become clear that the composition of this community is associated with various pathological conditions. As this community plays a role in host metabolism, alterations to its membership, distribution, or activity may impact metabolic-related disorders such as obesity, insulin resistance, and type 2 diabetes (T2D) (13, 21, 29, 32, 33, 41). The microbiota state can be influenced by many factors, particularly host dietary composition and intake pattern. Other environmental and lifestyle factors such as medication, exposure to pollutants, sleep, and physical activity are also known microbiota modulators. Indeed, numerous studies in humans and animal models have reported microbiota changes with exercise and in athletes (2, 3, 5, 6, 10, 12, 20, 26, 27, 30, 34, 35, 39). Yet the precise beneficial or negative effects of exercise-induced microbiota alteration to the host remain largely unknown.

Exercise training (ET) is widely accepted as a therapeutic intervention and protective against the development of obesity, insulin resistance, and T2D (7). However, many individuals, especially the injured, frail, and/or elderly, cannot exercise on a regular basis. Of those who can, rates of compliance are low. Therefore, identifying therapeutics that target pathways regulated by exercise and/or identifying modes of exercise giving maximal metabolic improvement are of interest. With exercise conferring many metabolic benefits through numerous mechanisms, it is unclear (and indeed difficult to discern) whether the microbiota is a conduit for such exercise-induced benefits.

Fecal microbiota transplantation (FMT) strives to engineer improvements in gut function and/or systemic health through the introduction of crude stool preparations from healthy donors. It has proven successful in treating chronic *Clostridium difficile* infections (40). Experimentally, FMT can reveal whether given effects are mediated through fecal components and are thus transmittable. From a metabolic perspective, FMT has demonstrated some metabolism-related phenotypes to be fecal component-modulated in experimental models and hu-

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man pilot trials; however, the corresponding mechanistic foundations remain unknown (9, 23, 24, 37). FMT effects are rarely apportioned between fecal components of live cells, microbial cell components, or metabolic factors of either microbe or host origin. The host is sensitive to all these components (36), and we postulate that any of them could exert a regulatory effect on metabolism.

Here, we assessed whether the metabolic benefits exercise confers on the host are mediated through fecal factors. Specifically, we assessed whether transfer of feces from exercise-trained (ET) or sedentary donor mice, on either a normal or high caloric diet, alters microbiota composition, body composition, and glucose metabolism in sedentary, normal chow-fed recipients. Our use of recipients on a chow diet was to provide the greatest opportunity for microbiota transfer to elicit an effect without the confounding factor of a microbiota-modulating high-energy diet in the recipients. We hypothesized that recipients of FMT from high-fat, high-sucrose (HFHS)-fed, sedentary donor mice (HFHS-Sed) would have increased adiposity and disrupted glucose tolerance compared with mice receiving FMT from mice fed a chow diet. Furthermore, we hypothesized that compared with mice that were sedentary, ET in the donors would protect the metabolic profile of recipients when they were transplanted with fecal matter from HFHS-fed mice. Whereas the FMT was able to transmit HFHS-induced microbiota changes and disrupt glucose metabolism in recipient mice independently of any change in adiposity, FMT from ET donor mice elicited no protective effect on glucose metabolism.

MATERIALS AND METHODS

Animals

C57BL/6J mice were sourced from Alfred Medical Research and Education Precinct (AMREP) Animal Services. All animals were fed a normal chow diet (NC) (14.0 MJ/kg, 75.2% kJ from carbohydrate, 4.8% from fat, 20% from protein: Specialty Feeds, WA, Australia) until the studies were initiated at 7–8 wk of age. Thereafter, depending on their allocated group, mice were fed NC or a high-fat, high-sucrose diet (HFHS) [19 MJ/kg, 36% kJ from carbohydrate (17% sucrose), 43% from fat 21% from protein; Specialty Feeds, WA, Australia] until study end point. The NC diet had a 5.2% total crude fiber composition and the HFHS diet 5.4%. Food and water access were unrestricted (except for experimental fasting periods). Mice were maintained at 22 ± 1°C on a 12-h light-dark cycle. The study was approved by the AMREP Animal Ethics Committee and conducted in accordance with National Health and Medical Research Council of Australia guidelines. Experimental procedure flowcharts (11) are provided in Figs. 1 and 2.

Study Design

The study involved both recipient and donor groups of male mice run in parallel (see Fig. 3). Two cohorts were used to limit experimental burden per mouse, and only one sex was used to remove the confounding physiological gender differences in this initial study. C57BL/6J mice were bred in two rounds, first producing pups for the donor groups and thereafter for recipient groups. At weaning, mice were rehoused across cages to remove cage-mate gut microbiome correlations (12) as a confounding factor. After 4 wk of NC or HFHS diet [a time frame we have previously demonstrated induces full glucose intolerance and insulin resistance from HFHS diet (42)], donor mice commenced 6 wk of treadmill running ET (or Sed

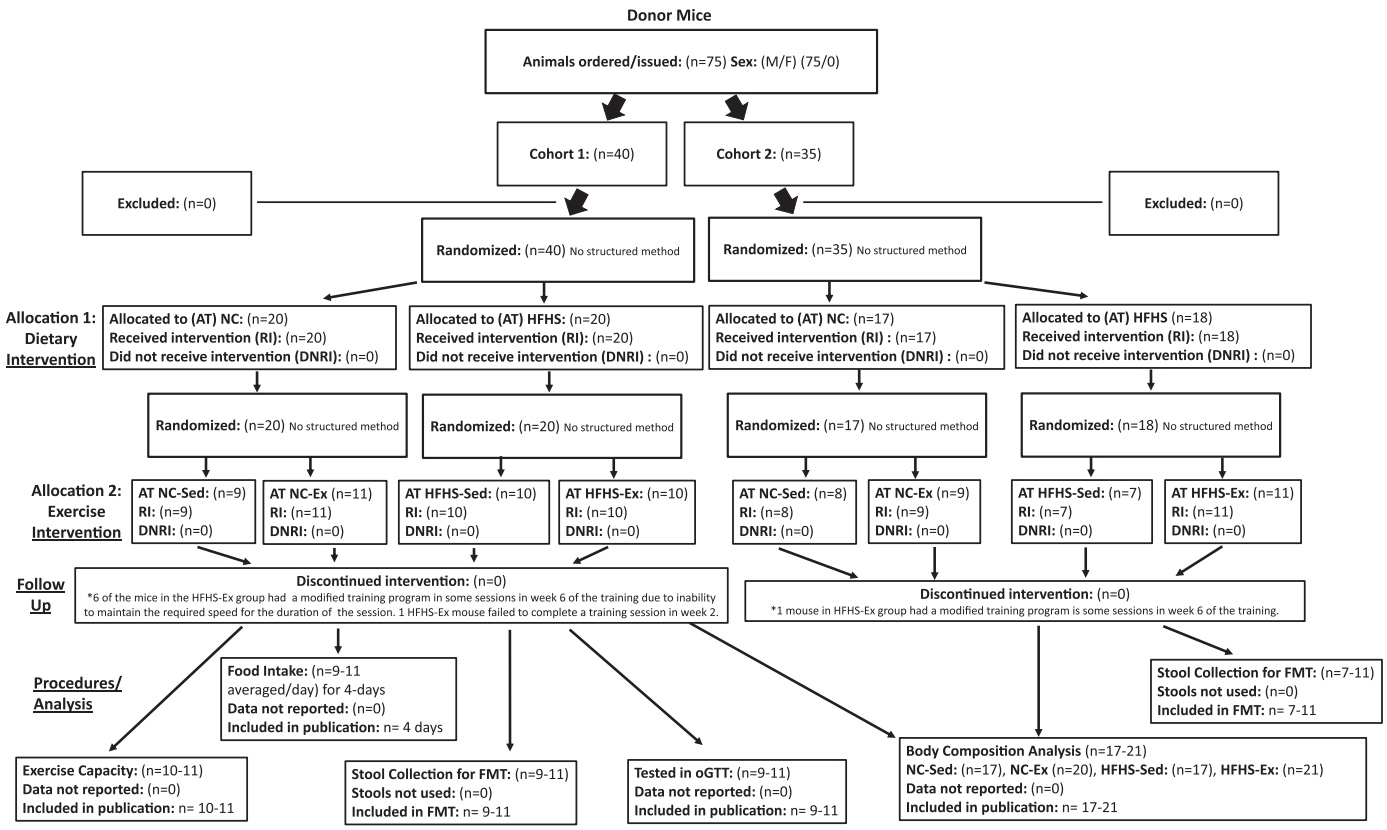

Fig. 1. Flow diagram of animal use and analysis for the donor groups based on the Consolidated Standards or Animal Experiment ReporTing (CONSAERT) template. FMT, fecal microbiota transplantation; HFHS, high-fat, high-sugar; HFHS-Ex, high-fat, high-sugar exercise-trained; HFHS-Sed, high-fat, high-sugar sedentary; NC, normal chow; NC-Ex, normal chow exercise-trained; NC-Sed, normal chow sedentary; oGTT, oral glucose tolerance test.

