

## Voluntary and forced exercise differentially alters the gut microbiome in C57BL/6J mice

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**Allen JM, Miller ME, Pence BD, Whitlock K, Nehra V, Gaskins HR, White BA, Fryer JD, Woods JA.** Voluntary and forced exercise differentially alters the gut microbiome in C57BL/6J mice. *J Appl Physiol* 118: 1059–1066, 2015. First published February 12, 2015; doi:10.1152/jappphysiol.01077.2014.—We have previously shown that voluntary wheel running (VWR) attenuates, whereas forced treadmill running (FTR) exacerbates, intestinal inflammation and clinical outcomes in a mouse model of colitis. As the gut microbiome is implicated in colitis, we hypothesized that VWR and FTR would differentially affect the gut microbiome. Mice (9–10/treatment) were randomly assigned to VWR, FTR, or sedentary home cage control (SED) for 6 wk. VWR were given running wheel access, whereas FTR ran on a treadmill for 40 min/day at 8–12 m/min, 5% grade. Forty-eight hours after the last exercise session, DNA was isolated from the fecal pellets and cecal contents, and the conserved bacterial 16S rRNA gene was amplified and sequenced using the Illumina Miseq platform. Permutational multivariate analysis of variance based on weighted UniFrac distance matrix revealed different bacterial clusters between feces and cecal contents in all groups ( $P < 0.01$ ). Interestingly, the community structures of the three treatment groups clustered separately from each other in both gut regions ( $P < 0.05$ ). Contrary to our hypothesis, the  $\alpha$ -diversity metric, Chao1, indicated that VWR led to reduced bacterial richness compared with FTR or SED ( $P < 0.05$ ). Taxonomic evaluation revealed that both VWR and FTR altered many individual bacterial taxa. Of particular interest, *Turicibacter* spp., which has been strongly associated with immune function and bowel disease, was significantly lower in VWR vs. SED/FTR. These data indicate that VWR and FTR differentially alter the intestinal microbiome of mice. These effects were observed in both the feces and cecum despite vastly different community structures between each intestinal region.

exercise; microbiome; gut; running; forced treadmill; voluntary wheel

THE GASTROINTESTINAL (GI) microbiome represents a dense and complex network of trillions of microbes, mainly bacteria, which coordinate in a bidirectional manner to regulate host physiology (19). Notably, the microbiome has a well-recognized role in the initiation and the progression of many chronic diseases, both within and beyond the gut environment (28, 44). A large body of work has been aimed at understanding the environmental impacts, especially diet, on the GI microbiome (7, 17, 45). These data have provided insight into potential therapies (e.g., probiotics and prebiotics) that may improve GI

health and aid in the prevention of inflammatory bowel disease (IBD) (3, 23, 30). However, the role that other environmental factors, such as physical exercise, play in influencing the gut microbiome remains understudied.

A few recent studies have provided some compelling evidence that exercise has robust effects on gut microbial communities (13, 14, 20, 26, 36, 41, 51). In 2008, Matsumoto et al. (36) first found that voluntary wheel running (VWR) altered the community structure of the microbiome in the cecum of rats. Intriguingly, the authors demonstrated that exercise increased the cecal concentration of the short-chain fatty acid, *n*-butyrate. Butyrate is produced by colonic and cecal bacteria during polysaccharide fermentation and exerts various beneficial effects on epithelial and host immune cells in the GI tract (37). Additionally, we and others have shown that exercise-induced changes in the gut microbiome correlate strongly to changes in host physiology, such as improved insulin sensitivity, satiety, and cognition in mice (20, 26, 41). Microbial changes brought about by exercise may also improve resilience to inflammatory insults, such as high-fat diets and environmental toxins (13, 20, 26). It was also recently shown that exercise may alter the fecal microbiome in humans (14).

These observations provide intriguing insight into microbiome regulation and also reveal potential new avenues by which exercise can improve host health. However, a simple portrayal of how exercise alters the gut microbiome may fail to illustrate the complexity of the exercise-gut microbiome relationship. Indeed, physiologists have long known that the modality and conditions (e.g., intensity and duration) under which exercise takes place influences its effects on host physiology, especially within the GI tract (24, 47). These effects are highlighted by the paradoxical, yet prominent, epidemiological evidence demonstrating a link between strenuous, prolonged exercise and GI disturbances (e.g., diarrhea, bloody stools, and ischemic colitis) in endurance athletes (39, 46). Findings such as these contrast evidence that regular, moderate physical activity significantly reduces the occurrence of colorectal cancer and IBDs in humans (29, 35, 42).

Recently, our laboratory demonstrated that two exercise training modalities cause vastly different effects within the gut in response to colitis in mice (15). We found that 6 wk of forced treadmill running (FTR) exacerbated, whereas 6 wk of VWR attenuated, clinical symptoms and inflammation in response to acute administration of dextran sodium sulfate (DSS) (15), one of the most common animal models of ulcerative colitis (UC) (33). As gut microbiota have been linked to UC

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and Crohn's disease, we sought to define the effects of these two commonly utilized exercise-training modalities (e.g., VWR and FTR) on the gut microbiome in C57BL/6J mice. Because unique metabolic and immune interactions occur in different regions of the GI tract, we also investigated the microbiome at two different intestinal sites, the distal colon (through fecal sampling) and the cecal contents. On the basis of our outcomes from our colitis study, we hypothesized that both exercise modalities would induce differential changes in diversity (higher richness in VWR and lower richness in FTR), community structure, and specific taxonomy of the microbiome at both intestinal sites compared with sedentary controls (SED).

