

EXERCISE IS MORE EFFECTIVE AT PRODUCING ROBUST, LASTING CHANGES IN
GUT MICROBIAL COMPOSITION IN JUVENILE THAN IN ADULT MALE F344 RATS

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Exercise is more effective at producing robust, lasting changes in gut microbial composition in juvenile than in adult male F344 rats

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The mammalian intestine harbors a complex microbial ecosystem that affects many aspects of host physiology. Exposure to specific microbes early in development can significantly impact host metabolism, immune function, and behavior across the lifespan. Just as the physiology of the developing organism undergoes a period of plasticity, the developing microbial ecosystem is characterized by instability and may be more sensitive to environmentally evoked change. Early life thus presents a window of opportunity for manipulations that produce adaptive changes in microbial composition. Recent insights have revealed that increasing physical activity status can increase the abundance of some beneficial microbial organisms. We therefore investigated whether exercise initiated in the juvenile period (P24) would produce more robust and stable changes in microbial communities versus exercise initiated in adulthood (P70) in male F344 rats. 16S rRNA gene sequencing was used to characterize the microbial composition of juvenile versus adult runners and their sedentary counterparts across multiple time points during exercise and following exercise cessation. Alpha diversity measures revealed that the microbial communities of young runners were less even and less diverse, a community structure that reflects volatility and malleability. Juvenile onset exercise altered the relative abundance of several phyla, and notably, increased Bacteroidetes and decreased Firmicutes, a configuration associated with leanness. Analyses of bacteria at the genus level using supervised learning approaches along with ANOVA also revealed potential functionally significant changes,

and indicated that juvenile onset exercise dramatically changed the abundance and presence of genera important for metabolism and emotional behavior. Among these genera, juvenile onset exercise increased *Lactobacillus spp.* just three days following exercise onset. A follow up investigation utilizing bacterial culture methods and *Lactobacillus spp.* selective media confirmed these results, and also showed that at the species level, juvenile onset exercise can induce early increases in *L. Rhamnosus*, a species shown to modulate a diverse array of host functions including carbohydrate metabolism and anxiety. Given the potential of these phyla and genus level changes to contribute to a lean phenotype, we chose to examine body composition in juvenile versus adult runners. Interestingly, exercise increased lean mass measured using chemical carcass analyses and *in vivo* MRI, in juvenile but not adult runners. Taken together, these results indicate that the impact of exercise on gut microbial composition as well as body composition depends on the developmental stage during which exercise is initiated. Furthermore, early life exercise produced robust, adaptive changes in bacteria associated with body composition, host metabolism and behavior, suggesting that a microbiome altered by exercise during early life could contribute to life-long improvements in mental and physical health.

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CHAPTER I: INTRODUCTION

The mammalian gut contains an estimated 100 trillion commensal microorganisms that have collectively evolved to enhance host physiology (Eckburg et al., 2005). A large body of work decisively demonstrates that these microorganisms are critical for the development and function of many physiological systems. Studies comparing germ-free mice (GF mice; mice bred in sterile conditions and lacking gut microbiota) to conventional mice have revealed that bacterial colonization of the intestine is important for nutrient synthesis and uptake (Conly, Stein, Worobetz, & Rutledge-Harding, 1994; Hill, 1997; Sommer & Backhed, 2013), immune regulation/tolerance (Hrncir, Stepankova, Kozakova, Hudcovic, & Tlaskalova-Hogenova, 2008; Tlaskalova-Hogenova et al., 2004), the development of primary and secondary lymphoid tissues (Hooper, Stappenbeck, Hong, & Gordon, 2003; Ohara et al., 2000), and an intact gastrointestinal barrier (Berg & Garlington, 1979).

In addition to the importance of an intact gut microbiota, the overall phylogenetic composition and the presence of specific species can confer benefits on host health. At the phylum level, there is evidence by some (Backhed et al., 2004; Ridaura et al., 2013; Turnbaugh et al., 2009; Turnbaugh et al., 2006) but not all (Duncan et al., 2008; Schwartz et al., 2010) researchers that an increased Bacteroidetes to Firmicutes ratio is linked to a lean phenotype (Backhed et al., 2004; Ridaura et al., 2013; Turnbaugh et al., 2009; Turnbaugh et al., 2006) and increases in the production of short-chain fatty acids (SCFAs) that promote energy expenditure. At the species level, some *Bacteroides spp.* (Round & Mazmanian, 2010) and indigenous *Clostridium spp.* (Atarashi et al., 2011) can facilitate T regulatory cell differentiation and induce anti-inflammatory immune responses. Recently, evidence has revealed that specific microorganisms can even influence brain plasticity and emotional behavior. For example,

Bifidobacteria and *Lactobacillus spp.* can attenuate anxiety and depressive-like behavior in rodents (Bravo et al., 2011; Messaoudi et al., 2011), as well as humans (Rao et al., 2009), and produce adaptations within brain regions regulating emotional behavior (Bravo et al., 2011). Thus, given that specific microorganisms at both the phyla and species levels can promote aspects of host health, the microbial composition of the intact gut is important to consider.

Interestingly, recent research demonstrates that the impact of the gut microbiota on host physiology can be age dependent. Studies using GF mouse models have revealed an early sensitive period during which the absence of an intact gut microbiota results in irreversible behavioral and physiological consequences. For instance, exaggerated HPA-responses exhibited by GF mice only can be partially normalized with *Bifidobacteria infantis* if given at 6 weeks but not 8 weeks of life (Sudo et al., 2004). Similarly, GF mice have altered anxiety behavior in the elevated plus-maze that can be normalized by exposure to microbial populations from conventionally raised mice if inoculation early but not late in life (Diaz Heijtz et al., 2011). Early exposure to certain microorganisms can also program the immune system. For example, inoculation with *Bifidobacteria spp.* produced oral tolerance in GF mice only if administered in early life (Sudo et al., 1997). In addition to the physiological consequences of conventionalizing GF mice in early life, evidence from humans suggests that bacterial community structure and composition during development can have long-lasting phenotypic impacts. For example, one study in humans revealed that infant microbial composition was predictive of childhood obesity (Kalliomaki, Collado, Salminen, & Isolauri, 2008). These findings reveal that the presence and composition of the gut microbiota during early life can program host health throughout the lifespan.