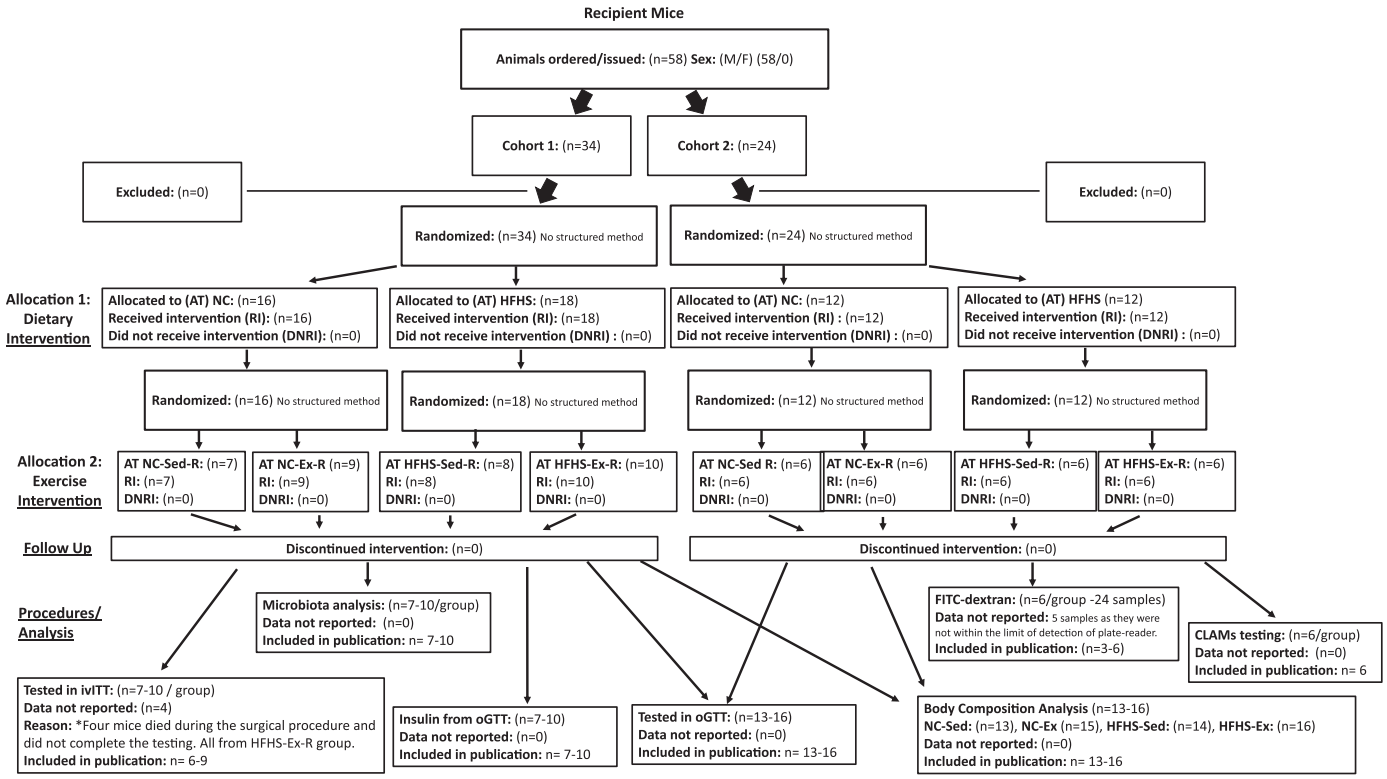

Fig. 2. Flow diagram of animal use and analysis for the recipient groups based on the Consolidated Standards or Animal Experiment ReporTing (CONSAERT) template. CLAMS, Comprehensive Laboratory Animal Monitoring System; HFHS, high-fat, high-sugar; HFHS-Ex-R, high-fat, high-sugar exercise-trained recipient; HFHS-Sed-R, high-fat, high-sugar sedentary recipient; ivITT, intravenous insulin tolerance test; NC, normal chow; NC-Ex-R, normal chow exercise-trained recipient; NC-Sed-R, normal chow sedentary recipient; oGTT, oral glucose tolerance test.

control); NC or HFHS allocations were retained throughout. Four donor groups resulted: NC sedentary (NC-Sed), NC exercise-trained (NC-ET), HFHS sedentary (HFHS-Sed), and HFHS exercise-trained (HFHS-ET). At both initiation of diet and exercise interventions, mouse body weights were analyzed to exclude biases resulting from randomized group allocations (Fig. 4, *A* and *E*). Feces were collected from each donor mouse once weekly during the ET period for FMT.

Recipient mice were NC-fed and sedentary. Groups were randomly assigned to receive FMT from each of the four donor groups: NC sedentary recipient (NC-Sed-R), NC exercise-trained recipient (NC-ET-R), HFHS sedentary recipient (HFHS-Sed-R), and HFHS exercise-trained recipient (HFHS-ET-R). Recipient baseline body weight analysis discounted any randomization-induced bias (Fig. 4*A*). Mice were housed only with mice receiving the same treatment and received one gavage of fecal slurry per week for 6 wk; we assessed metabolic parameters.

Fecal Microbiota Transplantation

One freshly voided stool per donor mouse was collected each week and combined with other stools from the same group. Stool pellets

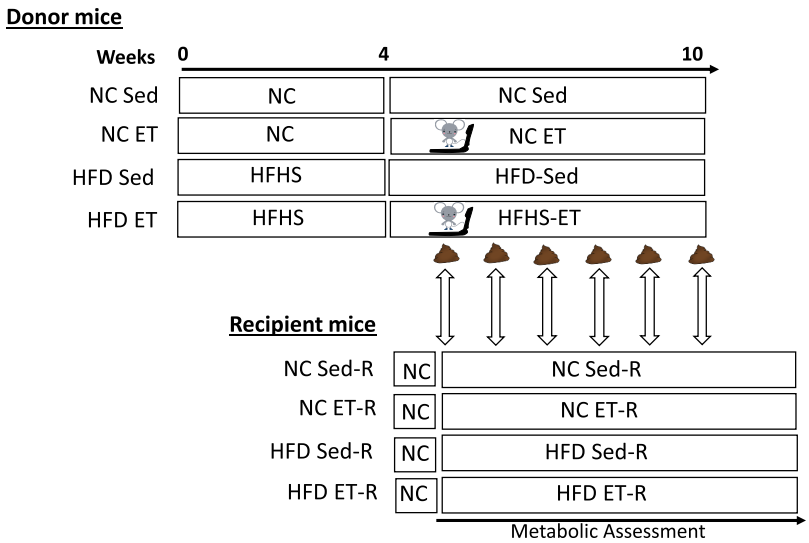

Fig. 3. Study design for donor and recipient groups. HFD ET-R, high-fat diet exercise-trained recipient; HFD-Sed, high-fat diet sedentary; HFD Sed-R, high-fat diet sedentary recipient; HFHS, high-fat, high-sugar; HFHS-ET, high-fat, high-sugar exercise-trained; NC, normal chow; NC ET, normal chow exercise-trained; NC Sed, normal chow sedentary; NC ET-R, normal chow exercise-trained recipient; NC Sed-R, normal chow sedentary recipient.

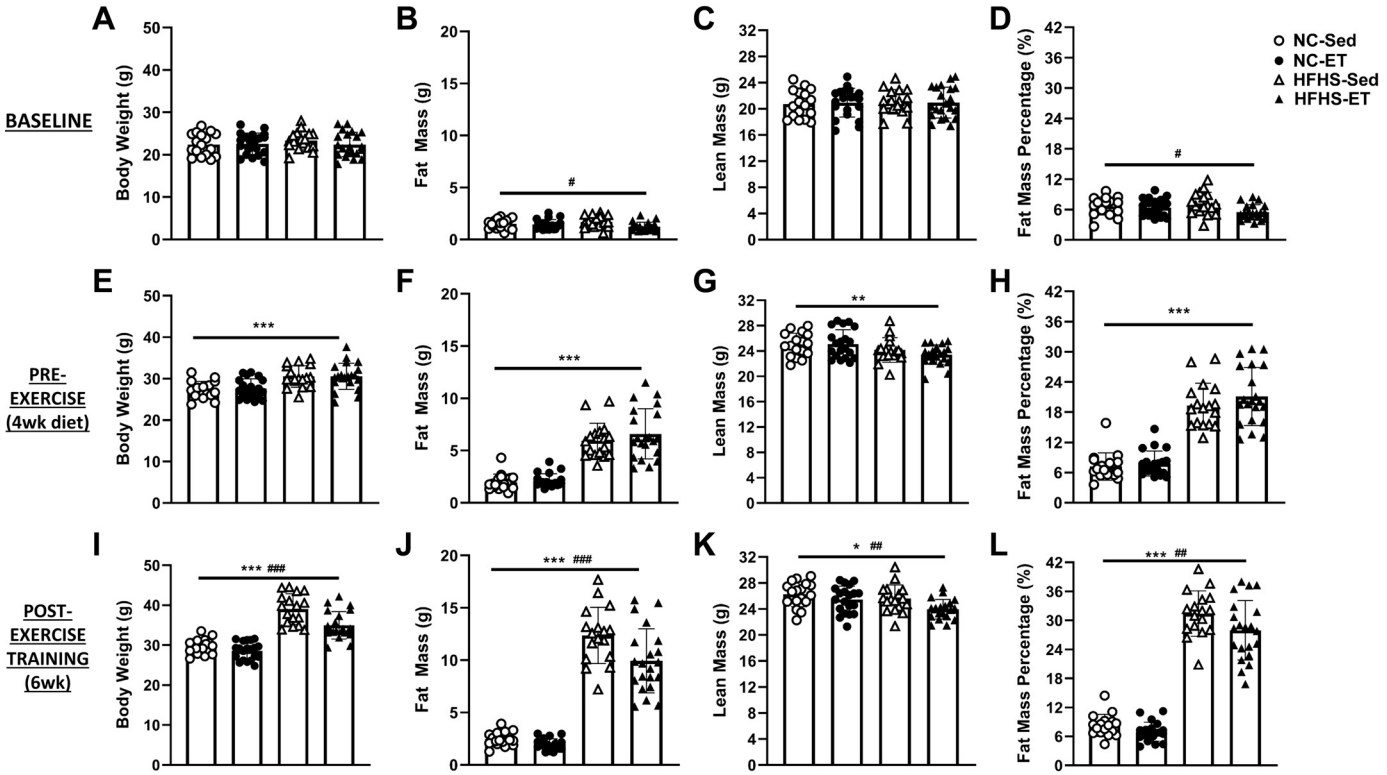

Fig. 4. Body composition analysis in fecal donor cohort. Body weight, fat mass, lean mass, and body fat percentage at baseline (7–8 wk of age) (*A–D*), following 4 wk of dietary intervention (pre-exercise) (*E–H*), and following exercise training (ET) intervention (*I–L*). Normal chow sedentary (NC-Sed), $n = 17$; normal chow exercise-trained (NC-ET), $n = 20$; high-fat, high-sugar sedentary (HFHS-Sed), $n = 17$; high-fat, high-sugar exercise-trained (HFHS-ET), $n = 21$. 2-way ANOVA. $*P \leq 0.05$; $**P \leq 0.01$, $***P \leq 0.001$ for dietary main effect; $\#P \leq 0.05$, $\#\#P \leq 0.01$, $\#\#\#P \leq 0.001$ for ET main effect; n = no. of mice.

were sliced with a scalpel and resuspended in 1 mL phosphate-buffered saline (PBS) per stool. Stools were homogenized, then twice vortexed and incubated for 15 min at 37°C, then vortexed again and spun at 800 rpm for 3 min. The crude aqueous fecal extract was oral gavaged to recipients (200 μL each) based on previous protocols (43). By performing weekly collections and gavages as the exercise training program progressed, we were attempting to simulate likely changes in the microbiota population over the course of a training program and transferring these to the recipients to assess any impact on metabolic readouts.

