High intensity exercise training increases the diversity and metabolic capacity of the mouse distal gut microbiota during diet-induced obesity

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ABSTRACT
Diet and exercise underpin the risk of obesity-related metabolic disease. Diet alters the gut microbiota, which contributes to aspects of metabolic disease during obesity. Repeated exercise provides metabolic benefits during obesity. We assessed if exercise could oppose changes in the taxonomic and predicted metagenomic characteristics of the gut microbiota during diet-induced obesity. We hypothesized that high intensity interval training (HIIT) would counteract high fat diet (HFD)-induced changes in the microbiota without altering obesity in mice. Compared to chow-fed mice, an obesity-causing HFD decreased the Bacteroidetes to Firmicutes ratio and decreased the genetic capacity in the fecal microbiota for metabolic pathways such as the tricarboxylic acid (TCA) cycle. After HFD-induced obesity was established, a sub-set of mice were HIIT for 6 weeks, which increased host aerobic capacity, but did not alter body or adipose tissue mass. The effects of exercise training on the microbiota were gut segment-dependent and more extensive in the distal gut. HIIT increased the alpha diversity and Bacteroidetes to Firmicutes ratio of the distal gut and fecal microbiota during diet-induced obesity. Exercise training increased the predicted genetic capacity related to the TCA cycle among other aspects of metabolism. Strikingly, the same microbial metabolism indices that were increased by exercise were all decreased in HFD-fed versus chow diet-fed mice. Therefore, exercise training directly opposed some of the obesity-related changes in gut microbiota, including lower metagenomic indices of metabolism. Some host and microbial pathways appear similarly affected by exercise. These exercise and diet-induced microbiota interactions can be captured in feces.
HIGHLIGHTS

- Obesity decreased the predicted genetic capacity for many metabolic pathways in the microbiota
- Exercise training increased the genetic capacity for metabolic pathways such as the TCA cycle in the microbiota during obesity
- Exercise-induced changes of the predicted metagenome can be captured in the fecal microbiota
- Exercise training altered the microbiota in a gut segment-dependent manner
- Diet had more profound effects than exercise on the microbiota
INTRODUCTION

The mammalian gut contains a large and diverse community of bacteria that have been implicated in infectious and chronic diseases, which can manifest in the gastrointestinal tract and systemically (9, 16, 31). Colonization of gut is influenced by complex environmental factors that include host genetics, age, diet, lifestyle, diseases and antibiotic use (12, 15, 53). The reciprocal relationship between the gut microbiota and host metabolism can influence disease risk. Understanding these connections may yield avenues to manipulate resident microbes to mitigate metabolic disease risk or severity including obesity and insulin resistance.

Two major factors contributing to the development of obesity and obesity-related insulin resistance are diet and exercise. Genetic models of hyperphagia-induced obesity and an obesity-causing high fat diet (HFD) alter the gut microbiota (35, 58). The microbes in this relationship are not just a marker of obesity or metabolic disease because transmissible components of the fecal microbial community in obese individuals can increase adiposity independently of host genetics (50, 57). In comparison to diet, little is known about how exercise may influence the microbiota. However, some connections between the microbiota, exercise status and daily activity levels are emerging. There are a limited number of studies associating exercise and gut microbiota alterations in rodent models. Studies have found that exercise increases the Bacteroidetes to Firmicutes ratio in the absence of obesity. For example, voluntary access to exercise (i.e. wheel running) in rats on a standard diet increased Bacteroidetes and decreased Firmicutes in the feces (49). In addition, voluntary exercise in juvenile chow-fed rats increased Bacteroidetes and decreased Firmicutes in the feces (44). In HFD-fed mice, the extent of voluntary exercise correlated with a change in Bacteroidetes to Firmicutes ratio (20). The effect
of exercise on the gut microbiota depends on the mode of exercise performed such as volitional wheel running compared to forced treadmill training (1). A targeted assessment found that low-intensity exercise can influence the cecal microbiome in obese, hyperglycemic mice (33). However, a complicating factor of all of the above studies is that these types of exercise can lower body mass and/or prevent weight gain during obesity, which may not allow microbial changes to be ascribed to exercise versus obesity or adiposity. It is currently unknown whether exercise training can overcome changes to the microbiota that occur during diet-induced obesity, independently of body or fat mass. It is also important to discern that acute effects of an exercise session versus repeated exercise training.