## MATERIALS AND METHODS

**Mice and exercise/diet protocols.** Adult male, 6-wk-old C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in an AALAC-accredited facility. After a 2-wk acclimation period, mice were randomly assigned to one of three groups: FTR ( $n = 10$ ), VWR ( $n = 10$ ), or SED ( $n = 9$ ). FTR mice performed 6 wk (5 days/wk; total 30 sessions) of forced moderate treadmill running (8–12 m/min; 5% grade; ~480 m of running per session) for 40 min per day at the beginning of the dark cycle (~9 AM of reverse light/dark cycle). FTR mice ran under a red light while researchers used gentle hand prodding (with gloves) to ensure mice continued to exercise. Soft sponges were placed at the back of the treadmill lanes to provide tactile sensation while reducing risk for injury. The VWR mice were housed in cages with free access to telemetered running wheels (Respironics, Bend, OR) for 30 days. The SED mice remained in singly housed cages for the study duration. To control for stress associated with treadmill running, we handled all groups for an equal amount of time on the treadmill intervention days (5 days/wk). We also controlled for the number of days of exposure to exercise ( $n = 30$ ) but did not control precisely for exercise intensity or volume between the two different exercise modalities, as mice chose to perform VWR. Mice were fed a commercial diet (Teklad 8640; Harlan Laboratories, Indianapolis, IN) throughout the study, and food and water intake was recorded daily. The University of Illinois Urbana-Champaign IACUC approved all experiments.

**Euthanasia and sample collection.** To minimize the influence of possible acute exercise-induced changes in the gut microbiome, mice were euthanized via rapid CO<sub>2</sub> asphyxiation followed by cervical dislocation 48 h after the last exercise session in both FTR and VWR. Cecal and colon contents were removed from the animals and dissected longitudinally. Fecal samples were collected directly from the distal colon at <2 cm from the rectum, snap frozen by liquid N<sub>2</sub>, and stored at -80°C. Cecal contents were collected directly from ceca, snap frozen, and stored at -80°C.

**DNA isolation, preparation, and sequencing.** Fecal and cecal content DNA was extracted and isolated using a Powersoil kit (MoBio, Carlsbad, CA) according to directions provided by the manufacturer. The V3 and V5 regions of the bacterial 16S rRNA gene were targeted using primers 357F (AATGATACGGCGACCACCGAGATCTA-CACTATGGTAATTGTCCTACGGGAGGCAGCAG) and 926R (CAAGCAGAAGACGGCATAACGAGAT-NNNNNNNNNNNN-AGTCAGTCAGCCCCGTCAATTCMTTTRAGT) with barcodes 1–60 from Caporaso et al. (9). These regions were then amplified via PCR using Kapa HiFi HotStart ReadyMix (Kapa Biosystems, Boston, MA). PCR was performed according to the following cycles: 98°C for 45 s, then 25 cycles of 98°C for 15 s, 65°C for 30 s, 72°C for 30 s; final extension of 72°C for 2 min. Electrophoresis of samples was used to verify amplicon specificity. Samples were then purified using a Qiagen QIAquick PCR Purification kit (Qiagen, Valencia, CA). PCR amplicons were pooled and then diluted to 10 mM for sequencing on a MiSeq (Illumina, San Diego, CA). Paired-end sequencing

with read lengths of 250 base pairs (2 × 250) was completed with the following primers: 357F926R\_read1 (TATGGTAATTGTCCTACGGGAGGCAGCAG), 357F926R\_read2 (AGTCAGTCAGCCCCGTCAATTCMTTTRAGT), and 357F926R\_index (ACTYAAAKGAATTGACGGGGCTGACTGACT).

**Sequencing outcome.** After quality assessment whereby reads with fewer than 187 bp in both R1 and R2 were discarded, ~7,987,231 total sequence reads with a mean of 137,710 reads/sample were deemed suitable for analysis. One cecal sample from the SED group was exempted from analysis because of a very low number of reads (21). After these steps, the range of sequences/sample was 16,081 to 1,110,585.