Exercise Capacity Test

An acute incremental exercise test of donors was performed at commencement and completion of the ET intervention. Mice performed a 3-day familiarization protocol in which intensity and durations of treadmill running (Model Exer-3/6 Treadmill, Columbus Instruments, OH) were progressively increased. The test began at 10 m/min for 3 min. The velocity was increased by 4 m/min every 3 min until fatigue. This was defined as spending >10 s at the base of the treadmill despite manual encouragement.

Exercise Training

Each session consisted of interval training, alternating 2 min of active running with 2 min of rest. Each session lasted for 60 min repeated 3 times per week. The initial speed was 16.0 m/min. Each week the speed was increased by 1 m/min as progressive overload. Sedentary mice were removed from their holding room and their cages placed next to the treadmill for the duration of the running to control for the activity of removing them from their environment. To control for any acute exercise effects, training was withheld for at least 48 h before glucose tolerance testing.

Body Composition Analysis

Fat mass and lean mass were measured with a 4-in-1 EchoMRI body composition analyzer (EchoMRI, Houston, TX) and standard laboratory scales were used for total body mass (Mettler Toledo, Greifensee, Switzerland) as previously described in full (28).

Oral Glucose Tolerance Test

Oral glucose tolerance tests (oGTT) were performed on fasted (6 h) mice. Mice received an oral gavage of 2 g glucose/kg lean body mass (25% glucose solution), and blood glucose levels were measured via a glucometer (AccuCheck, NSW, Australia) at the indicated times on blood that was collected from the tail.

Metabolic Caging Analysis (CLAMS)

A Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) was utilized to measure various aspects of metabolism as previously described (28). Mice were individually housed and oxygen consumption ($\dot{V}O_2$), respiratory exchange ratio (RER), energy expenditure (heat), and total movement (beam breaks) were recorded over a 48-h period. The first 24 h served as an acclimatization period, and the 24- to 48-h period was analyzed.

In Vivo Intestinal Permeability

As a marker of gut permeability, a 500 mg/kg bolus (125 mg/mL) of fluorescein isothiocyanate–conjugated dextran (FITC-labeled dextran, Sigma-Aldrich, St. Louis, MO) was administered by oral gavage to fasting mice. Blood was obtained from the tail into a heparinized capillary tube and plasma read on a fluorescent plate reader (Ex 490 mm, Em 520 mm).

Intravenous Insulin Tolerance Test

Intravenous insulin tolerance tests with combined glucose tracer were performed as previously described (17). Briefly, mice were anesthetized and the jugular vein cannulated. Following basal glucose measurements, a single bolus injection of insulin (0.6 U/kg lean body mass) also containing [^{3}H]2-deoxyglucose (2-DG; 10 μCi) (PerkinElmer, Waltham, MA) was injected down the line. Blood was sampled at 0, 2, 5, 10, 20, and 30 min for determination of blood glucose and 2, 10, 20, and 30 min for plasma radioactivity of [^{3}H]2-DG. At end point, the organs were excised and snap frozen and stored at −80°C.

Determination of Plasma and Tissue Radioactivity

The collected blood samples (10 μL) were deproteinized with barium hydroxide and zinc sulfate liquid scintillation fluid added and [^{3}H]2-DG radioactivity determined. Accumulation of [^{3}H]2-DG radioactivity in the tissue samples was determined by scintillation counting in an aqueous extract of the tissue after a homogenization process. Free and phosphorylated [^{3}H]2-DG were separated by ion exchange chromatography on Dowex 1-X8 columns. The area under the tracer disappearance curve for [^{3}H]2-DG and the radioactivity for the phosphorylated [^{3}H]2-DG from the organs were used to calculate the tissue-specific glucose clearance Kg′ as previously described (8, 18).

Fecal DNA Extraction and Sequencing

DNA was extracted from the feces using a FastDNA Spin Kit for feces (MP Biomedicals, Irvine, CA) and sequencing conducted using a 16S V1-3 (27F/519R) amplicon on an Illumina MiSeq v3 at the Ramaciotti Centre for Genomics, Sydney, Australia.

Microbial Ecology Profiling

Microbial ecology analyses were performed using R; all analysis code is publicly available at https://github.com/marknormanread/henstridge-2019. Raw sequence reads were clustered into amplicon sequence variants (ASVs) with inferred taxonomies using the DADA2 R package (4). Microbiota composition, α-diversity, and sequencing depth analyses were performed with the phyloseq R package (31). α-Diversity and sequencing depth statistical comparisons were performed using PERMANOVA [adonis function from the vegan R package (33a)]. Rarefaction analysis was performed using custom code. Principal component analyses (PCA) were performed using the mixomics R package (38); data were transformed using the isometric logratio transformation. Isometric and centered logratio transformations reduce biases inherent in the compositional nature of microbial sequencing data (15). Microbes *Bacteriodales S24–7* were renamed *Bacteriodales Muribaculaceae* in accordance with recent characterizations thereof (25).

Statistical Significance of Microbiota Clusterings by Experimental Group

This analysis was performed with custom code. We employ the Aitchison metric (Euclidean distance between centered logratio transformed data) to calculate the pairwise distance between all samples. Thereafter, the distributions of within-group and between-group sample distances are contrasted using a one-sided Kolmogorov–Smirnov statistic; if the latter exceeds the former, then the groups have statistically significantly different microbiota compositions. *P* values were corrected for multiple comparisons using the Bonferroni method.

Identifying Microbial Signatures through Supervised Machine Learning

Models were trained that predict experimental groups from microbiota profiles; the taxa employed by models form microbial signatures of experimental interventions. We employed the leave-one-out cross validation (LOO-CV) methodology to build predictive models with square discriminant analysis (sPLS-DA), using the mixomics R package (38). The available data is repeatedly partitioned into a building portion used in model construction and a validation portion used to assess model performance. Under LOO-CV, each sample is retained as the sole validation portion member exactly once, with all remaining samples forming the building portion; hence, all available data are ultimately used (once) in assessing model performance. Model predictions of validation sample class membership (experimental group) are through mahalanobis distance. Classification accuracies are reported for each class individually. Because the number of samples differs across experimental groups, models were trained to minimize the balanced error rate (the mean average error rate across classes; each class is equally important regardless of size) rather than the overall error rate (% of total errors, biased toward larger classes). The error rate is 1-accuracy. Microbial ecology sequencing data are typically noisy and encompass more numerous taxa than samples, which together risk spurious associations of taxa with experimental groups. Our methodology mitigates this risk by constructing models on the building portions of repeated bifurcations of our data and uses these models to predict the experimental group of the withheld samples. The noise in building and validation portions differs; hence, well-performing microbial signatures likely represent genuine signal and not noise.

The statistical significance of sPLS-DA model classification accuracy is estimated through permutation testing (reported in Supplemental Fig. S4*G*). The available samples are randomly reassigned into classes of equal number and size as the real data. The sPLS-DA pipeline (including selecting sPLS-DA parameters for the maximum number of components investigated and the number of features to include in each component) is applied to the randomized data, and the most accurate result is recorded. This process is repeated 50 times. The estimated *P* value corresponds to the count of randomized accuracies surpassing that of the real data; 50 replicates yields a *P* value granularity of 0.02. Through this approach, we gauge the potential for model overfitting: the likelihood that model accuracies reflect random chance or have identified sPLS-DA model parameters that serendipitously proffer superior performance despite the use of LOO-CV. This is unavoidable when limited data are available and is evidenced by randomized data prediction accuracies greater than the e.g., 50% that would be expected when employing two classes.

The sPLS-DA ordinations show the two components capturing the most variance in the data; sPLS-DA models can include additional components (as indicated in figures). These ordinations represent the building of an sPLS-DA model on all available data using optimal parameters as determined through LOO-CV. For taxa comprising signatures that distinguish experimental groups, we report only those taxa that were included in >90% of models built under LOO-CV; tables employed in each sPLS-DA component are available at https://github.com/marknormanread/henstridge-2019. The experimental groups these taxa associate with are derived from the final model constructed over all available data (and reported in the ordinations).

Biochemical Analysis

Insulin. Insulin concentrations were measured with a mouse ultrasensitive Insulin ELISA (ALPCO Immunoassays, Salem, NH). Plasma samples were read in a 96-well plate on a FLUOstar Omega microplate reader (BMG Labtech, VIC, Australia). Absorbance was measured at a wavelength of 450 nm.

Lipopolysaccharide-binding protein. Lipopolysaccharide-binding protein (LBP) was measured via ELISA with the absorbance measured at 450 nm with a spectrophotometer (Hycultec, Beutelsbach, Germany).