Further, gut segment-dependent changes in the microbiome due to exercise are ill-defined. We thought it was important to investigate how the stress of exercise could alter gut-segment and fecal differences in the microbial communities that include differences in anaerobic and aerobic bacteria, particularly since exercise reduces splenic and intestinal blood flow (26, 45). In fact, exercise can alter blood flow to different parts of gastrointestinal tract, which is modified by repeated exercise training. For example, an acute exercise session reduces blood flow to the large and small intestine in foxhounds, but after 8-12 weeks of exercise training, blood flow was not lower in the small intestine (46). Exercise training is also linked to functional changes in the gastrointestinal tract, such as increased mouth to colon transit time without altering intestinal absorption (27). It is important to characterize exercise-induced alterations in the intestinal microbiome as a first step toward understanding if microbial changes are responding to the stress of exercise and if microbes are related to functional outcomes.
Endurance exercise training alters a myriad of host responses in both lean and obese individuals including improvements in insulin sensitivity and increased cardiorespiratory fitness; effects associated with increased aerobic respiration (VO₂) (52) and muscle mitochondrial enzymes such as those involved in the tricarboxylic acid (TCA) cycle (28, 29). High intensity interval training (HIIT) has been shown to induce very similar metabolic effects to that elicited by endurance exercise training, including improvements in insulin sensitivity (25, 36). The mechanisms by which HIIT improves insulin sensitivity are not well understood since obesity status is usually not altered and recent studies in HFD-fed mice have demonstrated that HIIT improves both adipose and liver insulin sensitivity without changing body mass or adiposity (38). Remarkably, these changes in liver and adipose tissue insulin sensitivity occurred independently of liver lipid content or adipose tissue inflammation suggesting that other factors may have been responsible for the insulin sensitizing effects of HIIT.

Given the known connections between the gut microbiota and insulin sensitivity we sought to determine whether HIIT alters the microbiota in mice after the establishment of obesity. We assessed if HIIT influenced the taxonomy and predicted functional metagenomics of the murine gut and fecal microbiota during a HFD. The results show that HIIT opposed some of the effects of a HFD to reduce the predicted metabolic genetic capacity of the fecal microbiota, independently of obesity. These data suggest that HIIT alters similar metabolic pathways in both the host and microbiota and raises the possibility that this type of exercise training may elicit some of its beneficial effects on metabolism through alterations in the gut microbiome.

MATERIALS AND METHODS
Mice: All experiments were approved by the McMaster University Animal Research Ethics Board (Hamilton, Canada). All mice were born at McMaster University. Littermate mice were randomly assigned to exercise trained or untrained conditions using male offspring from a given in-house breeding pair of C57 BL/6 mice. This resulted in a mix of different mothers, breeding pairs and cages in each experimental condition, which was done in order to limit any environmental or inherited influence on the results obtained for microbiota analysis. Eight week old, male mice were maintained on a 12 h light/dark cycle and fed a HFD (45% kcal from fat, D12451, Research Diets; New Brunswick, NJ) for 12 weeks. After 6 weeks of HFD feeding, mice were either exercise trained (described in detail below) or left untrained. Untrained mice were exposed to the treadmill environment for equal periods of time as the exercise trained group. After a 3 day acclimation to treadmill running (19), exercise capacity was measured using a graded exercise test during which mice began running at 8 m/min on a 5% grade and treadmill speed was gradually increased by 1 m/min every 2 min until exhaustion, as described previously (38, 54). This test was also repeated after 6 weeks of exercise training, which involved treadmill running 3 days per week for 1-hour each day, as previously described (38). Specifically, the treadmill running involved running for 2 min at 17 m/min at a 5% grade (100% of average pre-trained maximal running speed/exercise capacity) and then resting for 2 min. The treadmill running speed was increased by 1m/min/week so that by the end of the training protocol, mice were running at 22 m/min. After 6 weeks of exercise training, and 24 hours after an exercise session, a 6 hour fasted insulin tolerance test was conducted (ITT). Tail vein blood glucose was measured using a glucometer during an insulin tolerance test (1 IU/kg), and epididymal adipose tissue was removed and massed, as described (30, 51). Two additional groups of mice were included as a comparison for microbiota analysis. Littermate male mice from multiple in-house
breeding pairs were randomly assigned to standard chow diet feeding (n = 7 mice) or at 6-7
weeks of age a subset of mice were fed the HFD (n = 9 mice) for 12 weeks. The purpose of these
additional groups was to provide context to the comparison between untrained and HIIT gut
microbes.