**Sequence analysis and statistics.** Sequence reads were aligned using the Illinois-Mayo Taxon Operations for RNA Dataset Organization (IM-TORNADO), a custom alignment tool designed by Jeraldo et al. (24a). IM-TORNADO merges paired end reads into a single multiple alignment, obtains taxa calls, and clusters sequences into operational taxonomic units (OTUs) using AbundantOTU+ (43, 50). Further analysis and visualization were performed using Quantitative Insights into Microbial Ecology (QIIME) version 1.8.0 (8). Two classifications of bacterial diversity are termed  $\alpha$  and  $\beta$ . On the basis of  $\alpha$ -diversity rarefaction plots (not shown), we used the minimum sequences/sample (16,081) as our sequencing depth for all analyses comparing cecal contents and feces. For comparing exercise interventions within each GI area, the lowest number of sequences per sample at each respective intestinal site (77,984; fecal) and (16,081; cecal contents) was used for sequencing depths. Analysis of  $\alpha$ -diversity (i.e., Chao1 and Shannon index) and taxonomy (i.e., phyla, OTUs) was performed by a one-way ANOVA or a nonparametric Kruskal-Wallis test. Kruskal-Wallis tests were used in place of ANOVA if variances were found to be unequal between groups. All variances were assessed through a Brown-Forsythe equality-of-variances test. Multiple comparisons of taxonomy were corrected by the Benjamini and Hochberg false discovery rate (FDR) correction factor at  $\alpha = 0.05$  (4). Correlations of taxa between the cecum and feces were carried out by Spearman rank correlation coefficient (Spearman Rho). Community structure ( $\beta$ -diversity) of the weighted and unweighted UniFrac distance metrics was generated from QIIME, visualized using *EMPeror*, and analyzed by permutational multivariate analysis of variance (PERMANOVA) (34, 48). The  $\alpha$  (statistical) was set a priori at 0.05 for all tests of significance.

## RESULTS

**Body weight changes and running distances.** Body weight change (%) was similar among groups ( $P > 0.05$ ) in response to the interventions ( $9.50 \pm 2.90$ ,  $8.00 \pm 1.90$ , and  $6.25 \pm 2.20$  for SED, VWR, and FTR, respectively). The VWR group averaged  $5,836 \pm 132$  m/night (means  $\pm$  SE) (range: 5,123–7,205 m/night) over the 30-day period.

**Differences in microbial community structure between feces and cecal contents.** PERMANOVA of the weighted UniFrac distance metric displayed that vastly different bacterial community structures existed between the feces and the cecal contents ( $P < 0.01$ ). Visualization of these communities via principle coordinate analysis (PCoA) displays site-specific clustering of these bacterial communities (Fig. 1).

**VWR and FTR differentially alter richness and evenness of gut bacterial populations.** The  $\alpha$ -diversity metric, Chao1, revealed differences in species richness among the three groups (SED, FTR, VWR) in both the feces (Kruskal-Wallis = 5.82,  $P < 0.05$ ) and the cecal contents (Kruskal-Wallis = 9.870,  $P < 0.01$ ) (Fig. 2, A and B). Contrary to our hypothesis, VWR exhibited the lowest species richness of the three groups in both areas of the GI tract. However, the Shannon index, a

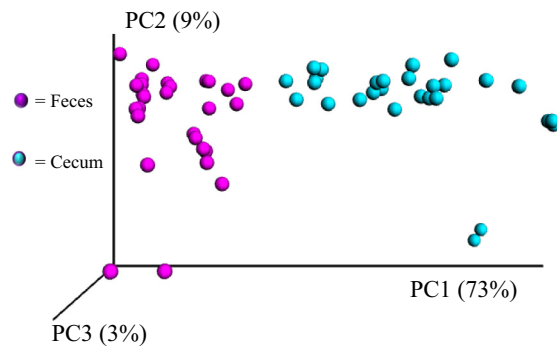


Fig. 1. Three-dimensional principle coordinate (PC) analysis (PCoA) based on the weighted UniFrac distance of all sequenced samples. Clustering reveals significant differences in community structure between feces and the cecal contents (PERMANOVA,  $P < 0.01$ ). Axes represent percentage of data explained by each coordinate dimension.

measurement of community richness and evenness, depicts a different view of  $\alpha$ -diversity. Contrary to the Chao1 index, the groups did not differ when measured by the Shannon index in the feces ( $F_{2,26}$ : 2.11;  $P = 0.141$ ) (Fig. 2C), whereas VWR trended higher vs. SED and FTR in cecal contents ( $F_{2,25}$ : 2.91;  $P = 0.07$ ) (Fig. 2D).

VWR and FTR differentially regulate community structure of the gut microbiome. PCoA based on weighted (Fig. 3A) and unweighted UniFrac (Fig. 3B) distance metrics revealed that exercise interventions differentially altered the community structure in the feces and the cecum (PERMANOVA,  $P < 0.05$ ). Notably, however, the two phylogenetic distance metrics (unweighted vs. weighted) reveal distinctive views of community structure. The unweighted UniFrac exposes

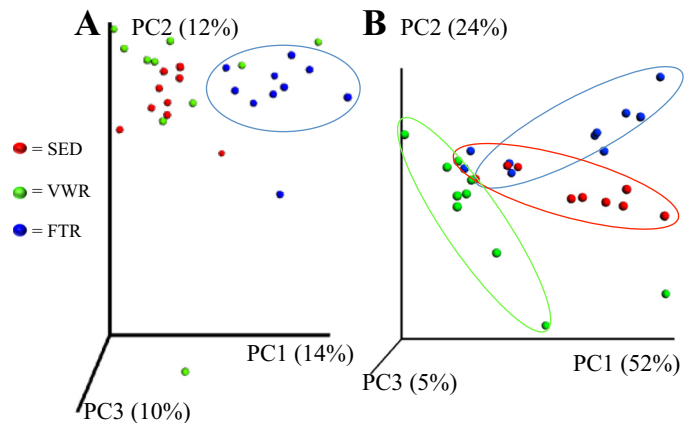


Fig. 3. PCoA based on unweighted UniFrac (A) and weighted UniFrac (B) distance metrics within the feces of mice. Clustering reveals significant differences between groups (PERMANOVA,  $P < 0.05$ ). Axes represent percentage of data explained by each coordinate dimension.  $\beta$ -Diversity determines the phylogenetic distance shared between samples and effectively measures the number of distinct communities within a given region. The ovals in the figure do not represent any statistical significance but rather serve a visual guide to group differences.