Complete blood count. For hematological assessment, 20 μL of whole blood was diluted 1:7 in Sysmex CELLPACK (Sysmex, Japan)

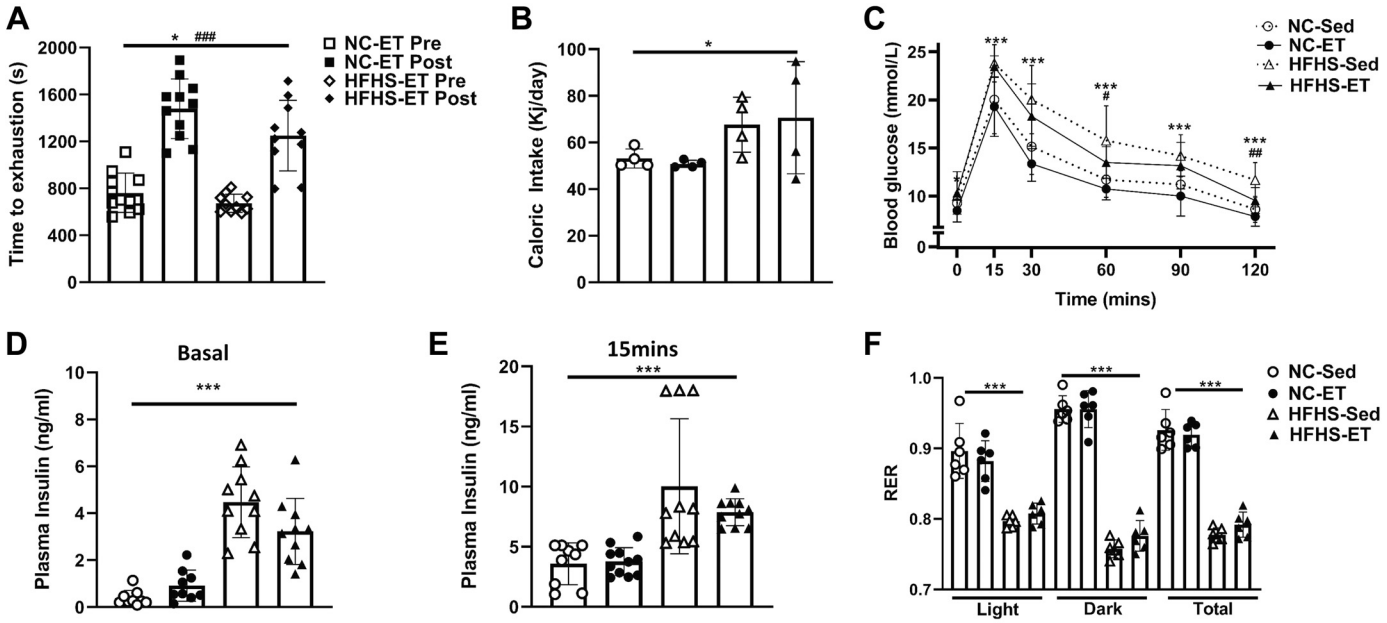

Fig. 5. *A*: exercise capacity from an incremental exercise test pre- and posttraining. Normal chow exercise-trained (NC-ET) pre and post, $n = 11$; high-fat, high-sugar exercise-trained (HFHS-ET) pre and post, $n = 10$. *B*: caloric intake per day during training period (measured and averaged per 24 h over 4 days). *C*: glucose excursions from an oral glucose tolerance test (oGTT). Normal chow sedentary (NC-Sed), $n = 9$; NC-ET, $n = 11$; high-fat, high-sugar sedentary (HFHS-Sed), $n = 10$; HFHS-ET, $n = 10$. *D* and *E*: plasma insulin levels at baseline and 15 min into the oGTT. NC-Sed, $n = 9$; NC-ET, $n = 11$; HFHS-Sed, $n = 10$; HFHS-ET, $n = 10$. *F*: respiratory exchange ratio (RER) of donor cohorts as measured in metabolic caging experiments; $n = 6$ per group. 2-way ANOVA. $^*P \leq 0.05$, $^{***}P \leq 0.001$ for dietary main effect; $\#P \leq 0.05$, $\#\#P \leq 0.01$, $\#\#\#P \leq 0.001$ for ET main effect; n = no. of mice.

diluent and assessed using an automated hematology analyzer (Sysmex XS-1000*i*, Kobe, Japan).

Triacylglycerol assay. Tissue triacylglycerol (TAG) content was quantified using a colorimetric assay kit and calculated as micrograms per milligrams of tissue (Triglycerides GPO-PAP; Roche Diagnostics, NSW, Australia).

Statistical Analysis

Metabolic data were analyzed by three or two-way analysis of variance (ANOVA) and Tukey post hoc tests when there was a significant interaction. All data are presented as means ± SE. Statistical significance was tested at $P < 0.05$. Microbiota data were analyzed as described above.

RESULTS

Metabolic Characteristics of Donor Mice

Donor mice were indistinguishable for body weight and lean mass at baseline (Fig. 4, *A* and *C*); however, there was slightly less adiposity in the groups later designated for ET (Fig. 4, *B* and *D*). After 4 wk on their respective diets, the HFHS groups had greater body weight, fat mass, and fat mass percentage and less lean mass compared with NC (Fig. 4, *E–H*). Relative to Sed, ET resulted in lower body weight, fat mass, lean mass, and fat mass percentage (Fig. 4, *I–L*). ET led to improvements in exercise capacity on both diets, with mice on NC outperforming mice on the HFHS diet (Fig. 5*A*). HFHS-fed mice exhibited higher daily caloric intake, but ET (vs. Sed) proved inconsequential (Fig. 5*B*). The HFHS diet caused glucose intolerance and ET countered it (Fig. 5*C*), likely due to improved insulin sensitivity given there was no ET effect in basal or glucose-induced insulin secretion (Fig. 5, *D* and *E*). RER values were lower in mice fed a HFHS diet, but ET had no effect (Fig. 5*F*). Summarily, the donor mice from which fecal material were collected for FMT displayed increased adiposity, glucose intolerance, caloric intake, and hyperinsulinemia in the HFHS groups, with ET reducing adiposity and improving exercise capacity and decreasing glucose levels during an oGTT.

FMT Recipient Mice Do Not Display Changes in Body Composition

Prior to commencing FMT, baseline body composition was assessed in the recipients with no difference observed for body weight, fat mass, lean mass, or fat mass percentage (Fig. 6, *A–D*). The FMT treatment period had no effect on the recipients' body composition with body weight, fat mass, lean mass, and fat mass percentage no different between groups in the week of the last gavage (Fig. 6, *E–H*) or at the end of the study ~3 wk later (Fig. 6, *I–L*). Consequently, any alteration in microbiota delivered to the recipients did not impact body composition, and any subsequent effect was independent of adiposity. The potential exists for microbiota to influence feeding behavior (22); however, consistent with the body composition data, no change was detected in recipient intakes (Fig. 7*A*).

FMT Recipient Mice Display Higher Glucose Levels When Challenged with a Glucose Load When Treated with HFHS Donor Microbiotas

Interestingly, despite the lack of an adiposity or food intake phenotype, mice administered FMT from HFHS donors, irrespective of donor ET status, had a spike in their blood glucose levels at early timepoints following an oGTT (Fig. 7*B*), sug-