Microbiome Sampling: Feces were collected directly from the anus of mice into sterile tubes
which were immediately snap frozen in liquid nitrogen before the mice were placed on the
treadmill. Before repeated exercise training was initiated, the acute effects of a single exercise
session were assessed by collecting feces within one hour and one week after the initial graded
exercise capacity test. To test the chronic effects of repeated exercise training fecal samples were
collected 3 days after the last exercise training session following 6 weeks of exercise training
with gut segments collected the following week 1 day after the final exercise session. At the
completion of the 6 week exercise training protocol, the duodenum plus jejunum, ileum, cecum
and colon were snap frozen in liquid nitrogen and stored at -80 °C. Fecal pellets were removed
before processing any intestinal segment.

Bacterial profiling: Genomic DNA was extracted from fecal and gut segment samples. PCR
amplification of the variable 3 (V3) region of the 16S rRNA gene was done on each sample,
which included Illumina compatible adapter sequences and barcoding for multiplexing. DNA
products of this PCR amplification were sequenced using the MiSeq platform followed by
preliminary analysis by the McMaster Genome Center (McMaster University). A custom in-
house pipeline was used to process the FASTQ files as described (17). Cutadapt was used to trim
sequences beyond the 16S rRNA V3 region and PANDAseq was used to align paired-end
sequences (39, 40). AbundantOTU+ grouped reads into Operational Taxonomic Units (OTUs) based on 97% similarity (41, 60). Taxonomy was assigned to OTUs Ribosomal Database Project (RDP) classifier in Quantitative Insights Into Microbial Ecology (QIIME) (6) against the 2011 version of the Greengenes reference database (18). QIIME was used to calculate the diversity within communities (alpha diversity) and between community diversity (beta-diversity), as previously described (6, 17). At the genus level, OTUs were assigned to the corresponding genus and represented to the closest root of the phylogenetic tree. This can result in different OTUs being assigned to the same classification. Principal coordinates analysis (PCoA) used the Bray-Curtis dissimilarity values to position the points relative to each other. Sequencing characteristics are described in Table 1. Prediction of metagenome functional content from 16S rDNA library was developed using PICRUSt software and PICRUSt predictions were categorized as level 1 to 3 into KEGG pathways (34). QIIME was used to visualize the predicted functions within KEGG pathways.

Statistical analysis: Results were analyzed by an unpaired, two-tailed Student’s t-test where two means are compared or ANOVA (for more than two means) using GraphPad Prism 6 software. Subsequently, false discovery rate (FDR) was accounted for via implementation of the Benjamini-Hochberg multiple testing adjustment procedure using R, where FDR-corrected p-values were estimated for all taxonomic data or predicted metagenomic data within a specific PICRUSt level. Statistical significance was accepted at p<0.05 after adjustment for FDR.

RESULTS

HFD alters the gut and fecal microbiota:
Consistent with previous findings (17, 58), we observed a lower Bacteroidetes to Firmicutes ratio in the distal gut and feces in HFD-fed mice compared to chow-fed mice (Fig. 1A). This comparison of chow fed mice (average body mass $32 \pm 0.5$ g) to HFD-fed mice (average body mass $50 \pm 0.7$ g) was separate from all exercise-related experiments. Compared to chow-fed mice, HFD-fed mice were insulin and glucose intolerant (data not shown). We found no evidence that the type of diet changed the overall alpha diversity (data not shown), but we found lower alpha diversity (i.e. Shannon index) within the phylum Bacteroidetes in both the colon and feces of HFD-fed mice (Fig. 1B). Additional differences in the colonic and fecal microbiome taxonomy from chow-fed versus HFD-fed mice are shown in Supplemental Table S1.

Exercise training during obesity improves insulin tolerance independently of adiposity:
Mice were on an obesity-causing HFD for 12 weeks with HIIT during the final 6 weeks (Fig. 2A). HIIT was initiated after the establishment of obesity and this type of repeated exercise training did not alter body mass in HFD-fed mice (Fig. 2A). As expected, HIIT increased the time to exhaustion and running speed at the completion of a graded exercise test (Fig. 2B-C). Exercise training also increased insulin tolerance, but did not alter fasting blood glucose and did not alter epididymal adipose tissue mass in HFD-fed mice (Fig. 2D-F). We recently demonstrated that this HIIT exercise protocol increased oxygen consumption ($VO_2$), carbon dioxide consumption ($VCO_2$), respiratory exchange ratio (RER), food intake and water intake in HFD-fed mice (38). We also published that this HIIT exercise protocol does not alter body mass, whole body adiposity, adipose mass, liver mass or heart mass (38). Importantly, we made every effort to control for different environmental microbial exposures in the mice, including placing
all of the untrained mice on the exercise treadmill (which was turned off) for an equal duration that corresponded with each exercise training session.