clustering of the FTR group. Conversely, PCoA of the weighted UniFrac reveals distinct clusters representing all three groups (FTR, VWR, and SED). A similar phenomenon is also evident when measuring the community structure of the cecal contents. Here, the unweighted UniFrac again reveals clustering of the FTR group (Fig. 4A), whereas the weighted UniFrac displays clustering of the VWR group but not SED and FTR. (Fig. 4B).

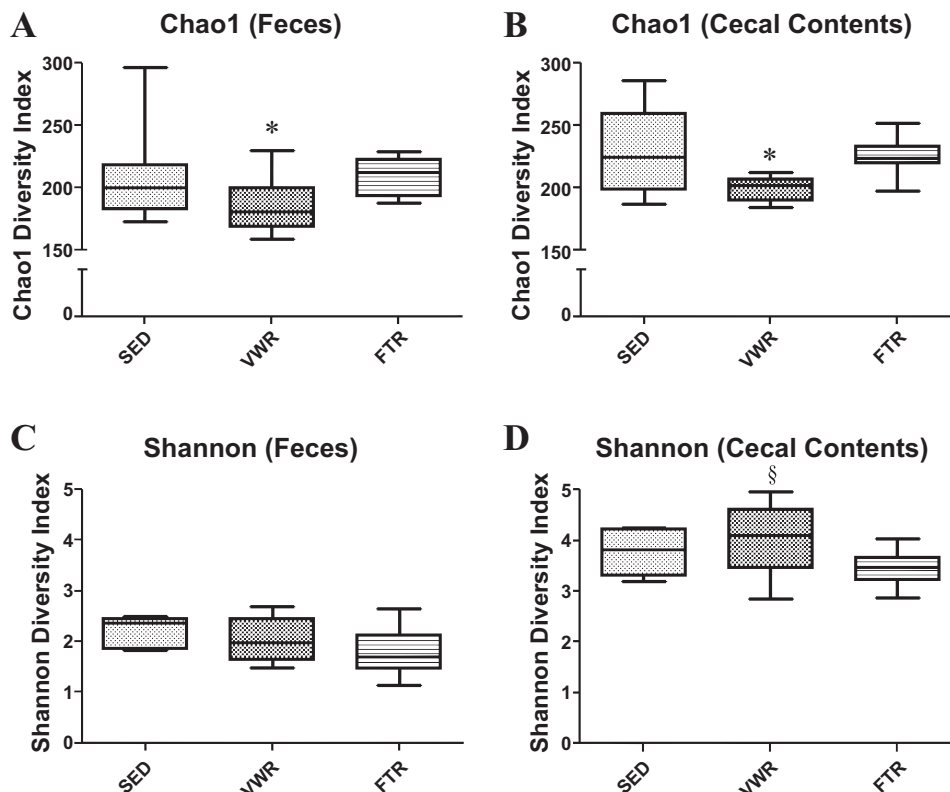


Fig. 2.  $\alpha$ -Diversity analysis of bacterial communities in the feces (A and C) and cecal contents (B and D) of mice. The richness metric, Chao 1, reveals a significant reduction (\*) in species richness in the voluntary wheel running (VWR) group vs. sedentary control (SED)/forced treadmill running (FTR) (Dunn's post hoc) at both intestinal sites. A: feces: Kruskal Wallis = 5.82,  $P < 0.05$ . B: cecum: Kruskal Wallis = 9.870,  $P < 0.01$ . Meanwhile, measurements of community richness and evenness by the Shannon index reveal no difference in diversity. C: feces:  $F_{2,26} = 2.11$ ,  $P = 0.14$ . D: cecum:  $F_{2,25} = 2.910$ ,  $P = 0.07$ ;  $\$0.05 < P < 0.10$  vs. FTR.  $\alpha$ -Diversity determines the richness and evenness within bacterial populations.



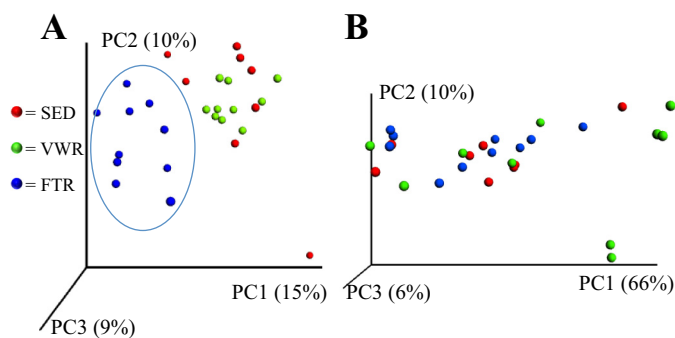


Fig. 4. PCoA based on unweighted (A) and weighted (B) UniFrac distance metrics of the cecal contents. Clustering reveals significant differences between groups (PERMANOVA,  $P < 0.05$ ). Axes represent percentage of data explained by each coordinate dimension. The ovals in the figure do not represent any statistical significance but rather serve a visual guide to group differences.