Physiological systems of the developing organism are highly malleable and sensitive to change, and the gut microbial community is similarly more plastic and volatile early in life (Koenig et al., 2011; O'Toole & Claesson, 2010; Palmer, Bik, DiGiulio, Relman, & Brown, 2007; Wall et al., 2009; Yatsunenکو et al., 2012). To our knowledge, the most comprehensive study to date investigating microbial diversity across age included samples from infants, children, adolescents, and adults and demonstrated that interpersonal variation in microbial composition was significantly greater in children versus adults (Yatsunenکو et al., 2012). Importantly, this work also revealed that bacterial diversity increased across age. It has been suggested that the increased stability and complexity of the adult microbiota provides resistance against long-term changes in composition (Wall et al., 2009). The early life microbial ecosystem therefore may be more sensitive to environmental change because it is less stable and diverse than the adult microbiota. Thus, environmental manipulations that produce adaptive changes in community structure could potentially have a greater and more lasting impact on the microbiota if implemented in early life.

Exercise is one such environmental manipulation capable of changing gut microbial composition in a manner that could potentially benefit the host. Indeed, previous studies have shown that exercise can increase specific bacterial taxa that have been linked to adaptive changes in metabolism, body composition and behavior. For instance, six days of wheel running exercise increased *Bifidobacterium* and *Lactobacillus spp.* (Queipo-Ortuno et al., 2013), two bacteria associated with positive mental and physical health outcomes. Another group reported that wheel running significantly altered overall microbial composition and increased n-butyrate concentrations (Matsumoto et al., 2008). This particular SCFA is capable of increasing host energy expenditure (Gao et al., 2009) and conferring protection against pathological conditions

such as inflammatory bowel disease (Perrin et al., 2001). Notably, in another study, wheel running prevented high-fat diet associated weight gain and produced a microbial composition similar to lean mice (Evans et al., 2014). Although some evidence suggests that exercise can adaptively alter the gut microbial composition, no research to date has considered the developmental stage of exercise initiation, nor investigated the stability of these exercise-induced changes across the lifespan. Given the state of plasticity of the developing gut and the therapeutic potential of manipulating bacterial composition in early life, it is important to investigate whether exercise-induced changes in composition are greater and more stable if exercise is initiated earlier in development.

To explore this, adult (PND 70) and juvenile (PND 24) male F344 rats were housed in standard cages with or without running wheels for six weeks. Wheel running in rodents is rewarding (Greenwood et al., 2011) and produces a plethora of beneficial effects including increased endurance (Mann et al., 2010), decreased visceral adiposity (Speaker et al., 2014), and increased stress robustness (Greenwood & Fleshner, 2008; Greenwood, Foley, Burhans, Maier, & Fleshner, 2005; Greenwood et al., 2003; Greenwood, Loughridge, Sadaoui, Christianson, & Fleshner, 2012). Additionally, wheel running is a natural behavior as rodents in the wild will choose to run on wheels if given access (Meijer & Robbers, 2014).

In the present study, gut microbial composition was assessed using 16S rRNA gene sequencing in fecal samples from juvenile and adult runners and their sedentary counterparts. Samples were collected after three days and six weeks of wheel running, and 25 days after running had stopped. 16S rRNA gene sequencing provides a comprehensive measure of microbial ecology without the bias of traditional culture methods. To assess possible physiological consequences associated with a microbial configuration altered by early life

exercise, long lasting adaptations in body composition were also investigated in separate cohorts of rats using chemical carcass composition analysis and *in vivo* MRI. We hypothesize that exercise initiated during the juvenile period will produce more robust and stable changes in gut microbial composition than exercise initiated in adulthood, and that these changes will impact bacteria associated with adaptive changes in body composition and emotional stress robustness.

CHAPTER II: MATERIALS AND METHODS

i. Subjects and Housing

Juvenile, PND 24 (postnatal day; n=18) and adult, PND 70 (n=18), male F344 rats (Harlan, Indiana) were housed in a temperature (22 °C) and humidity controlled environment and maintained on a 12:12-hr light: dark cycle. All rats were pair housed in Nalgene Plexiglas cages (45 x 25.2 x 14.7 cm). Pair housing was necessary in these experiments due to the stressful nature of single housing juveniles (Takemoto, Suzuki, & Miyama, 1975). Care was taken to minimize discomfort during all procedures, and all experimental protocols were approved by the University of Colorado Animal Care and Use Committee. All rats were weighed weekly, and had *ad libitum* access to food and water.

ii. Voluntary Exercise

Immediately upon arrival, juvenile and adult rats were randomly assigned to either remain sedentary in standard cages (Juvenile sed; n=9/Adult sed; n=9) or were housed in standard cages equipped with running wheels and allowed voluntary wheel access for six weeks (Juvenile run; n=9/Adult run; n=9). Following six weeks of wheel access, wheels were rendered immobile with metal stakes for 25 days (Figure 1). Daily wheel revolutions were recorded using Vital View software (Mini Mitter, Bend, OR, USA) and running distance was calculated by multiplying the number of wheel revolutions by circumference of the wheel (1.081 m). Running distance data are represented as weekly totals. Since rats were pair-housed, values for individual rats were estimated by dividing the total weekly distance by two.

iii. Fecal Sample Collection

Fecal samples were collected from each animal at three different time points: following three days of exercise, following six weeks of exercise, and 25 days after wheels were locked (Figure 1). On each of the sample collection days, at approximately 0900 hours, each rat was placed into a sterile Nalgene Plexiglas cage devoid of bedding. Exposure to a novel environment has been shown to induce defecation in rats (Restrepo & Armario, 1987). Following defecation, samples were obtained with sterilized forceps and placed into 1.5mL sterile, screw cap tubes (USA Scientific, FL), and immediately placed on ice. Forceps were sterilized with 100% ethanol between samples. Immediately following sample collection, rats were placed back into their home cages and samples were frozen at -80°C until later processing.