Exercise training during obesity alters the microbiota in a gut segment-dependent manner:

Exercise training of HFD-fed, obese mice increased the overall alpha diversity of the microbiota in the colon (Fig. 3A). Exercise training increased the alpha diversity within the phylum Bacteroidetes in both the cecum and colon (Fig. 3B). Exercise training increased the Bacteroidetes to Firmicutes ratio in the cecum and a similar trend (P = 0.06) was seen in the colon (Fig. 3C). No clear pattern of exercise training-related changes could be seen after PCoA (Fig. 3D). Exercise training did not significantly alter any other phylum-level microbiota characteristics as depicted by relative quantity of each phylum in various gut segments (Fig. 3E).

These data indicate that exercise training opposed the effects of the HFD, since a HFD decreased, but exercise training increased the Bacteroidetes to Firmicutes ratio and diversity within Bacteroidetes in the distal gut.

At the OTU level, exercise training during the final 6 weeks of the HFD had more profound effects in the distal gut. Figure 4A depicts the average relative abundance of genera in various gut segments of HFD-fed mice that were exercise trained or untrained. Figure 4B-E depicts all of the statistically significant genus-level changes associated with exercise that occurred in at least one of the gut segments after correction for FDR. Based on the relative quantity of each OTU that was detected, exercise training was associated with a significant difference in 1 OTU in the duodenum plus jejunum (Actinobacteria (c)) and 1 OTU in the ileum (Lactobacillus) compared to 3-5 significantly different OTUs in the cecum or colon (Fig. 4B-E). Also at the
OTU level, exercise training increased Bacteroidales (o) (~1% of the relative abundance) in both the cecum and colon (Fig. 4D-E). Overall, this data shows that repeated exercise training altered the constituents of the microbiome more profoundly in the distal gut during HFD-induced obesity and that HIIT consistently increased Bacteroidales (o) in both the cecum and colon of obese mice.

Exercise training during obesity alters the fecal microbiota:

Feces are easier to obtain for biomarker assessments and use of feces allows comparison within the same individual/mouse across time. Hence, we analyzed the feces of all HFD-fed mice before exercise (i.e. PreTreadmill) compared to samples collected after 6 additional weeks from HFD-fed mice that were exercise trained and age-matched HFD-fed untrained mice. Analysis of the fecal samples collected before mice were allocated to trained or untrained groups (i.e. direct comparison within the PreTreadmill groups) showed that the exercise training-induced differences in microbiota characteristics were not derived from an existing difference in these microbiome measurements before mice initiated exercise training (data not shown). Within subjects analysis at the phylum and genus levels (Fig. 5A-B) revealed that 6 weeks of exercise training significantly increased only 1 OTU in the feces. Bacteroidales (o) was higher in exercise trained versus pre-treadmill fecal samples (Fig. 5C). This is consistent with exercise training-induced effects in the cecum and colon. Exercise training increased the alpha diversity as measured by a higher Shannon index in trained versus untrained fecal samples (Fig. 5D), but it did not change the Shannon index within Bacteroidetes (data not shown). Exercise training also increased the Bacteroidetes to Firmicutes ratio compared to pre-treadmill fecal samples (Fig. 5E). Again, there was no discernable pattern based on PCoA of exercise trained, untrained and
PreTreadmill fecal samples (data not shown). These data indicate that exercise training increased Bacteriodales (o) and diversity in the feces, which is reflective of exercise-induced changes in distal gut of HFD-fed mice.

We next sought to determine if these changes in the taxonomy of the microbiota during repeated exercise training were independent of an acute bout of exercise. In feces collected within 1 hour and 1 week after a single acute bout of exercise, we found no phylum level changes (Fig. 6A-B). Only one genus (Lactococcus) was decreased 1 hour after acute exercise (Fig. 6C) and this change did not persist 1 week after acute exercise. Finally, there was no change in the overall alpha diversity (i.e. Shannon index) or alpha diversity within Bacteroidetes or Bacteroidetes to Firmicutes ratio of the feces at 1 hour or 1 week after a single exercise session compared to pre-treadmill values (Fig. 6D-F). Overall, these results show that the changes observed in repeated exercise training could not be explained by the effect of a single exercise session.