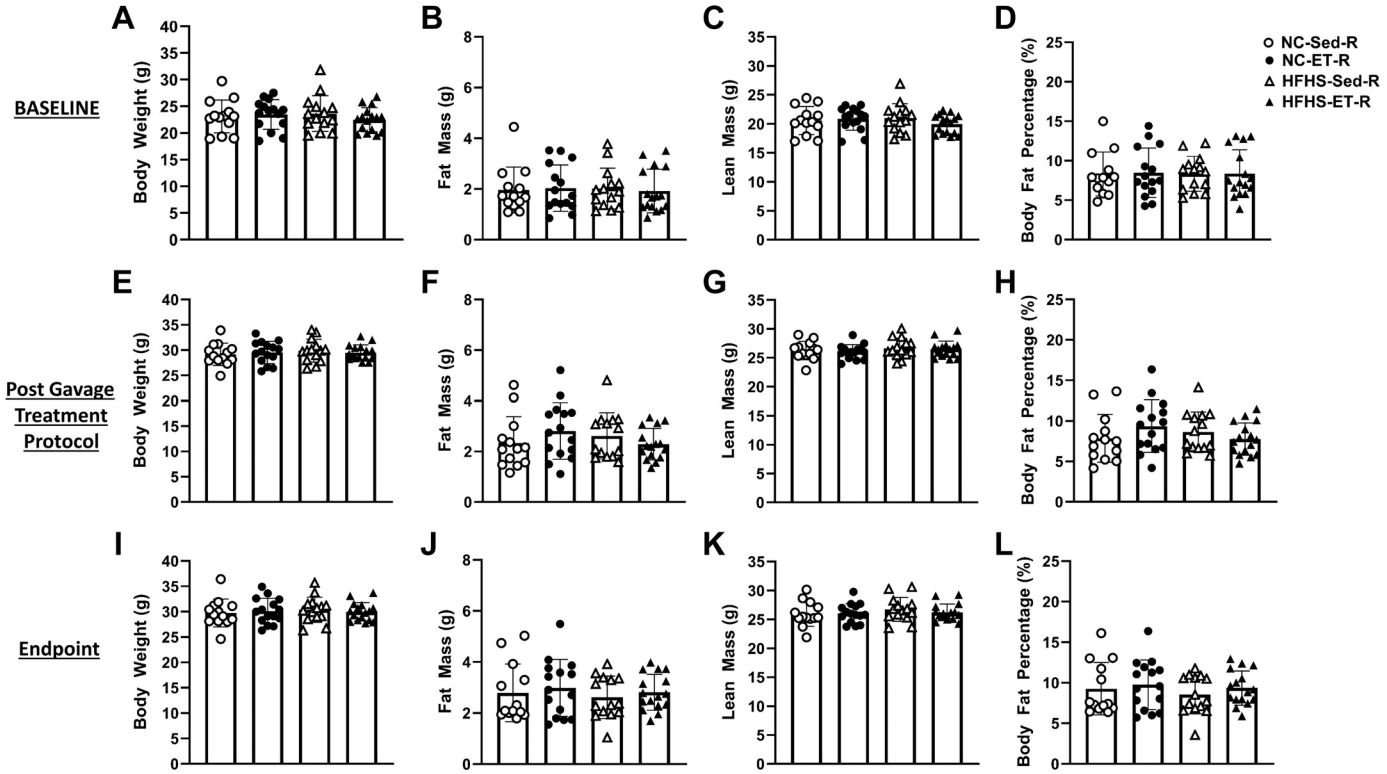

Fig. 6. Body composition of fecal microbiota transplantation (FMT)-recipient mice. Body weight, fat mass, lean mass, and body fat percentage at baseline (7–8 wk of age) (*A–D*), following 6 wk of weekly FMT intervention (*E–H*), and at end point of study ~3 wk after last gavage (*I–L*). Normal chow sedentary recipient (NC-Sed-R), $n = 13$; normal chow exercise-trained recipient (NC-ET-R), $n = 15$; high-fat, high-sugar sedentary recipient (HFHS-Sed-R), $n = 14$; high-fat, high-sugar exercise-trained recipient (HFHS-ET-R), $n = 16$; n = no. of mice.

gesting a defect in the ability to clear glucose or inhibit endogenous glucose production. Calculation of incremental area under the curve for the glucose curve demonstrated a significant increase in the mice receiving HFHS-FMT out to 90 min (Fig. 7*C*) that was reduced to a trend by 120 min ($P = 0.081$, Fig. 7*D*). As higher glucose levels could result from a pancreatic phenotype (if less insulin is secreted), we assessed basal and glucose-stimulated insulin secretion (15-min) when the greatest difference in glucose was observed in the oGTT. There was no difference between the groups at either timepoint (Fig. 7, *E* and *F*). As insulin levels were unchanged, we next assessed whether there was a difference in insulin-stimulated glucose clearance into tissues. Although we found no change in insulin-stimulated glucose clearance into skeletal muscle or brown adipose tissue (Fig. 7, *G* and *H*), we did detect a decrease in glucose clearance into epididymal white adipose tissue (WAT) in recipients of FMT from HFHS donors (Fig. 7*I*). We also assessed sections of the gastrointestinal tract, relevant given FMT, finding a decrease in the glucose clearance into the large but not small intestine under HFHS donation (Fig. 7, *J* and *K*).

Glucose Tolerance Defect in HFHS-R Mice is Not Due to Lipid Accumulation, Physical Activity, or Energy Expenditure

Given the alteration to glucose levels during the oGTT in the HFHS-recipient mice, we assessed possible mechanisms of action. Despite no difference in adiposity, it is possible that there had been alterations to the partitioning of lipids within organs, which can disrupt glucose metabolism. We assessed triacylglycerol levels in the large intestine and WAT (locations of insulin-stimulated glucose clearance defects) but found no difference between recipient groups (Fig. 7, *L* and *M*). As hepatic steatosis is linked to disrupted glucose handling (42), we also measured triacylglycerol levels in the liver, but again observed no difference (Fig. 7*N*).

It is possible that alterations to physical activity, substrate utilization, or energy expenditure could drive a change in glucose disposal before such changes manifest in the body composition data. Indirect calorimetry studies could not detect a difference in oxygen consumption, energy expenditure, or physical activity levels between the groups (Fig. 8, *A*, *B*, and *D*). There was a decrease in the RER in the NC-ET-R group (Fig. 8*C*), suggesting a preference toward fatty acid utilization; however, we reiterate, a decrease in triacylglyceride content was not evident in the three tissues we assessed (Fig. 7, *J–L*). This finding of a decrease in RER in the NC-ET-R group was independent of donor RER given there was not a difference in donor RER with exercise on the NC diet (Fig. 5*F*).

Glucose Tolerance Defect in HFHS-R Mice is Not Due to Leaky Gut or Changes to Blood Profile or Inflammation

A well-characterized mechanism of diet-induced change in host-microbiota interaction is the inflammophile positive feedback loop, wherein microbes capable of anaerobic growth on inflammatory factors promote inflammation to gain a competitive ecological advantage (16). To assess this, we measured gut permeability and lipopolysaccharide-binding

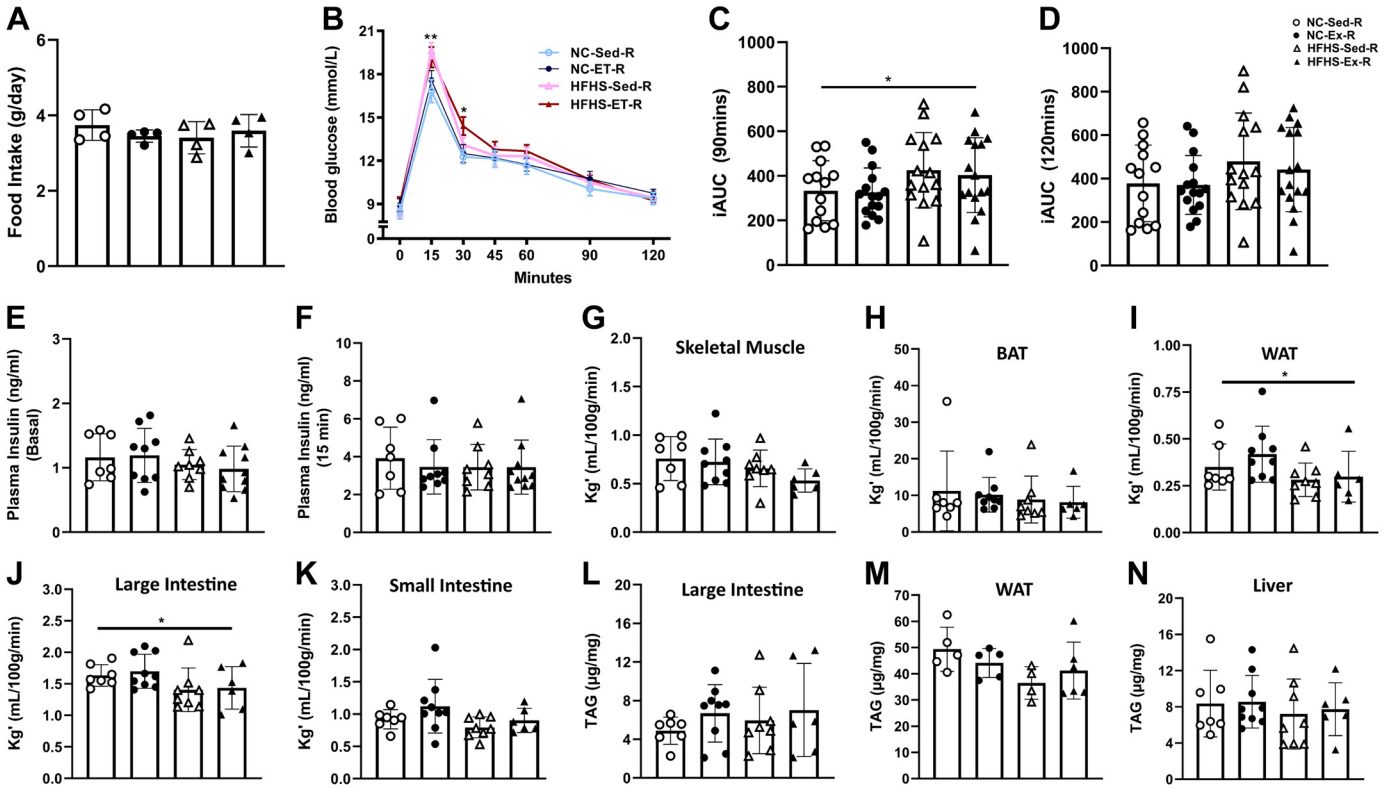

Fig. 7. Metabolic characteristics of fecal microbiota transplantation (FMT)-recipient mice. *A*: food intake per day during gavage period (measured and averaged per 24 h over 4 days). *B*: plasma glucose excursion curves from an oral glucose tolerance test (oGTT). Normal chow sedentary recipient (NC-Sed-R), $n = 13$; normal chow exercise-trained recipient (NC-ET-R), $n = 15$; high-fat, high-sugar sedentary recipient (HFHS-Sed-R), $n = 14$; high-fat, high-sugar exercise-trained recipient (HFHS-ET-R), $n = 16$. *C* and *D*: incremental area under the glucose curve (iAUC) analysis. NC-Sed-R, $n = 13$; NC-ET-R, $n = 15$; HFHS-Sed-R, $n = 14$; HFHS-ET-R, $n = 16$. *E–F*: plasma insulin concentration before and during the oGTT. NC-Sed-R, $n = 7$; NC-ET-R, $n = 9$; HFHS-Sed-R, $n = 8$; HFHS-ET-R, $n = 10$. *G–K*: insulin-stimulated glucose clearance into skeletal muscle (*G*), brown adipose tissue (BAT; *H*), white adipose tissue (WAT; *I*), large intestine (*J*), and small intestine (*K*). NC-Sed-R, $n = 7$; NC-ET-R, $n = 9$; HFHS-Sed-R, $n = 8$; HFHS-ET-R, $n = 6$. *L–N*: triacylglycerol (TAG) levels in large intestine (NC-Sed-R, $n = 7$; NC-ET-R, $n = 9$; HFHS-Sed-R, $n = 8$; HFHS-ET-R, $n = 6$) (*L*), WAT (NC-Sed-R, $n = 5$; NC-ET-R, $n = 5$; HFHS-Sed-R, $n = 4$; HFHS-ET-R, $n = 6$) (*M*), and liver (NC-Sed-R, $n = 7$; NC-ET-R, $n = 9$; HFHS-Sed-R, $n = 8$; HFHS-ET-R, $n = 6$) (*N*). 3- or 2-way ANOVA. $^*P \leq 0.05$, $^{**}P \leq 0.01$ for diet effect; n = no. of mice.

protein (LBP), an acute phase protein produced by the liver that recognizes lipopolysaccharide. We detected no difference between recipient groups under either measure (Fig. 8, *E* and *F*). Finally, we conducted a complete blood count to determine any effect of the FMT on circulating cells. White blood cells, platelets, and red blood cells were all unchanged in the recipients (Fig. 8*G*).