FIGURE 1: EXPERIMENTAL TIMELINE

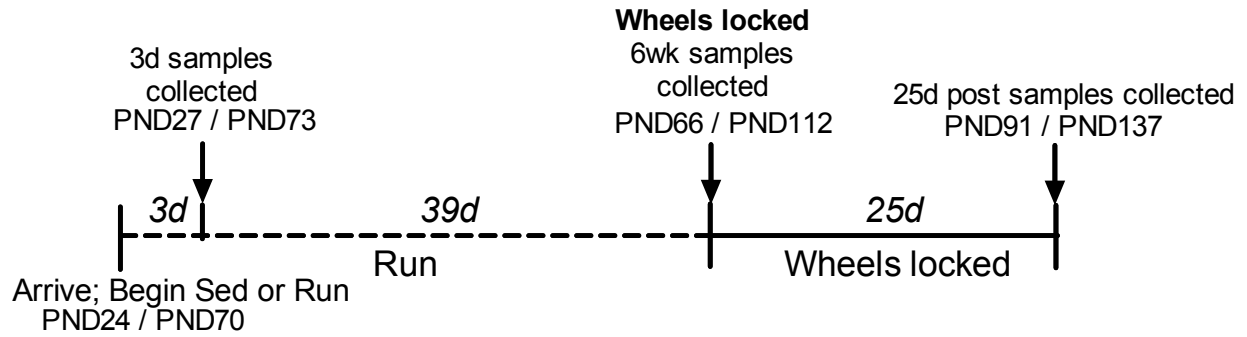


Figure 1 depicts the experimental timeline. Juvenile and adult male F344 rats were either given access to voluntary running wheels immediately upon arrival, or were housed in sedentary cages. Following the six weeks of exercise, wheels were rendered immobile for 25 days. Fecal samples were collected three days and six weeks following the start of exercise, as well as 25 days after exercise cessation.

iv. 16s rRNA Gene Sequencing and Microbial Composition Analysis

Samples were prepared for sequencing using established protocols (Caporaso, Lauber, Costello, et al., 2011; Maslanik et al., 2012). After sample preparation, variable region 4 (V4) of 16S rRNA genes present in each sample was PCR-amplified with forward and reverse primers (F515/R806). The reverse primer is barcoded with an error-correcting 12-base Golay code to facilitate demultiplexing of up to 1,500 samples (Caporaso, Lauber, Walters, et al., 2011). Following purification and precipitation to remove PCR artifacts, samples were subjected to multiple sequencing on an Illumina Genome Analyzer Iix. Operational taxonomic units (OTUs) were picked using a ‘closed reference’ approach (Navas-Molina et al., 2013). In brief, this approach takes sequenced reads and compares them to a reference database. A sequence is considered a ‘hit’ if it matches something in the reference database at greater than 97% sequence identity. If an experimental sequence failed to match any member of the reference collection, it was discarded. Closed-reference picking is preferable to ‘de-novo’ or ‘open-reference’ picking in well characterized rat gut communities because the curated reference database acts as a filter; low quality or noisy sequences which get past the quality control steps, but do not actually represent novel OTUs, are eliminated. GreenGenes May 2013 version was the reference database used (McDonald et al., 2012), and all sequence processing was done with QIIME v 1.8.0 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010). Taxonomy and phylogeny were taken from the GreenGenes reference collection. The current experiment generated 5,787,335 sequences, of which 1,132,569 were discarded because of uncorrectable barcode errors, low quality, or for being too short (using the default parameters in the QIIME script ‘split_libraries_fastq.py’). The remaining 4,654,766 sequences of median length 151 nucleotides were clustered. The resulting OTU table was rarefied at 8468 sequences/sample to correct for

uneven sequencing depth due to amplification differences between samples. Rarefaction is a conservative approach that normalizes library size to prevent type I errors in a variety of techniques applied by QIIME. Recent literature has questioned the ‘statistical admissibility’ of rarefaction (McMurdie & Holmes, 2014) in the context of differential abundance testing (e.g. ANOVA), but provide a superior method for only the basic two-way comparison. PCoA, supervised learning, and other methods perform poorly without rarefaction when sequencing depth differs between samples. To check that our selected rarefaction depth was not responsible for erroneous conclusions, these data were also rarefied at higher levels to check that patterns were not artifacts of low sequence coverage. PCoA visualizations were done using the Emperor software package (Vazquez-Baeza, Pirrung, Gonzalez, & Knight, 2013).

Final group sizes at each time point are as follows: Adult sed at 3 days (n=9), 6 weeks (n=7), and 25 days (n=5); Adult run at 3 days (n=9), 6 weeks (n=9), and 25 days (n=6); Juvenile sed at 3 days (n=9), 6 weeks (n=9), and 25 days (n=6); Juvenile run at 3 days (n=9), 6 weeks (n=9), and 25 days (n=6). Rats were excluded from sequencing/ subsequent analyses if insufficient fecal materials were obtained, or if a particular sample yielded less than 8468 sequences (see rarefaction description above).

FIGURE 2A: SELECTIVE *LACTOBACILLUS SPP.* ANALYSIS EXPERIMENTAL

TIMELINE

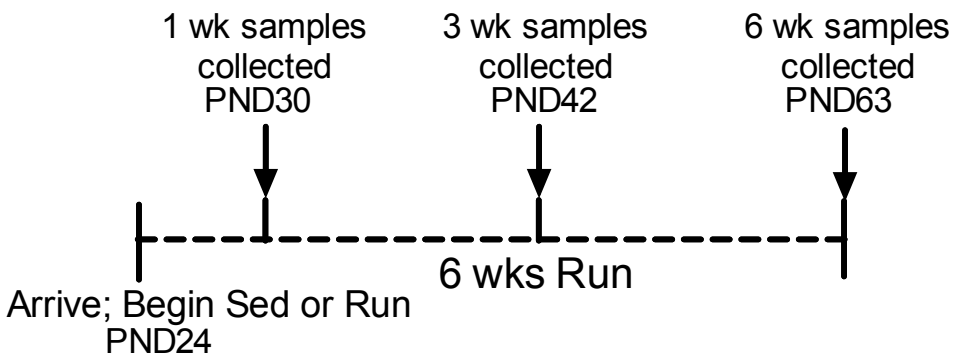


Figure 2A depicts the experimental timeline for the examination of *Lactobacillus spp.* using M-RTL V agar. Fecal samples were collected from a separate cohort of juvenile runners and seds over the course of 6 weeks of exercise. Samples were specifically collected following one, three, and six weeks of exercise and plated on Lactobacillus-specific M-RTL V agar.