**Exercise training during obesity alters the predicted function of the fecal microbiota:**

We next sought to determine if exercise training was associated with predicted changes in the genetic capacity for microbial functions in the feces. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis showed a significant increase in the microbial genetic material that is KEGG-annotated to metabolism after exercise training (Fig. 7A). This directly opposes a decrease in the genetic capacity related to metabolism during a HFD compared to chow diet (Fig. 7B). A HFD also increased the predicted genetic capacity related to Environmental IP (Fig 7B). Further analysis revealed that exercise training only altered predicted pathways within the KEGG pathways assigned to metabolism. Exercise training increased the
fecal microbial genes predicted to be involved in glycan biosynthesis and metabolism, carbon
domination and the TCA/citrate cycle (Fig. 7C). Again, this directly opposes a subset of the changes
during a HFD where all of these KEGG-assigned metabolic pathways were decreased compared
to chow fed mice (Fig. 7D). A single, acute exercise session did not alter these or any predicted
metagenomic characteristics (data not shown).

The effects of HFD (compared to chow diet) included over 30 changes within the metabolism
annotation (Supplemental Table S1). Therefore, the effects of diet on the microbiota were more
extensive compared to exercise. Nevertheless, our results show that exercise and an obesity-causing diet shift the predicted metagenomic characteristics of the fecal microbiota in opposite
directions. Further, our results show that exercise during diet-induced obesity directly opposes
some of the obesity-related functional characteristics of the microbiota despite no change in body
mass.

DISCUSSION

Diet and exercise are two factors involved in obesity. Obesity is associated with altered gut
microbiota. We show that HIIT can oppose some of the microbiota changes characteristic of diet-
induced obesity even though there is no change in body mass or adipose tissue mass during this
type of exercise training. These exercise-related changes in the microbiota include expansion of
the predicted genetic capacity related to various pathways in metabolism.

Diet-induced changes in the microbiota have emerged as a contributor to host metabolism
relevant to obesity-related disease. Gut-derived microbial factors can influence metabolic disease
characteristics (4, 10). Multiple diet-related factors influence obesity, including caloric intake and macronutrient composition. Diets high in fat promote obesity and alter the taxonomic and metagenomic characteristics of the gut microbiome (58). The characteristic changes in the gut microbiota during an obesity-causing HFD include a lower relative level of Bacteroidetes and a relative expansion of Firmicutes (35, 59). A high fat/sugar diet is the dominant factor influencing the gut microbiota when compared to host genetics (7). Further, the fecal microbiota from lean and obese twins can influence fat mass when transferred to germ free mice (50). Therefore, diet and/or obesity appear to be a powerful factor influencing the metabolic effects of the microbiota. Our results confirm that diet (i.e. HFD versus chow diet) is a dominant factor in shaping the microbiota, including its predicted functional genetic characteristics. Diet interacts with many host and environmental factors that can potentially influence the microbiota and obesity. We sought to determine if exercise was a factor that could influence or overcome dietary shifts in the microbiota. Exercise can counterbalance many host metabolic processes during obesity, but little is known about how exercise influences the microbiota during diet-induced obesity. We found that exercise training (i.e. HIIT) during obesity promoted changes in the distal gut and fecal microbiota that were opposite to those characteristic of obesity and/or a HFD. For example, exercise training increased the Bacteroidetes to Firmicutes ratio and also increased the alpha diversity (within the Bacteroidetes phylum) of the microbiota. HIIT could overcome the influence of a HFD in the distal gut and feces, since exercise training opposed some of the taxonomic and predicted metagenomic changes caused by diet-induced obesity. Altered host obesity/adiposity appears not to be a major driver of these exercise-induced changes in the microbiota, since the type of exercise used in our study did not alter body mass or adipose tissue
mass during the HFD. Adaptations to repeated exercise sessions appear to be required for
diversity, taxonomic and predicted metagenomic microbiota characteristics, since acute exercise
did not alter these indices. A limitation of our study is the inability to segregate improved insulin
tolerance caused by repeated exercise as a factor that could contribute to alterations in the
microbiota.