The Microbiotas of HFHS-R Mice are Distinguishable from NC-R Mice with Limited Effect of ET Status

We next profiled the gut microbiotas of recipient mice via fecal 16S ribosomal RNA sequencing before and after their FMT, confirming that each donor intervention induced distinct and transmissible microbial communities and seeking a potential explanation for the elevated glucose during the oGTT. Recipient mice microbiotas were dominated with members of the *Bacteriodetes* and *Firmicutes* phyla, (Fig. 9, *A* and *B*). We discounted insufficient or differential sequencing depth as confounding factors for subsequent analysis (Fig. 9, *C* and *D*). As expected, pre-FMT microbiotas exhibited no clustering by experimental group (Fig. 9*E*). However, post-FMT microbiotas did cluster by donor diet but not donor exercise state (Figs. 9*F*, Fig. 10, *A* and *B*). Microbiota ecological diversities also differed with donor diet (Fig. 10*C*); diversity was higher in recipients of NC donors and highest in the NC-ET-R group. In summary, donor diet explained much of the variation between samples and led to less diverse microbiota communities in the HFHS recipients; this was not so for donor exercise state.

Next, we sought signatures, comprising subsets of taxa, in recipient microbiotas that were distinguishing of donor diets and exercise states through supervised machine learning (Fig. 10*D*). We derived a model representing signatures correctly indicative of sample donor group (NC-Sed, NC-ET, HF-Sed, HF-ET) on average 80% of the time (Fig. 10*E*). For context, as a baseline, guessing the majority group for all samples would yield an accuracy of 29% (majority group is HF-Ex, representing 10 of the total 34 samples). We sought to explicitly test and discount model overfitting as a driver of accuracy; the attained 80% exceeded all 50 applications of an identical methodology to random experimental group reassignments among these samples, indicating $P < 0.02$ (Fig. 11). The model was relatively complex, encompassing 35 unique amplicon sequence variants (ASVs) to distinguish the 4 groups (Supplemental Table S1; all Supplemental tables are available at https://github.com/marknormanread/henstridge-2019/tree/master/supplementary_data). Interestingly, distinct strains of identical phylotypic classification associated with multiple experimen-

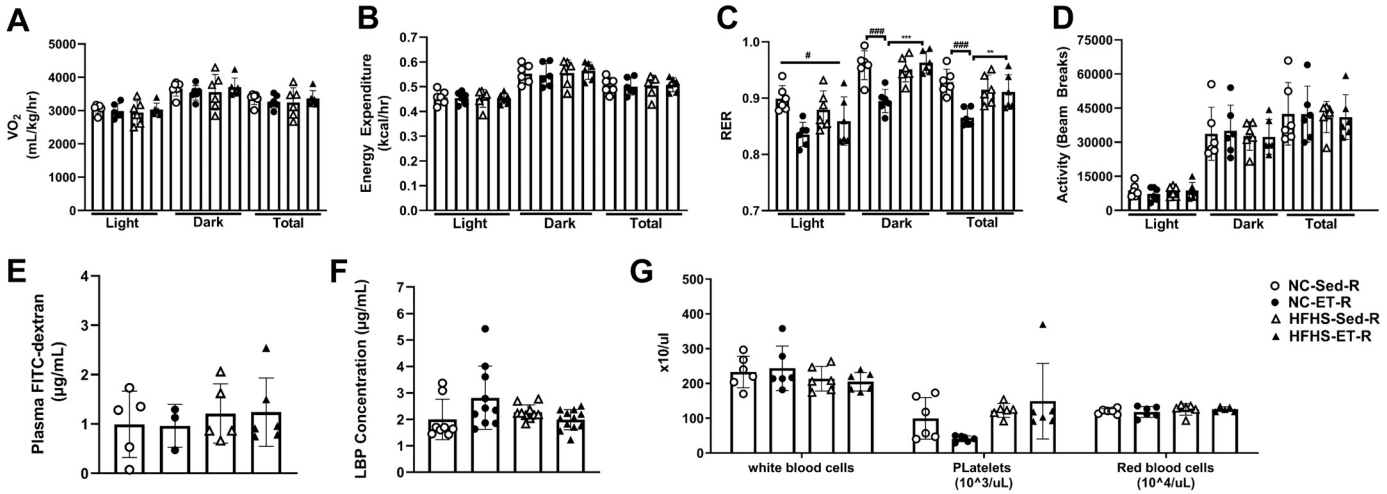

Fig. 8. Metabolic measures in fecal microbiota transplantation (FMT)-recipient mice. Oxygen consumption ($\dot{V}O_2$, *A*), energy expenditure (*B*), respiratory exchange ratio (RER; *C*), and physical activity levels (*D*) from metabolic caging experiments, $n = 6$/group. *E*: plasma FITC-dextran measurement as a measure of gut permeability. Normal chow sedentary recipient (NC-Sed-R), $n = 5$; normal chow exercise-trained recipient (NC-ET-R), $n = 3$; high- fat, high-sugar sedentary recipient (HFHS-Sed-R), $n = 5$; high-fat, high-sugar exercise-trained recipient (HFHS-ET-R), $n = 6$. *F*: plasma lipopolysaccharide-binding protein (LBP) concentration as a marker of systemic inflammation. NC-Sed-R, $n = 8$; NC-ET-R, $n = 10$; HFHS-Sed-R, $n = 10$; HFHS-ET-R, $n = 12$. Plasma analysis in FMT-recipient mice. *G*: total white blood cells, platelets, and red blood cell counts; $n = 6$/group. 2-way ANOVA with Tukey post hoc for interaction (when between 2 bars). #$P \leq 0.05$, ###$P \leq 0.001$ for ET effect; **$P \leq 0.01$, ***$P \leq 0.001$ for diet effect; n = no. of mice.

tal groups, suggesting strain-level functional differences. Examples include strains classifying as *Erysipelotrichaceae Allobaculum*, *Bacteroidales Muribaculaceae*, and *Lachnospiraceae*.

Microbial signatures reliably indicative of donor diet, irrespective of donor exercise state, were also derived (Figs. 10*F* and 11, Supplemental Table S2). ASVs classifying as *Erysipelotrichaceae Allobaculum* and *Bacteroidales Muribaculaceae* were overrepresented in FMT recipients of NC-fed donors. Interestingly, other ASVs also classifying as *Bacteroidales Muribaculaceae* were overrepresented in the HFHS-R, further suggesting strain-level functional differences. ASVs classifying as *Cyanobacteria 4C0d-2 YS2* were likewise overrepresented in HFHS-R, as was a *Lachnospiraceae* of undetermined genus.

Although we found experimental groups receiving ET and Sed FMT on the same diet to be statistically indistinguishable at the whole microbiota level (Fig. 10*C*), machine learning does identify microbial signatures indicative of donor exercise state (Fig. 10, *D* and *G*; Fig. 11, Supplemental Table S3), although not as robustly as for donor diet (Fig. 10*F*). Associated with the FMT of sedentary donors were ASVs classifying as *Lactobacillaceae lactobacillus*, *Alcaligenaceae sutterella*, *Bacteroidales Muribaculaceae* (genus undetermined), *Prevotellaceae prevotella*, and *Lachnospiraceae* (genus undetermined). Associating exercised donors were ASVs classified as *Alphaproteobacteria RF32* (genus undetermined), other *Bacteroidales Muribaculaceae* (genus undetermined), and *Porphyromonadaceae parabacteroides*.

DISCUSSION

Altered gut microbiota composition has emerged as a component of the ET state. Indeed, recent studies even suggest that FMT could be used to increase endurance exercise performance (39). The precise roles these changes have on host metabolism are largely unknown and are difficult to characterize given the multiple mechanisms through which exercise alters metabolic processes. Whether these changes are metabolically protective in an environment of over-nutrition is of interest, given the worldwide need to offset the growing incidence of obesity, insulin resistance, and T2D. Through FMT, we investigated whether exercise conferred microbiota-mediated effects of metabolic value to the host. Although we confirm in non-germ-free, non-antibiotic-treated mice that FMT from HFHS-fed donors is, at least in part, capable of negatively impacting recipient glucose levels during an oGTT, we find no evidence that FMT from ET donors alters recipients' metabolic profile.

Transfer of gut microbiota to recipient mice caused a glucose spike during an oGTT if the donor was fed a HFHS diet, independently of donor exercise status. To put the magnitude of the effect into perspective, the average oGTT blood glucose difference at the 15-min mark between the NC-Sed and HFHS-Sed donor groups was 3.78 mmol/L (Fig. 5*C*). The difference at the same 15-min mark between FMT recipients of NC-Sed and HFHS-Sed was 2.81 mmol/L (Fig. 7*B*). Hence, contrasted with mice fed a HFHS diet for ~2 mo, simple FMT from these animals into NC-fed recipients transfers over 70% of the early glucose level defect. This finding is of interest given: *1*) the recipient mice experienced no increase in adiposity, so it was not an adipose-driven phenomenon, *2*) it was independent of a leaky gut-inflammatory environment, and *3*) the HFHS-ET-R group phenocopied the HFHS-Sed-R group, meaning that donors being exercised, having reduced adiposity and improved glucose levels during an oGTT, conferred no protective effect. This suggests that the "Fat but Fit paradox" may not extend to the gut microbiota and associated host glucose metabolism regulation.