FIGURE 2B: M-RTL V AGAR

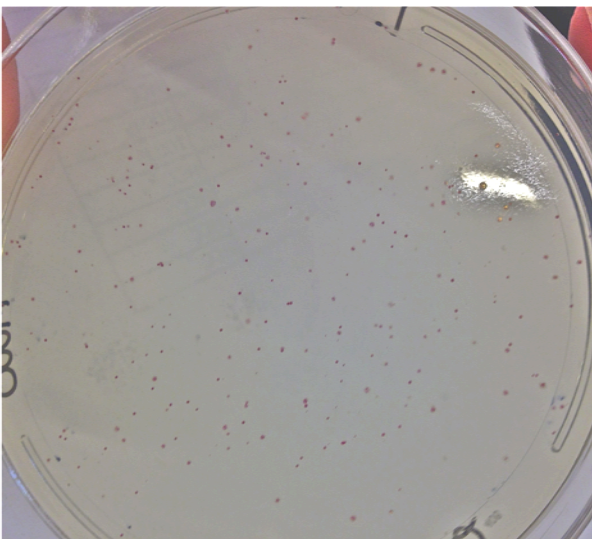
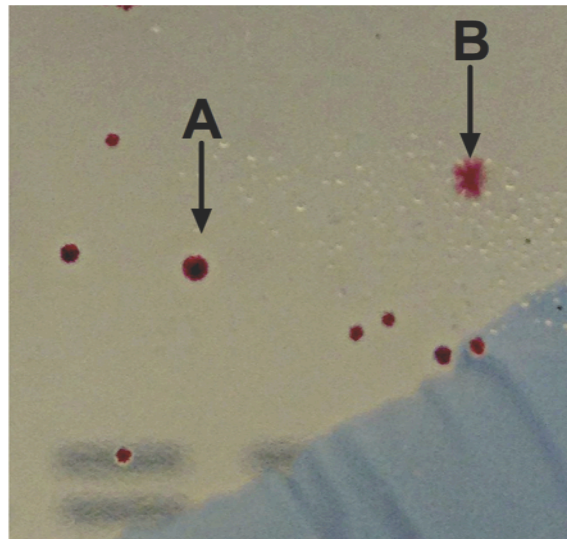
FIGURE 2C: *L. RHAMNOSUS*

Figure 2B depicts a representative M-RTL V agar plate growing distinguishable colonies of *Lactobacillus spp.* Figure 2C depicts a zoomed in M-RTL V plate growing colonies of *Lactobacillus spp.* other than *L. rhamnosus*. (A) along with clearly distinguishable colonies of *L. rhamnosus* (B).

v. Quantification of *Lactobacillus spp.* using M-RTL V agar

To verify the early increases in *Lactobacillus spp.* observed with 16S, fecal samples were collected from a separate cohort of juvenile runners and seds over the course of 6 weeks of exercise (Figure 2A; 2 groups: juvenile run/juvenile sed; n= 6/grp at each timepoint). Samples collected following one, three, and six weeks of exercise were plated on *Lactobacillus*-specific media. Briefly, these samples were homogenized in 2.0 ml phosphate buffered saline, then homogenates were further diluted at 1:5000 before plating on media selective for the growth of *Lactobacillus spp.* *Lactobacillus*-specific media (modified-rhamnose-2,3,5-triphenyltetrazolium chloride-LBS-vancomycin agar, or M-RTL V-agar) was made in accordance to published protocols (Sakai et al., 2010). This media allows for the selective growth of vancomycin-resistant *Lactobacillus spp.* This media also allows for the ability to distinguish *L. rhamnosus* colony-forming units (CFU) from CFUs of other *Lactobacillus spp.* through the addition of TTC, a salt that forms a deep red precipitate when reduced, and L-rhamnose, a deoxy sugar. *L. rhamnosus* can ferment L-rhamnose in order to produce lactic acid, whereas other species of *Lactobacillus* cannot complete this fermentation process. In the presence of TTC, bacteria that are able to ferment L-rhamnose will appear lighter because the acidic conditions inhibit the reduction of TTC, whereas bacteria that are incapable of fermenting L-rhamnose will appear red. Specifically, *L. rhamnosus* colony-forming units appear pinkish or white in color and asymmetrical in shape in contrast to the deep red, round, symmetrical colonies characteristic of all other *Lactobacillus spp.* growing on the plate (Figure 2B/2C). Briefly, M-RTL V agar was prepared by combining 0.4 g/ml of L-rhamnose, 30.0 mg/ml of 2,3,5 triphenyltetrazolium chloride (TTC), 10 mg/ml of vancomycin, and 10.0 mg/ml of metronidazole with nutrient agar. Plated samples were incubated at 37.0°C for 48 hours in anaerobic conditions. Anaerobic

conditions were created using a BD GasPak EZ Anaerobe Container System Sachets with the indicator placed inside a BD GasPak EZ Large Incubation Container (33.35 x 16.51 x 17.145 cm). The sachets create an anaerobic atmosphere inside the incubation chambers through activation of inorganic carbonate, activated carbon, ascorbic acid and water once exposed to air. Following incubation, CFUs of bacteria were counted using a cell counter (Scienceware Electronic Colony Counter) and dilution-corrected averages were calculated and analyzed. A subset of plates was sent to Genewiz (South Plainfield, NJ) for 16S rRNA sequencing to verify the selective growth of *lactobacillus spp.*; sequencing results confirmed selectivity of the plates (data not shown).