There is evidence mounting that sustainable perturbations to the microbiota can alter obesity-
related metabolic disease characteristics. For example, a low dose of antibiotics given during an
early-life window can promote increased adiposity (11). The transient early-life dysbiosis caused
by certain antibiotics appears to interact with dietary stress, since it magnifies the obesity-
causing effect of a high fat diet (15). Antibiotics can also promote intestinal dysbiosis that is
sufficient to accelerate diabetes (3). Immune signals are positioned to connect microbial
dysbiosis to disease characteristics (2). Diet is a powerful factor influencing the microbiota,
which can influence immune underpinnings of disease (37). Unravelling the compartmentalized
responses and connections between diet, dysbiosis, metabolism and immunity is a complex
challenge (43). We have recently shown that diet-induced changes in intestinal immunity do not
necessarily parallel immune responses in adipose tissue (8). It is beyond the scope of this work to
determine the underlying immune or endocrine signals that are a potential cause or consequence
of exercise and diet-induced changes in the microbiota. Future goals of this type of work include
assessments of immune cell populations and inflammatory or endocrine mediators in the gut and
metabolic tissues. This will be important since there is a reciprocal relationship between
inflammatory mediators and cellular energy sensors involved in exercise responses (22, 55).
These connections could also engage adipokines and myokines and changes in bile acid
metabolism and could involve increased food/water intake coincident with exercise training. Intriguingly, different dietary lipids can promote dysbiosis during aging and infectious colitis (23, 24). Therefore, a key future goal is to expand the model of a HFD and assess if specific dietary lipids interact with the effects of exercise on microbiota and host responses. It is also important to extend this work to humans. Professional rugby players have increased diversity of the fecal microbiota (12). However, it appears very difficult to separate exercise and dietary influences on the human microbiome (48).

The evidence is mounting that exercise is a perturbation that can influence the microbiota. Our most consistent finding regarding taxonomy was an exercise training-related increase the levels of Bacteriodales (o) in distal gut (cecum, colon) and feces. In HFD-fed mice, we also found an increase in Dorea in the cecum and colon, which is consistent with other groups that employed forced treadmill running and found increased Dorea in the cecum and feces in chow-fed mice (1). Another previous study found that exercise and diet each altered the microbiota independently by investigating the interaction of exercise and non-exercised mice that were chow fed versus HFD-fed (32). This type of exercise caused decreased body mass in HFD-mice, which is different from the HIIT protocol in the current study. In fact, the mode of exercise could be very important in dictating changes in microbiome characteristics. As expertly reviewed, rodent models using different types of exercise have shown different gut microbiome characteristics (13). This may be related to differential immune responses in the gut. For example, macrophage number is higher in the colon of mice after forced treadmill exercise, an effect that does not occur with voluntary wheel running (13). This is an intriguing gut immune response that may relate to the exercise-related expansion of lactobacilli in the distal colon.
during voluntary running, but not forced treadmill running (13). These gut immune and microbe
changes correspond to functional outcomes such as increased inflammatory score and mortality
due to forced treadmill running if there is an additional stress such as a model of colitis in mice
(14). It remains to be determined what aspects of metabolic disease, such as obesity,
hyperlucemia or even specific dietary components interact with different modes of exercise to
influence the microbiome. This seems worthwhile, since it has already been shown that 6 weeks
of exercise increased Bifidobacterium in the cecum of control mice, but not in hyperglycemic
leptin receptor deficient (db/db) littermate mice (33).

The effects of exercise training on the microbiota during a HFD diet in our study were relatively
small when compared to changes in diet or other stimuli that can cause dysbiosis such as
antibiotics. This is not that surprising and we chose to characterize exercise because of the
overwhelming evidence of the host metabolic changes (and health benefits) induced by exercise.
This descriptive information may set the stage for understanding how to assess if microbes play a
role in the metabolic health benefits of exercise during obesity. Further, the altered microbial
signatures may be able to be used as a biomarker of exercise status or responsiveness. Our data
supports investigation of exercise-induced metagenomic characteristics in the feces.