An important factor in identifying host-microbe metabolic interactions is exposure timing. Foley and colleagues (14) demonstrated that mice exposed to feces from soiled cages from high-fat-fed mice in the short term (4 days) had similar glucose tolerance as compared with mice exposed to feces

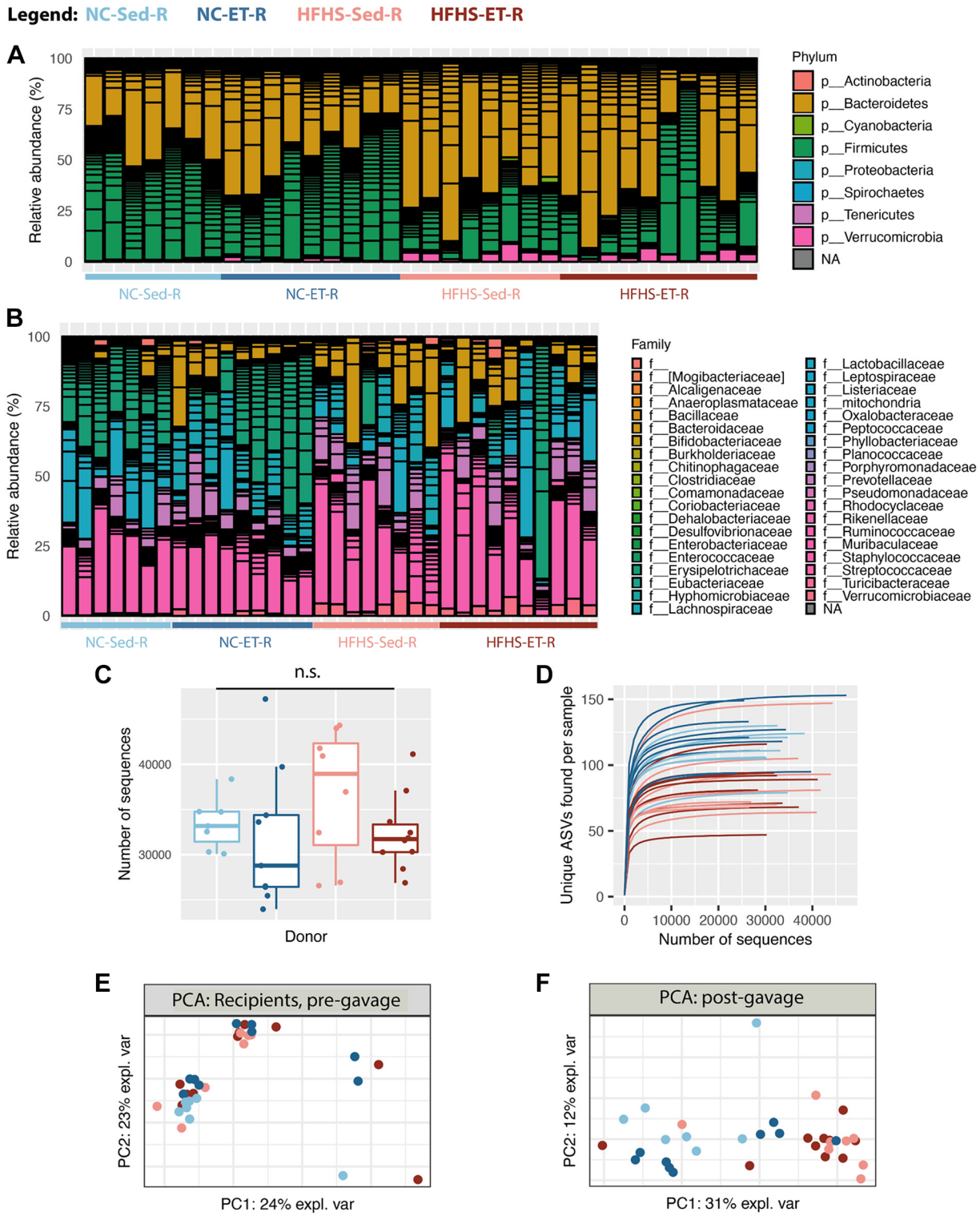

Fig. 9. Fecal gut microbiota composition in recipient mice post-fecal microbiota transplantation (FMT). Microbiota composition of recipients at the phylum (*A*) and family (*B*) levels, based on bacterial 16S rRNA sequencing. *C*: number of sequence reads per sample, collected by donor group. *D*: rarefaction curves quantifying distinct microbes (amplicon sequence variants, ASVs) identified when subsampling the available data; sequencing effort adequately captured the diversity of microbes present. Principal component analysis (PCA) ordinations showing recipient microbiota similarities of recipients at baseline (pre-FMT) (*E*) and following gavage treatment study conclusion (*F*). Normal chow sedentary recipient (NC-Sed-R), $n = 7$; normal chow exercise-trained recipient (NC-ET-R), $n = 9$; high-fat, high-sugar sedentary recipient (HFHS-Sed-R), $n = 8$; high-fat, high-sugar exercise-trained recipient (HFHS-ET-R), $n = 10$; n = no. of mice. n.s., not significant.

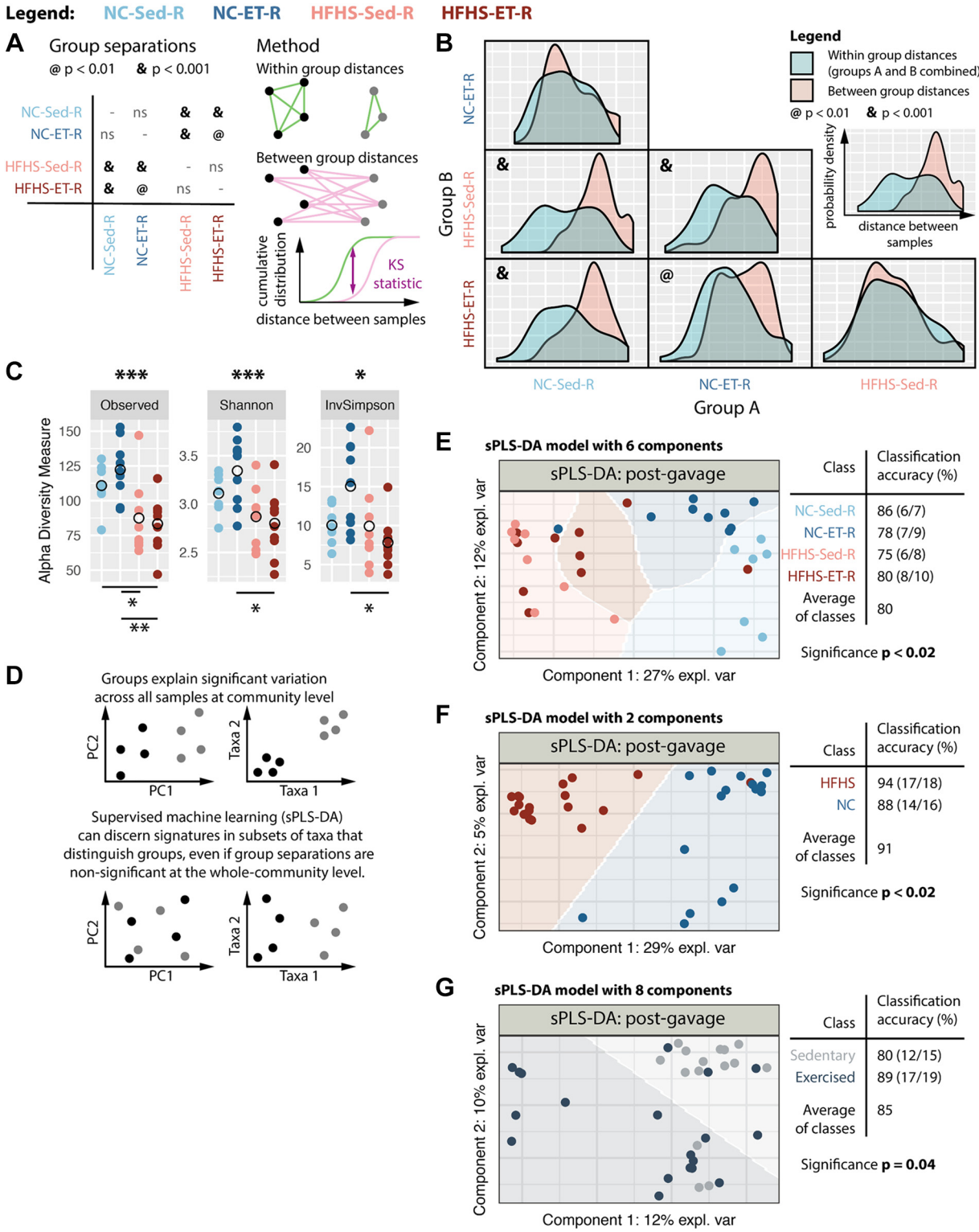

Fig. 10. Donor diets drive community-level differences in recipient microbiota, but both donor diet and exercise statuses induce distinguishing microbial signatures in subsets of taxa. Assessing the clustering of microbiotas by donor group by contrasting between- vs. within-group Aitchison distances (*A*); distance distributions (*B*). *C*: microbiota α-diversity quantifications: the number of distinct amplicon sequence variants observed and Shannon and Inverse Simpson metrics. Black circles indicate mean values. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ for dietary effect; no exercise training (ET) effects found; assessed through PERMANOVA. *D*: we assessed whether donor experimental statuses induce distinguishing alterations across subsets of recipient microbiota taxa (signatures) through supervised machine learning (sparse partial least squares discriminant analysis, sPLS-DA). Such signatures can emerge even in absence of significant whole community level effects, as for ET status. *E–G*: sPLS-DA models are constructed to distinguish recipients' microbiotas based on donor diets and ET states (*E*), diets irrespective of exercise state (*F*), and exercise states irrespective of diet (*G*). Normal chow sedentary recipient (NC-Sed-R), $n = 7$; normal chow exercise-trained recipient (NC-ET-R), $n = 9$; high-fat, high-sugar sedentary recipient (HFHS-Sed-R), $n = 8$; high-fat, high-sugar exercise-trained recipient (HFHS-ET-R), $n = 10$; n = no. of mice. ns, not significant.