FIGURE 3: BODY COMPOSITION EXPERIMENTAL TIMELINE

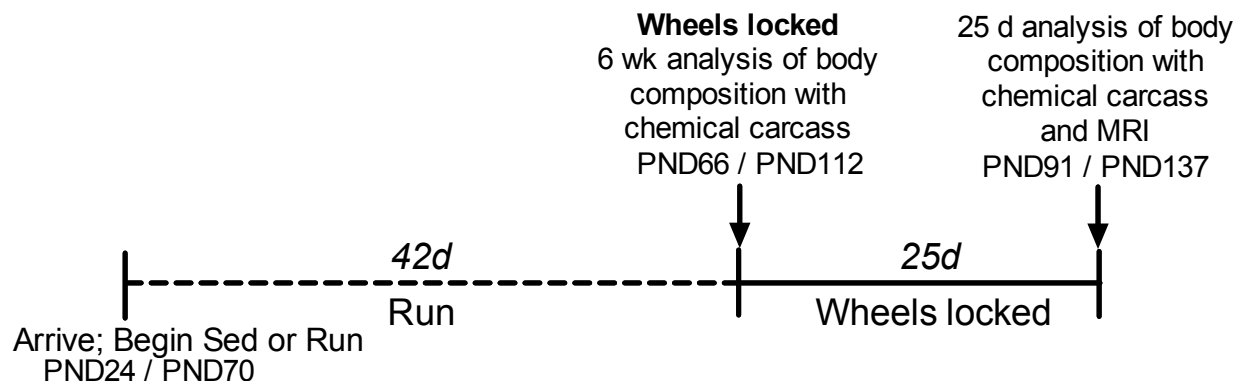


Figure 3 depicts the experimental timeline for body composition experiments. Juvenile and adult male F344 rats were either given access to voluntary running wheels immediately upon arrival, or were housed in sedentary cages. Following the six weeks of exercise, wheels were rendered immobile for 25 days. Body composition was measured following six weeks of exercise (chemical carcass analysis) and 25 days following exercise cessation (chemical carcass as well as MRI).

vi. Body Composition analysis

Chemical carcass analysis and MRI were utilized to examine the long-lasting impact of exercise on body composition using separate cohorts of rats exposed to the same protocols (Figure 3). Specifically, for chemical carcass procedures, another cohort of rats (n=48) was subjected to the same exercise protocol, wherein adult and juvenile rats were allowed voluntary access to running wheels for six weeks. Immediately following 6 weeks of exercise, rats were sacrificed via rapid decapitation and carcasses were frozen for later processing (4 groups: adult sed/adult run/juvenile sed/juvenile run; n=6/group). Wheels were locked for 25 days for the remainder of the rats (4 groups; n=6/grp). 25 days following exercise cessation, these rats were also sacrificed and carcasses were frozen for later carcass analysis. Chemical carcass analysis was performed on all rats in accordance to previously published protocols (Smith, Johnson, & Nagy, 2009) to determine total fat mass and total lean mass (total lean mass was calculated as fat free dry mass plus water content, minus ash content).

In attempt to replicate the long lasting effects of early life exercise on body composition derived from chemical carcass analysis, MRI scans (model 900, EchoMRI, Houston, TX) were employed. Another separate cohort of rats were subjected to the same exercise protocol, where rats were allowed voluntary access to running wheels for six weeks, then wheels were locked for 25 days (n=8/grp). Here, body composition using MRI was analyzed only 25 days after wheels were locked.

vii. Statistical Analysis

a. Running Distance, Body Weight, and Body Composition

Statistical analyses were conducted using the SPSS software package V.21 (SPSS, Chicago, IL). Running distance was compared using a 2 (age) by 6 (weeks of exercise) mixed design ANOVA, and body weight was compared using a 2 (age) by 2 (exercise status) by 9 (weeks) mixed design ANOVA. Chemical carcass body composition and body weight was analyzed using a 2 (age) by 2 (exercise status) by time point (6wks vs. 25d post), and MRI body composition and body weight was analyzed using a 2 (age) by 2 (exercise status) ANOVA.

b. Alpha Diversity and Relative Abundance of Microbial Taxa at the Phyla and Genus Level

Alpha diversity measures (Shannon Entropy, observed number of species, and phylogenetic diversity) as well as relative abundance of microbial taxa at the phylum and the genus levels were subjected to normality tests (Shapiro-Wilk), and all non-normal data were subsequently rank transformed. A 2 (age) x 2 (exercise status) x 3 (time point of fecal sample collection; 3d vs. 6wk vs. 25d post) mixed design ANOVA was then used to investigate measures of alpha diversity and relative abundance at the phylum and genus levels. At the genus level, taxa without an order classifier were excluded from analyses. Correction for multiple comparisons was conducted using the Benjamini Hochberg step down method (Benjamini & Hochberg, 1995) implemented in the QIIME 1.8.0. Significant interactions were further investigated with Fisher's PLSD, with alpha set to $p < .05$.

c. Lactobacillus spp. derived from M-RTL_V agar

Lactobacillus spp. and *L. rhamnosus* quantified using *M-RTL_V* agar were analyzed in juvenile runners vs. seds at each time point using independent samples t-tests.

d. Beta Diversity

Principal coordinates analysis (PCoA) using unweighted UniFrac distances with an explicit time axis was used to cluster microbial communities of juvenile versus adults at each time point. UniFrac is an algorithm that is used to determine if bacterial communities differ between samples based upon their mutual branch length on a phylogenetic tree (Lozupone & Knight, 2005). Specifically, Unifrac is used to calculate a distance metric between pairs of samples. To generate a Unifrac score for a sample pair, all taxa established within both samples are positioned on a phylogenetic tree. Branches of the phylogenetic tree that lead to taxa that exist in one sample but not the other are marked as unique or unshared branches, whereas branches that lead to taxa that exist in both samples are marked as mutual or shared branches. Distance is then calculated as the sum of unshared branch lengths /the sum of both shared + unshared branch lengths (i.e. total branch length). Weighted Unifrac takes into account the abundance of particular taxa found within each sample, whereas unweighted Unifrac just quantifies the presence or absence of particular taxa. In the current study, a distance matrix was created for each sample pair, then PCoA was used as means to visualize the similarity between all samples in relation to one another. Briefly, PCoA is a multidimensional scaling technique that can be utilized to visually display data derived from distance matrices.