Exercise is well known to increase oxidative capacity, mitochondria and proteins involved in the
TCA cycle (28, 29, 47). Our results show that exercise training increased the predicted
metagenomic capacity for metabolism and the TCA cycle in the fecal microbiota. Our data only
provide a prediction of the genetic capacity of the microbial community for these KEGG
pathways. Caution is warranted in assuming that these genetic indices influence the same host
pathways by producing chemical messengers or altering the metabolism of substrates. It is not yet clear which of the many host effects of exercise relate to microbiota changes. Nevertheless, it is enticing to speculate about the exercise-induced functional changes in the microbiota, which only occurred in the metabolism annotation. The observed changes in poorly classified glycan biosynthesis could be linked to the regulation of mucin O-glycan or biosynthesis of lipopolysaccharide or peptidoglycan. All of these pathways have been implicated in aspects of host metabolic disease (4, 10, 17, 21, 51, 56). The observed changes in carbon fixation and the TCA cycle pathways could be related to Acetyl-CoA and short chain fatty acid regulation, which have been implicated as microbial ligands/metabolites that influence host metabolism (5). For example exercise has been shown to increase cecal butyrate levels in rats (42).

A HFD had a more profound effect on the predicted metagenomic characteristics of the fecal microbiota, which corresponds with diet dominating other factors (7). Intriguingly, all of the exercise-induced changes in predicted microbial function occurred in the opposite direction compared to those associated with a HFD. Our data suggests that exercise counterbalances a subset of the changes in microbial function caused by obesity or obesity-causing diets. It is not clear how repeated exercise training elicits a change in the microbiota of the distal gut, but intestinal motility should be considered. Further, exercise reduces blood flow to the colon to a much greater degree compared to other parts of the intestine. This reduced blood flow might equate to a hypoxic state in the resident microbiota, which could dictate expansion of microbes with a greater metabolic or suitable respiratory capacity. It is probable that the exercise-induced changes include bacteria where the respiratory chain proteins do not necessarily require oxygen as the terminal electron acceptor. Nevertheless, future studies testing different exercise regimes
(low and high-intensity training), altered intestinal blood flow and hypoxia will be important to
delineate whether these environmental cues alter the gut microbiome.

In summary, our results show that repeated exercise training can overcome a distinct subset of
the changes in the distal gut and fecal microbiota caused by HFD-induced obesity, independently
of changes in body mass or fat mass. In the fecal microbiota, an obesity-causing diet decreased,
whereas repeated exercise training increased, several predicted metagenomic traits involved in
metabolism, including the TCA/citric acid cycle. Exercise training is well known to regulate
these host metabolic pathways and it is enticing to speculate that the physiological response to
exercise also includes changes in analogous microbial pathways.

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**FIGURE LEGENDS**

**Figure 1. Phylum and diversity changes in chow-fed and HFD-fed mice.** Relative Bacteroidetes to Firmicutes ratio (A) and alpha diversity (i.e. Shannon index) within Bacteroidetes (B) in the colon and fecal microbiota from chow-fed and HFD-fed mice. Data are mean ± SEM. *P<0.05* Chow versus HFD. The number of mice analyzed for each condition are shown in brackets.

**Figure 2. Host physiology associated with HIIT in HFD-fed mice.** Weekly body mass in HFD-fed mice that were high intensity interval trained (HIIT) 3 times a week or untrained (A, n = 8 mice per group). Time to exhaustion (B) and running speed at exhaustion (C) during a graded treadmill exercise test in HFD-fed mice that were exercise trained for 6 weeks versus untrained mice. Epididymal adipose tissue mass in HFD-fed mice that were exercise trained for 6 weeks or untrained (D). Blood glucose (E) and cumulative area under the curve (AUC) (F) during an insulin tolerance test (ITT; 1 IU/kg) in HFD-fed mice that were exercise trained for 6 weeks or untrained. Data are mean ± SEM, *P<0.05* versus untrained. The number of mice analyzed for each condition are shown in brackets.
Figure 3. Phylum and diversity changes in the gut microbiome associated with repeated exercise in HFD-fed mice. Overall alpha diversity (i.e. Shannon index) in microbiota from various gut segments (A) and alpha diversity (i.e. Shannon index) within Bacteroidetes (B) in untrained and exercise trained mice that were all HFD-fed. Relative Bacteroidetes to Firmicutes ratio in various gut segments in HFD-fed untrained and exercise trained mice (C). Principal coordinates analysis (PCoA) performed on Bray-Curtis distances in the colon of exercise trained versus untrained mice (D). Average relative abundance of the major phyla in HFD-fed untrained and exercise trained mice (E). Panels A-C: Data are mean ± SEM. Panel E: Data are mean values. #P<0.05 trained versus untrained. The number of mice analyzed for each condition are shown in brackets.