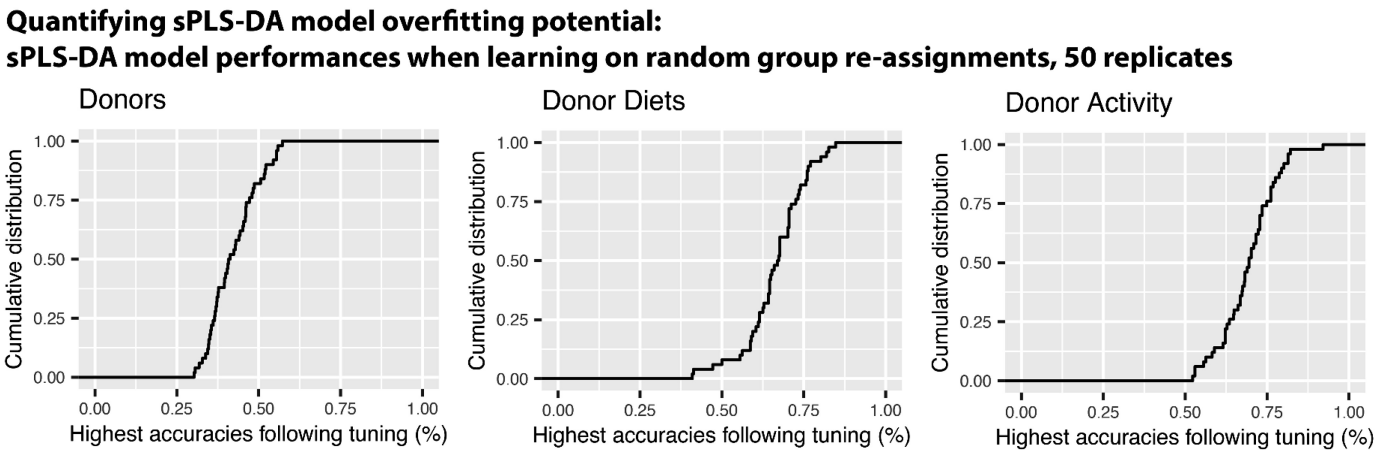

Fig. 11. Microbial signatures derived through square discriminant analysis (sPLS-DA) were robust and not the result of models overfitting to noise in the data, determined through repeated model retraining under permuted data. All sPLS-DA models on real data (Fig. 10, *E*–*G*) were statistically significant.

from chow-fed mice. However, after a longer term (45 days) of microbiota exposure, mice that were exposed to feces from high-fat-fed mice were more glucose intolerant compared with mice that received feces from chow-fed mice. Importantly, this is the same timeframe in which we assessed glucose control in our study. Given that this length of exposure time was significant enough to promote dysglycemia in both these studies in response to high-energy diet FMT/fecal exposure, we believe it is also long enough for any exercise-induced effects to manifest, although we cannot exclude that longer exposure time to the exercise-trained microbiota may be necessary to see an impact on glycemia.

One metabolic parameter that was assessed whereby there was an exercise effect on the recipients was the RER values for the NC-ET-R group, which showed lower values compared with the NC-Sed-R group (Fig. 8*C*). A lower RER is indicative of a drive toward fatty acid utilization/oxidation and linked to endurance training due to mitochondrial enzyme adaptations. However, in our hands we saw no difference with exercise training in the donors in relation to RER (Fig. 5*F*. Consequently, it was not the case of a direct effect of the NC-ET donors having a lower RER and transmitting that to the recipients and must have arisen due to more complicated mechanisms. Although we saw no effect on TAG levels in the large intestine, WAT, or liver of the NC-ET-R group, we cannot exclude the possibility that there were lipid alterations in other organs. The effect was only observed in the NC groups, suggesting that its transmission was nullified when the donors were fed a HFHS diet. Future studies designed to specifically elaborate on this finding may be warranted.

Our data also demonstrate that diet asserts dominance over physical activity in relation to gut microbial composition and penetrance of recipient metabolic phenotypes with FMT. In untargeted analyses, recipient microbiotas readily clustered by donor diet but donor exercise state was indistinguishable. Although targeted supervised machine learning did identify subsets of microbes that reliably identified exercise status, the models were more nuanced and encompassed a greater number of microbes than when distinguishing donor diet. This suggests that donor diet was readily determinable through few microbes, but exercise induced far more subtle patterns spanning multiple microbes.

During the preparation of this manuscript a paper was published (26) whereby the authors performed a similar set of experiments. In their study, administration of FMT from ET mice to antibiotic-treated high-fat-fed recipient mice (5 FMTs/wk for 12 wk) decreased adiposity and blood glucose levels in the recipients (26). Although this previous study suggested exercise conferred some microbiome-mediated effects, they were predicated on a fairly extreme protocol of treatment of antibiotics and many gavages. Our study suggests that such effects would be relatively minor under more realistically tempered contexts of people adhering to regular physical activity. The discordant results of their study with our own likely stem from methodological differences. The current study used NC recipients, whereas the previous study used high-fat-fed (60% fat) recipients. We avoided antibiotic pretreatment, whereas their mice received ciprofloxacin/metronidazole. The present study used 6 FMTs, and the other study performed 60. Also, we utilized a sham gavage control group (NC-Sed donors → NC-Sed recipients) to control for *1*) the stress of gavage treatment and for any potential metabolic impact of this and *2*) introducing new microbial constituents to the gut; conversely, the control group in the other paper was treatment naïve (26). It would be interesting in future studies to combine their approach with our method; that is, to test the effect of FMT from ET donors on high-fat-fed recipients but at less frequency, with the inclusion of a gavage control group and with and without antibiotic treatment. Another additional experimental design may be to test whether precolonization of recipient mice with microbes from ET-donor mice could protect against the effects of later, subsequent exposure of FMT from HFHS-fed mice.

The specific composition of high caloric diet may also limit whether exercised microbiota is transmissible and/or metabolically protective, as microbiota transmissibility has been previously shown to be noneffective when recipients are fed a diet high in fat (37). Consequently, looking at our study and other similar studies, the pathological or physiological metabolic state of the recipient may be an important determining factor in the effectiveness of FMT or the ability of FMT to assert a phenotype. This is an important factor for scientists to consider when conducting such studies.

Our study has some limitations worthy of consideration. Despite investigating numerous potential mechanisms via

which the FMT from HFHS donors may be causing the glucose spike during the oGTT in recipients (i.e., blood cell profile, leaky gut, lipid accumulation, inflammation, microbiota composition), we cannot provide a precise mechanism of action. Other mechanistic candidates for the effect include, but are not limited to, tissue crosstalk signaling, neural regulation, alteration to blood flow, and glucose transport regulation. Additionally, as our oGTTs did not contain glucose stable isotope tracers, we could not determine the relative contribution of the liver to the glucose spike phenotype. The study assessed only one method of delivering the FMT (orally). It is possible that rectal FMT may have yielded a different profile of viable microbes colonizing the gut, and thus a differing metabolic phenotype. We also cannot conclude whether the observed effects were due to the microbiota changes or other fecal-derived components that were transferred, or whether the effects require microbial engraftment, as gavages were administered periodically throughout. Determining the ratio of alive-to-dead bacteria that is transplanted with FMT in future studies may provide information in determining factors impacting on glucose metabolism following FMT. Furthermore, completely recolonizing the gut with FMT (i.e., like what occurs with the use of germ-free mice or broad-spectrum antibiotic treatment) may have yielded different results. Such procedures may promote the engraftment of microbes during and following on from the FMT process, when the intestine is devoid of microbiota and consequently the competition for engraftment and growth is lowered. We selected our approach to test our hypothesis with mice in their natural state to simulate as closely as possible the normal physiological system (i.e., a system where we tested FMT efficacy in mice that already harbored a natural microbiota composition; this would better reflect the microbiota of people commencing an exercise regime with diet-induced microbiota in place).

Together, our study demonstrates that the negative effects of HFHS diet on glucose levels during an oGTT can be transferred to recipient mice via FMT. This may be an important factor to consider when screening donors for FMT. Indeed, there are case reports of potential transmission of new-onset obesity via FMT (1), so donor-metabolic screening may need prioritization to protect the metabolic health of recipients. Our study also questions whether ET-induced changes in microbiota composition can dominate over normal diet-influenced composition to an extent to alter host metabolism because besides an alteration to RER (on normal chow diet only), no metabolic phenotype was observed in recipient mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.J.H., M.A.F., and D.C.H. conceived and designed research; J.Z., S.E.H., E.E., J.P.M., H.L.K., T.L.A., and D.C.H. performed experiments; J.Z., M.N.R., S.E.H., E.E., H.L.K., and D.C.H. analyzed data; M.N.R., S.E.H., E.E., H.L.K., A.J.H., M.A.F., and D.C.H. interpreted results of experiments; M.N.R. and D.C.H. prepared figures; M.N.R. and D.C.H. drafted manuscript; M.N.R., S.E.H., J.P.M., H.L.K., T.L.A., A.J.H., M.A.F., and D.C.H. edited and revised manuscript; J.Z., M.N.R., S.E.H., E.E., J.P.M., H.L.K., T.L.A., A.J.H., M.A.F., and D.C.H. approved final version of manuscript.

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