VWR and FTR alter multiple phyla, genera, and OTUs within both areas of the GI tract. The two major phyla of bacteria in the GI tract (Bacteroidetes and Firmicutes) were unaltered by exercise training at both intestinal sites. However, Tenericutes and Proteobacteria, two less abundant bacteria phyla, were elevated by FTR vs. SED/VWR in the feces (FDR,  $P < 0.05$ ) (Fig. 5). A total of eight bacterial genera were altered by some form of exercise training (FDR,  $P < 0.05$ ). Of these genera, three were altered at both intestinal sites (*Dorea*, *Turicibacter*, and *Anaerotruncus*). Table 1 displays the relative abundance vs. sedentary controls at the end of the exercise interventions. A total of 21 operational taxonomic units (OTUs) were altered by exercise training in the cecum, and five OTUs were altered in the feces (FDR,  $P < 0.05$ ). Table 2 displays the OTUs altered by exercise training.

Bacterial genera altered by exercise correlate between sequenced regions of the GI tract. Exercise alters specific bacterial genera, such that the relative abundance of these taxa are significantly correlated between intestinal areas. Figure 6 displays a strong relationship of two genera (*Turicibacter* and *Anaerotruncus*) between the feces and cecum, both of which were significantly altered by VWR.

## DISCUSSION

This study examined the effects of two common exercise modalities, FTR and VWR, on the GI microbiome in C57BL/6J mice. The novel findings of this study are manifold. 1) Exercise modalities differentially altered  $\alpha$ -diversity, as

measured by Chao1 index, implying that forced and voluntary exercise have contrasting effects on bacterial community richness. 2) PCoA, based on both weighted and unweighted UniFrac distance metrics, revealed that exercise modalities differentially altered bacterial community structure. 3) The distinct clustering of the FTR group, visible through the PCoA in the unweighted UniFrac, but not the weighted UniFrac, indicated that less abundant bacterial species may be evident in the bacterial communities of these FTR mice. 4) Many bacterial taxa, measured at the phyla, genus, and OTU level, were differentially altered by the exercise-training modalities, some of which may be related to intestinal immune function and implicated in inflammatory bowel diseases. 5) A portion of bacterial genera were similarly affected in both intestinal regions (feces and cecum) despite different community structures of these intestinal sites.

$\alpha$ -Diversity metric reveals differences in how exercise modalities altered the richness and the evenness within each bacterial community. In this study, analysis by the Chao1 index revealed that mice that voluntarily exercised had, on average, reduced community richness compared with mice that remained sedentary or were forced to exercise. These results were initially perplexing, as lower richness of bacterial populations has been associated with disease states, including IBD in humans and DSS colitis in mice (11, 38). Thus the lower richness of the VWR group did not match with the beneficial effects of VWR in the large intestine, which has been observed in our previous study examining UC (15), as well as other studies investigating exercise and the microbiome (15, 20, 36). However, a recent study showed that metformin, an antidiabetic drug, also reduced the  $\alpha$ -diversity (richness) of the gut microbiome when given to mice fed high-fat diets (31). Metformin, like exercise, activates intracellular AMP kinase in many cell types, improves skeletal muscle insulin sensitivity, and improves disorders related to metabolic regulation in mice and humans (16). Such similarities indicate that the changes in microbiome diversity may be related to the anti-inflammatory and antidiabetic actions of these two treatments. Despite these resemblances, however, it is still unclear why the two exercise modalities differentially alter  $\alpha$ -diversity.

Unlike the Chao1 index, which measures community richness, the Shannon index is an  $\alpha$ -diversity index that measures both community richness and evenness. Interestingly, unlike Chao1, diversity trended higher in the VWR compared with SED and FTR when analyzed by the Shannon index (Fig. 2D). Therefore, these data imply that, despite the lower richness of

Fig. 5. Relative abundance (as measured by percentage of total bacteria) of 2 bacteria phyla, Tenericutes (A) and Proteobacteria (B). \*False discovery rate (FDR),  $P < 0.05$ .

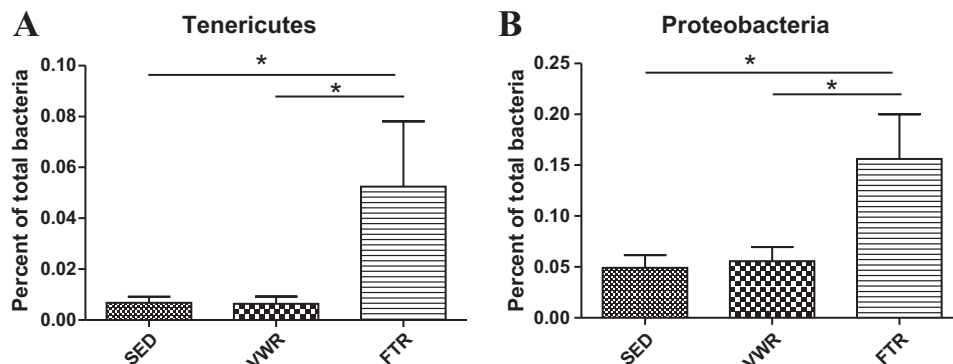


Table 1. Genera (% of total representation) significantly altered by 6 wk of exercise training