d. Supervised Learning

Supervised machine learning using random forests as implemented in QIIME were employed to classify and differentiate sample classes. Supervised learning is a type of machine learning approach that splits data into training and test sets to build predictive models of class (sample) labels given the features (OTUs) in those samples. Here, we employ the popular

random forests (RF) algorithm. In brief, the RF model utilizes a forest of decision trees to attempt to predict which experimental group a sample came from based upon the presence of certain features (OTUs and taxonomically grouped OTUs) within that sample. The supervised learning algorithm is allowed to train on a subset of samples, and is then used to classify the remainder of the samples. The success rate of this algorithm is defined by its classification accuracy, which is computed by the ratio of the percentage of mislabeled samples using random guesses / the percentage of mislabeled samples using the models of the decision trees. A classification accuracy value of 2.0 or higher indicates that particular OTU's can be used to predict what experimental group a sample came from with significantly higher accuracy than random chance, and can signify robust changes to the microbial population due to an experimental manipulation (Knights, Costello, & Knight, 2011).

CHAPTER III: RESULTS

i. Running Distance

Figure 4 depicts the mean total weekly running distance for each group, estimated per rat. Running distance increased for both adult and juveniles across six weeks ($F(5,80)=40.057$; $p<0.0001$), and age did not impact overall running distance ($F(1,16)=2.855$; $p=0.110$). A time by age interaction was observed ($F(5,80)=17.803$; $p<0.0001$). During the initial two weeks of exercise, adult onset runners ran significantly more than juvenile onset runners; however, during the second half of the exercise, juveniles ran significantly more than adults (see graph for detailed post-hoc comparisons).

FIGURE 4: RUNNING DISTANCE

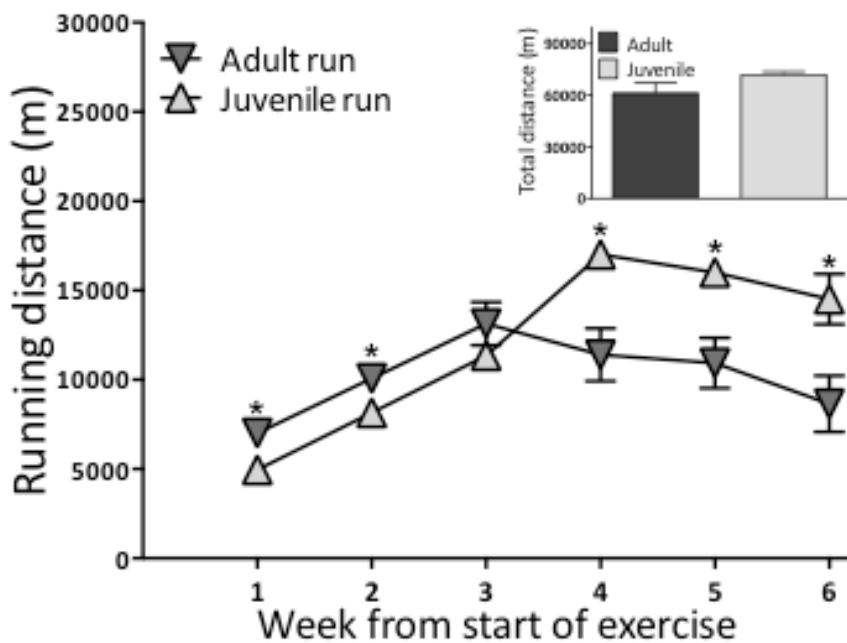
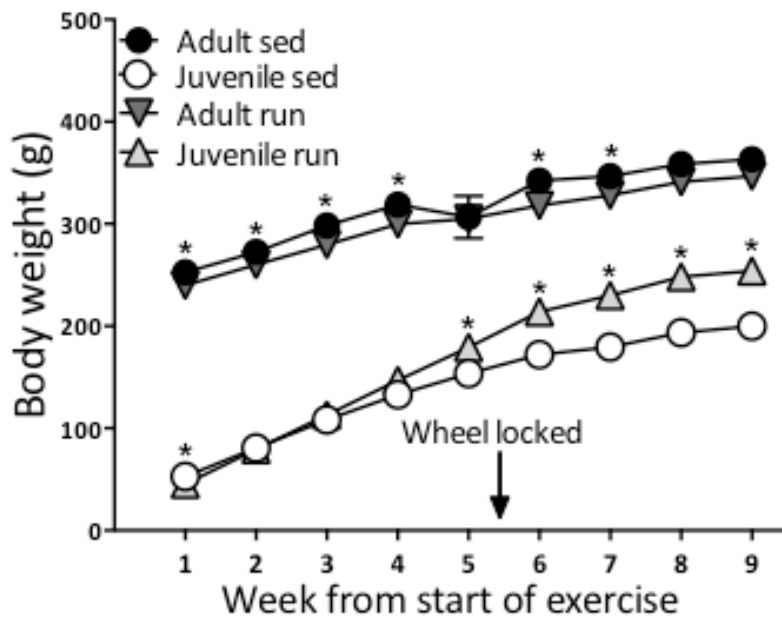


Figure 4 depicts weekly total running distance, estimated per rat. Adults ran more in the first half of exercise, whereas juveniles ran more during the second half of exercise, although average total weekly distance was not different between age groups. Data are represented as mean \pm SEM; * $p < 0.05$.

ii. Body Weight

As depicted in Figure 5, both adults and juveniles gained weight throughout the experiment ($F(8,160)=297.599$; $p<0.001$). Overall, the adults weighed more than the juveniles ($F(1,20)=1412.26$; $p<0.001$). ANOVA also revealed significant interactions between time and age ($F(8,160)=20.735$; $p<0.001$), time and exercise status ($F(8,160)=4.518$; $p<0.001$), as well as age and exercise status ($F(1,20)=26.044$; $p<0.001$). Post hoc comparisons revealed that adult onset runners weighed significantly less than their sedentary counterparts during exercise, and returned to sedentary levels one week following exercise cessation. In contrast to the pattern observed in the adults, juvenile onset runners began to weigh more during exercise, and continued to gain more weight than their sedentary counterparts following exercise cessation (see graph for detailed post-hoc comparisons).