Figure 4. Genus level changes of the gut microbiota associated with repeated exercise. Average relative abundance of genera in various gut segments of HFD-fed untrained and exercise trained mice (A). OTUs that were significantly different in the microbiota from any gut segment are shown from exercise trained versus untrained HFD-fed mice (B-E). Significant differences are noted in bold for taxonomic classification in duodenum plus jejunum (A), ileum (B), cecum (C) and colon (D). Panel A: Data are mean values. Panels B-E: Data are box-and-whisker plots. *P<0.05 trained versus untrained. The number of mice analyzed for each condition are shown in brackets.

Figure 5. Taxonomic changes in the fecal microbiome associated with repeated exercise. Average relative abundance of the major phyla (A) and genera (B) in the feces of HFD-fed untrained and exercise trained mice. Depiction of the only significantly different genus-level
change (Bacteriodales (o)) in the feces (C). Alpha diversity represented by the Shannon index in exercise trained versus untrained versus PreTreadmill conditions in feces from HFD-fed mice (D). Relative Bacteroidetes to Firmicutes ratio in exercise trained versus untrained versus PreTreadmill conditions in feces from HFD-fed mice (E). Panels A, B: Data are mean values. Panel C: Data are box-and whisker plots. Panels D-E: Data are mean ± SEM. #P<0.05 Trained versus Untrained. *P<0.05 Trained versus PreTreadmill. The taxonomic data for the PreTreadmill condition is presented in Figure 6. The number of mice analyzed for each condition are shown in brackets.

**Figure 6. Taxonomic changes in the fecal microbiome after acute exercise.** Average relative abundance of the major phyla (A) and genera (B) in the feces of HFD-fed (untrained) mice before exercise (PreTreadmill) and 1 hour (± 1 h) and 1 week (± 1 w) after a single acute exercise session. Depiction of the only significantly different genus-level change (C). Alpha diversity the feces represented by the Shannon index (D) and Shannon index within Bacteroidetes (E) in the feces of HFD-fed mice before exercise (PreTreadmill) and 1 hour or 1 week after an acute exercise session. Relative Bacteroidetes to Firmicutes ratio in the feces of HFD-fed mice before exercise (PreTreadmill) and 1 hour or 1 week after an acute exercise session (F). Panels A, B: Data are mean values. Panel C, Data are box-and whisker plots. Panels D-F: Data are mean ± SEM. *P<0.05 Acute Exercise versus PreTreadmill. The number of mice analyzed for each condition are shown in brackets.

**Figure 7. Predicted functional metagenomic changes in the fecal microbiome associated with diet and exercise.** Phylogenetic Investigation of Communities by Reconstruction of
Unobserved States (PICRUSt) was used to calculate and the relative proportion of genes predicted to be present in various KEGG pathways in feces from exercise trained and untrained mice that were all fed a HFD (A). This was compared to the relative proportion of genes predicted to be present in various KEGG pathways in feces from chow-fed and HFD-fed mice that were not exercise trained (B). Depicted are significant differences in feces from exercise trained and untrained mice (C) and a side-by-side comparison of a subset of those significant differences within the metabolic pathways in feces from chow-fed and HFD-fed mice (D). Data are mean ± SEM. *P<0.05 Trained versus Untrained. #P<0.05 Chow versus HFD. The number of mice analyzed for each condition are shown in brackets.
Table 1. QIIME 16S rDNA sequencing analysis

<table>
<thead>
<tr>
<th></th>
<th>Trained versus Untrained mouse gut segments</th>
<th>Trained versus Untrained mouse feces</th>
<th>Chow vs HFD-fed mouse feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Samples</td>
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<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Minimum Sequences Count</td>
<td>9881</td>
<td>92644</td>
<td>32649</td>
</tr>
<tr>
<td>Maximum Sequences Count</td>
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<td>131501</td>
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<td>Median Sequences Count</td>
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<td>100643</td>
<td>42021</td>
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<tr>
<td>Normalized Sequences#</td>
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<td>92640</td>
<td>32640</td>
</tr>
<tr>
<td>Number of 97% phylotypes</td>
<td>335</td>
<td>187</td>
<td>165</td>
</tr>
<tr>
<td>(genus level-assignable)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The number of sequences in each dataset (of a given sample) were normalized by rarefaction to allow for intra-sample comparisons of the datasets.*
Fig 1
Fig 2
Fig 6
Fig 7