Genus	Location(s)	Response vs. SED	Group	% of Total Representation Feces	% of Total Representation Cecum	False Discovery Rate
<i>Dorea</i>	Cecum and Feces	↑	FTR	SED: 0.02 FTR: 0.32 VWR: 0.08	SED: 0.10 FTR: 0.69 VWR: 0.14	*
<i>Turicibacter</i>	Cecum and Feces	↓	VWR	SED: 0.17 FTR: 0.26 VWR: 0.03	SED: 0.22 FTR: 0.16 VWR: 0.00	†
<i>Anaerotruncus</i>	Cecum and Feces	↑	VWR	SED: 0.01 FTR: 0.00 VWR: 0.46	SED: 0.07 FTR: 0.05 VWR: 0.85	*
<i>Moryella</i>	Cecum	↑	FTR & VWR	Not detected	SED: 1.43 FTR: 0.92 VWR: 0.80	*
<i>Nautilia</i>	Feces	↓	FTR	SED: 0.02 FTR: 0.52 VWR: 0.05	SED: 0.05 FTR: 0.12 VWR: 0.02	†
<i>Prevotella</i>	Feces	↓	VWR	SED: 0.02 FTR: 0.08 VWR: 0.00	Not detected	*
<i>Coprococcus</i>	Feces	↑	FTR	SED: 3.35 FTR: 4.95 VWR: 1.96	SED: 13.31 FTR: 20.37 VWR: 11.85	*
<i>Oscillospira</i>	Feces	↑	FTR	SED: 1.52 FTR: 2.27 VWR: 1.14	SED: 5.13 FTR: 6.80 VWR: 5.58	*

\*False discovery rate (FDR)  $P < 0.05$ . †FDR  $P < 0.01$  vs. sedentary control (SED). FTR, forced treadmill running; VWR, voluntary wheel running.

the microbial communities in the VWR group (Chao1), the VWR bacterial populations are more evenly distributed compared with FTR/SED (Shannon). These data also suggest that different exercise modalities (FTR and VWR) have unique roles in shaping bacterial community diversity.  $\beta$ -Diversity analysis, meanwhile, revealed differences in how exercise modalities shaped the community structure of the microbiome. Briefly,  $\beta$ -diversity determines how much phylogenetic distance is shared between samples and effectively measures the “number of distinct communities” within a given region (25). In this study, the most striking difference, revealed by PCoA, was the distinct clustering of the FTR groups at both intestinal sites, as measured by the unweighted UniFrac distance metric (Figs. 3A and 4B). This is in contrast to the weighted UniFrac metrics, which do not display distinct clustering of the FTR group (Figs. 3B and 4B). It is important to note that the unweighted UniFrac measures the presence and absence of particular bacteria taxa and is a more qualitative measure of  $\beta$ -diversity. Therefore, the unweighted UniFrac is more sensitive to rare species within a bacterial community than a similar

analysis performed with a weighted UniFrac (12). As such, the distinct clustering of the FTR group by the unweighted metric may indicate that rare bacteria are altering the community structure of this group. This assertion is also supported by the previously discussed  $\alpha$ -diversity analysis, in which community richness trended higher (Chao1), but evenness trended lower (Shannon) in the FTR group vs. SED controls. Together these separate, yet interrelated, diversity analyses may indicate an expansion of rare bacterial species within the large intestine of the FTR mice.

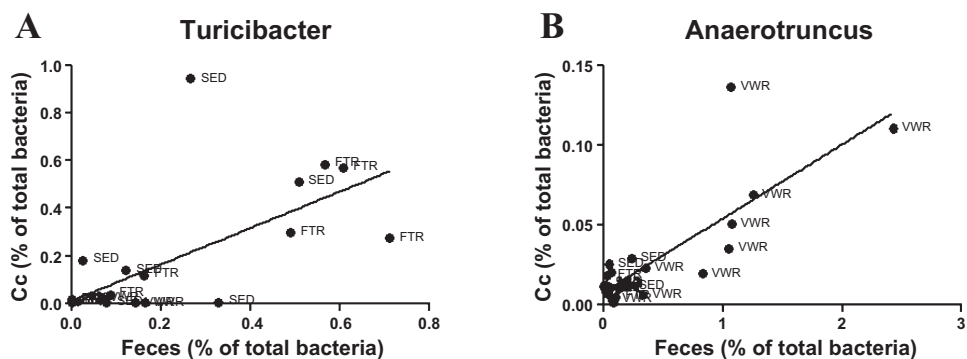
Meanwhile, the weighted UniFrac, a distance metric more sensitive to abundant lineages, provides a very different view of microbial community structures (12). Here, clustering of SED and VWR bacterial communities is more evident (Figs. 3B and 4B). This is perhaps best exemplified by distinct clustering of all intervention groups in the feces (SED, VWR, and FTR) (Fig. 4B). Regardless of the analytic tool, these data suggest that microbial communities are differentially altered by exercise modalities at both intestinal sites. Meanwhile, the distance metrics used to analyze the data (weighted and un-