FIGURE 5: BODY WEIGHT



Body weight is depicted in Figure 5; adult runners weighed less than their sedentary counterparts during exercise, then returned to sedentary levels following exercise cessation. Juvenile runners weighed more than their sedentary counterparts toward the end of exercise and continued to gain weight after exercise cessation. Data are represented as mean \pm SEM; * p <0.05.

iii. Alpha Diversity

a. Shannon Entropy

Three measures of alpha diversity were calculated for all samples: Shannon entropy (a measure of community evenness), number of species (a measure of species richness), and Phylogenetic Diversity (Faith, 1992). Analysis of Shannon entropy (Figure 6A), an indicator of an even and balanced community structure, revealed that adults had significantly higher Shannon entropy than juveniles ($F(1,18)=10.710$; $p<0.01$), indicating that their microbial communities were more balanced and evenly dispersed. Additionally, an exercise status by age interaction ($F(1,18)=5.371$; $p<0.05$) was observed, in that juvenile runners displayed decreased Shannon entropy overall, indicating that their microbial communities were less balanced and even compared with their sedentary counterparts. Analyses at each time point further revealed that Shannon entropy in juvenile runners was specifically decreased after six weeks of exercise compared to sedentary juveniles. Running had no impact on Shannon entropy in adults.

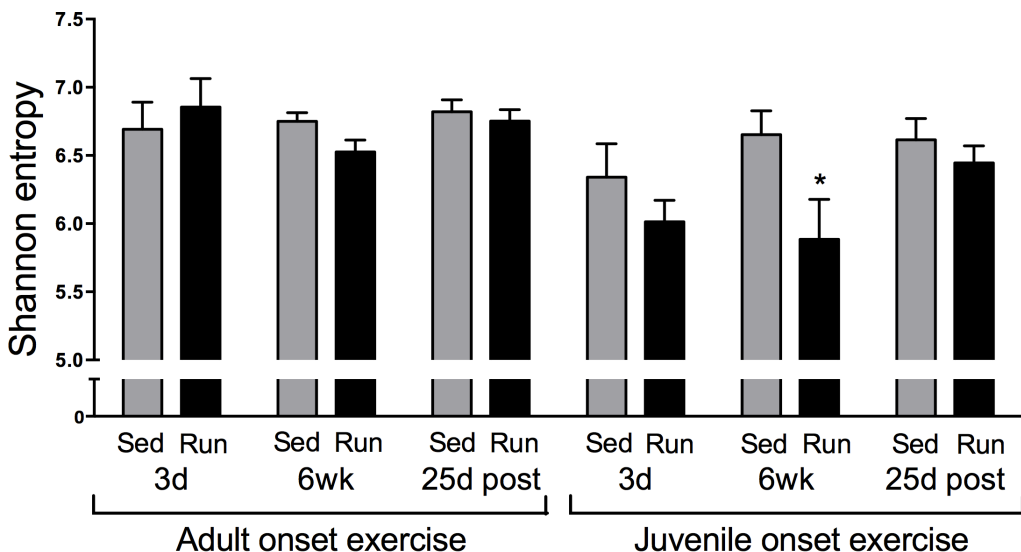
b. Number of Species

Examination of the number species (species richness; Figure 6B) revealed that adults exhibited more species overall than juveniles ($F(1,18)=8.074$; $p<0.05$). Furthermore, runners, regardless of age, had fewer species overall ($F(1,18)=4.506$; $p<0.05$). A time by run by age interaction was also observed ($F(2,36)=3.604$, $p<0.05$), and post-hoc analyses revealed that three days after beginning exercise, juvenile runners exhibited fewer species than juvenile sedentary rats. In contrast, no differences were observed between adult runners vs. adult sedentary rats at each time point.

c. Phylogenetic Diversity

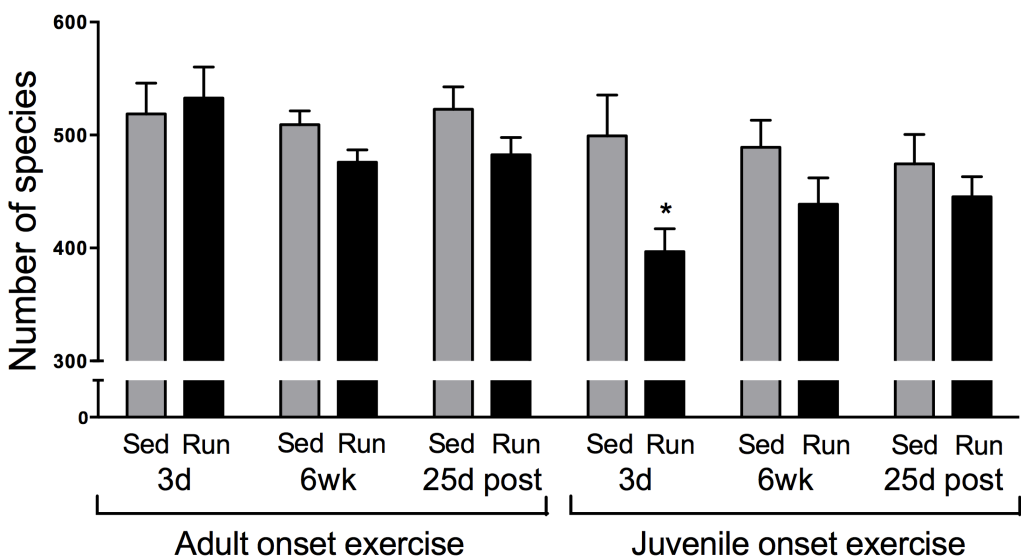
ANOVA revealed no statistically reliable group differences in phylogenetic diversity, i.e. the total descending branch length of the constructed phylogenetic tree for a given sample (data not shown).

FIGURE 6A: EARLY LIFE EXERCISE DECREASED SHANNON ENTROPY



Shannon entropy, an indicator of community evenness, was calculated for adult and juvenile run and sed rats across all three time points. Shannon was significantly higher in the adults than the juveniles. An exercise by age interaction revealed that juvenile runners displayed decreased Shannon entropy overall, and specifically after six weeks of exercise. Data are represented as mean \pm SEM; * $p < 0.05$.