Table 2. OTUs significantly altered by exercise training

OTU No.	Classification (family: f_;genus: g_;species: s_)	Intestinal Site	Fold Difference vs. SED	Group
18	f_Ruminococcaceae;g_unclassified;s_unclassified	Cecal contents	↑	10.18
28	g_Oscillospira;s_unclassified	Cecal contents	↑	1.82
48	g_Ruminococcus;s_Ruminococcus_gnavus	Cecal contents	↑	332.67
50	g_Coprococcus;s_unclassified	Cecal contents	↑	2.29
54	g_Coprococcus;s_unclassified	Feces	↑	32.45
70	g_Ruminococcus;s_Ruminococcus_gnavus	Cecal contents	↑	12.20
77	g_Oscillospira;s_unclassified	Cecal contents	↑	4.94
92	g_Ruminococcus;s_Ruminococcus_gnavus	Cecal contents	↑	257.7
111	g_Turicibacter;s_unclassified	Cecal contents	↓	43.65
113	g_Butyrvibrio;s_unclassified	Cecal contents	↑	11.60
126	g_Dorea;s_unclassified	Cecal contents	↑	27.67
130	g_Oscillospira;s_unclassified	Feces	↑	7.15
133	g_Ruminococcus;s_unclassified	Feces	↑	24.22
141	g_Coprococcus;s_unclassified	Cecal contents	↓	8.51, 2.59
142	g_Coprococcus;s_unclassified	Cecal contents	↓	63.92
153	g_Ruminococcus;s_Ruminococcus_gnavus	Feces	↑	359.20
156	g_Butyrvibrio;s_unclassified	Feces	↑	93.75
168	g_Nautilia;s_unclassified	Feces	↑	12.32
175	f_Mogibacteriaceae;g_unclassified;s_unclassified	Cecal contents	↑	6.85
184	g_Clostridium;s_unclassified	Cecal contents	↑	8.00
188	g_Clostridium;s_unclassified	Cecal contents	↑	3.78
219	f_Ruminococcaceae;g_unclassified;s_unclassified	Cecal contents	↑	8.14
233	g_Ruminococcus;s_unclassified	Cecal contents	↑	189.12
293	g_Oscillospira;s_unclassified	Cecal contents	↑	13.82
389	g_Coprococcus;s_unclassified	Cecal contents	↑	83.33, 141.87
477	g_Coprococcus;s_unclassified	Cecal contents	↑	28.60

Fold difference is based off of average number of operational taxonomic unit (OTU) reads per group vs. SED controls. OTUs are significantly different at FDR  $P < 0.05$ .

Fig. 6. Genus *Turicibacter* (A) and genus *Anaerotruncus* (B) relative abundance (% of total bacteria) significantly correlate between the two intestinal regions (feces vs. cecal contents, Cc) *Turicibacter*: Spearman Rho = 0.735;  $P < 0.01$ . *Anaerotruncus*: Spearman Rho = 0.453;  $P < 0.05$ .



weighted UniFrac) produced different visual representations of the microbiome community structures.

Contrary to another study examining exercise and the gut microbiome (20), this study revealed no differences in the two main bacteria phyla, Bacteroidetes and Firmicutes, by either of the training modalities. However, we did observe that two less abundant bacterial phyla, Tenericutes and Proteobacteria, were elevated in the feces of the FTR group. The bacteria that represent these phyla contain a Gram-negative cell wall and thus present a primary endotoxin, lipopolysaccharide, to gut epithelial cells and immune cells. An FTR-induced increase in these phyla may imply some form of pathogenesis (or risk thereof) within the GI tracts of FTR mice (10). Intriguingly, almost all of the Proteobacteria were represented by one family, Epsilonproteobacteria and one genus, *Nautilia* spp. Importantly, *Nautilia* spp. are closely related to other genera of Epsilonproteobacteria, such as *Helicobacter* spp. and *Campylobacter* spp., which have well established pathogenic roles in mammalian GI tracts (6). However, it is important to recognize that certain taxa of Epsilonproteobacteria have been recognized for other unique metabolic processes, which are not necessarily detrimental to the gut (6). Meanwhile, Tenericutes was represented by a single family, Mollicutes, which has been shown to increase with high-fat diets in mice and has also been associated with UC in humans (31, 49). On the contrary, another study has shown that phylum Tenericutes was significantly reduced in mice with colitis, possibly indicating an important role for this phylum in maintaining GI homeostasis (38). As such, the diverse functions and environmental responses that accompany these bacterial phyla highlight the complexity in relating changes in high-level taxonomy to host physiological parameters. Nevertheless, how the roles of Proteobacteria and Tenericutes relate to exercise and host physiology should be pursued in future studies.

Both forms of exercise training also altered numerous taxa of bacteria at the genus level. Of the genera altered by exercise, the genus *Turicibacter* was of particular interest. Notably, this particular genus was significantly lower in the VWR group compared with FTR/SED at both intestinal sites (Table 1). Moreover, the abundances of *Turicibacter* significantly correlated between the cecum and the feces (Fig. 6), further suggesting that this taxon may have important roles in GI physiology. The reduction of *Turicibacter* by VWR is similar to what was observed by Evans et al. (20), who also showed a significant decrease in the Turicibacteraceae family as a result of voluntary exercise in the feces of mice. Interestingly, there is also evidence that GI *Turicibacter* populations are reliant on

host immune cells and bacterial sensors for survival. To this point, Dimitriu et al. (18) showed that two immunodeficient mouse models [innate immune deficient (e.g., CD45 phosphatase deficient) and B and T cell deficient (e.g., RAG)] had completely abolished *Turicibacter* populations within the GI tract compared with wild-type, which had populations composed of 12% of total bacteria. Meanwhile, Kellermayer et al. (27) showed a completely abolished *Turicibacter* population in Toll-like receptor-2 knockout mice compared with wild-type mice, again suggesting an interaction between this bacterial population and host immune regulation (27). In addition, *Turicibacter* was strongly associated with the well-known pathogenic bacteria, *Clostridium perfringens*, in the pathogenesis of ileal pouchitis in humans with prior UC (21). In light of these data, it is reasonable to suggest that *Turicibacter* has a unique bidirectional relationship with the mammalian immune system and the pathogenesis of IBD. Thus, the reduction of *Turicibacter* populations by voluntary exercise, now shown by two independent studies, indicates that this bacterial population may be an important focal point for future studies examining exercise and GI health.

Exercise also induced noticeable changes at the OTU level of taxonomic rank, as 21 and 5 OTUs were altered in the cecum and feces, respectively (FDR,  $P < 0.05$ ). Importantly, 23 out of the 26 of these taxa were significantly higher in the FTR group compared with SED, suggesting that forced exercise has robust effects on how these bacteria replicate and survive. These included OTUs that mapped to *Ruminococcus gnavus*, *Butyrivibrio* spp., *Oscillospira* spp., and *Coprococcus* spp. Notably, these four OTUs were altered at both intestinal sites, which indicates that these bacterial species may be distinctly sensitive to the FTR exercise interventions. Of these specified bacteria, *Ruminococcus gnavus* has well defined roles in intestinal mucus degradation and has been directly implicated in IBD (5, 40).

It is important to note that, despite the fact that exercise alters some low-abundance genera and OTUs, it should not discredit the possible physiological significance of these changes. In fact, low-abundance microbes have been proposed to be “microbial seed banks,” which allow community adaptation to environmental changes (32). Moreover, it is also postulated that low-abundance microbes may harbor keystone species, which can have profound effects even at a low abundance (22).

On the basis of these OTU data and the previously discussed diversity analyses, it is our conjecture that FTR serves as a stressful stimulus to mice, thus altering the bacterial structure



within the GI tracts of these animals. FTR as a stressful paradigm is supported by previous data from our laboratory, which showed increased adrenal weights and reduced thymic weights in FTR mice (15). With regard to environmental regulation, other stressful stimuli, such as restraint stress and social disruption, can also alter bacterial ecology within the GI tracts of mice (1, 2). However, these stimuli induce different effects in the diversity and taxonomy of gut bacteria; thus drawing parallels between these stress paradigms is difficult. Moreover, many of the OTUs altered by forced exercise in this study remain unclassified (i.e., *Coprococcus* spp.), and thus the physiological relevance of these taxa is difficult to interpret. Regardless, a better understanding into the regulation of the microbiome by stressful encounters, including FTR, will likely reveal unique microbial-host interactions that are important for GI and systemic health.

In summary, this study provides the first evidence that forced and voluntary exercise induces distinct effects on the gut microbiome in mice. This is supported by different presentations of bacterial community diversity, structure, and taxonomy between the intervention groups. Moreover, these data provide evidence that exercise can alter the microbiome at more than one intestinal site, as changes were observed within the cecum and the feces located in the distal colons of these mice. These microbial changes brought about by exercise may also have implications in the pathogenesis of IBD, colorectal cancer, nutrient absorption, immune function, and host physiology. This is evidenced by differences in particular bacterial taxa between the intervention groups, such as *Turicibacter*, which has bidirectional relationships between host immune function and is also implicated in IBD in humans. We are aware of the overall descriptive nature and the inherent limitations of this study. Notably, this study did not address any transcriptional or protein changes within the bacterial communities; thus we are limited to assessing the genomic community structure of these bacteria populations. Nevertheless, data such as these are useful for hypothesis generation and future testing of interactions between the gut microbiota and the host.

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#### DISCLOSURES

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

#### AUTHOR CONTRIBUTIONS

Author contributions: J.M.A. and J.A.W. conception and design of research; J.M.A., M.E.B.M., and K.W. performed experiments; J.M.A., M.E.B.M., and K.W. analyzed data; J.M.A., M.E.B.M., B.D.P., B.A.W., and J.A.W. interpreted results of experiments; J.M.A. prepared figures; J.M.A. drafted manuscript; J.M.A., M.E.B.M., B.D.P., V.N., H.R.G., B.A.W., J.D.F., and J.A.W. edited and revised manuscript; J.M.A., M.E.B.M., B.D.P., V.N., H.R.G., B.A.W., J.D.F., and J.A.W. approved final version of manuscript.

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