FIGURE 6B. EARLY LIFE EXERCISE DECREASED SPECIES RICHNESS

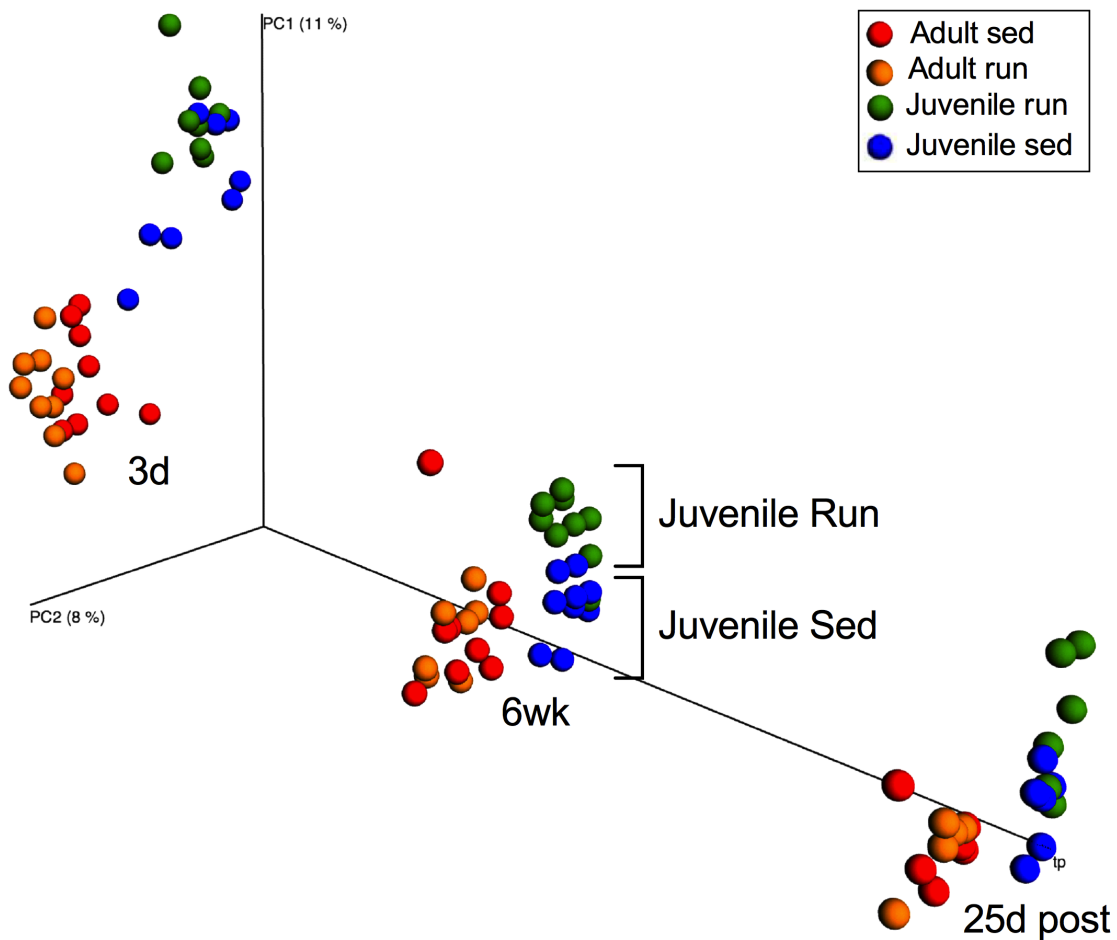


Number of species, i.e. species richness, found in a given sample, was calculated for adult and juvenile run and sed rats across all three time points. Overall, adults had more species than juveniles. Runners, regardless of age, had significantly fewer species overall than their sedentary counterparts. A time by age by exercise interaction revealed that juvenile runners had significantly fewer species than their sedentary counterparts 3d following the start of exercise. Data are represented as mean \pm SEM; * $p < 0.05$.

iv. Beta Diversity

Principle coordinate analysis (PCoA) using unweighted UniFrac distances with an explicit time axis revealed clustering of the microbial communities of juveniles versus adults at each time point (Figure 7). Briefly, UniFrac is an algorithm that determines how similar the microbial communities are between samples based upon their shared branch length on a phylogenetic tree (Lozupone & Knight, 2005). After six weeks of exercise, a clear clustering of the microbial communities of juvenile runners versus juvenile sedentary rats is evident, with no noticeable pattern within the adults.

FIGURE 7: AGE AND EARLY LIFE EXERCISE ALTERED BETA DIVERSITY



Principle coordinate analysis (PCoA) using unweighted UniFrac distances with an explicit time axis depicts a clear clustering of the microbial communities of juvenile runners v. juvenile seds after six weeks of exercise, with no noticeable pattern within the adults.

v. Relative Abundance of Microbial Taxa at the Phylum Level

Figure 8 depicts the relative abundance of 12 phyla. After controlling for false discovery rate, ANOVA revealed that only Deferribacteres changed significantly across time ($F(2,36)=6.078$; $p=0.0315$). A time by age interaction in Actinobacteria ($F(2,36)=7.089$; $p<0.05$) was also observed, in that higher levels were detected in the adults after six weeks. Significant differences due to exercise were only observed in juvenile runners within Euryarchaeota, Bacteroidetes, Firmicutes, and Proteobacteria (Run by age interaction; $F(1,18)=9.809$; $F(1,18)=8.052$; $F(1,18)=9.668$; $F(1,18)=12.575$; $p<0.05$, respectively). Specifically, juvenile onset exercise increased relative abundance of Euryarchaeota and Bacteroidetes overall, and analyses at each time point revealed that these phyla were significantly decreased compared to juvenile sedentary rats after six weeks of exercise. An opposite pattern was observed in Firmicutes and Proteobacteria, both overall and specifically after six weeks.

FIGURE 8: RELATIVE ABUNDANCE OF MICROBIAL TAXA AT THE PHYLUM LEVEL

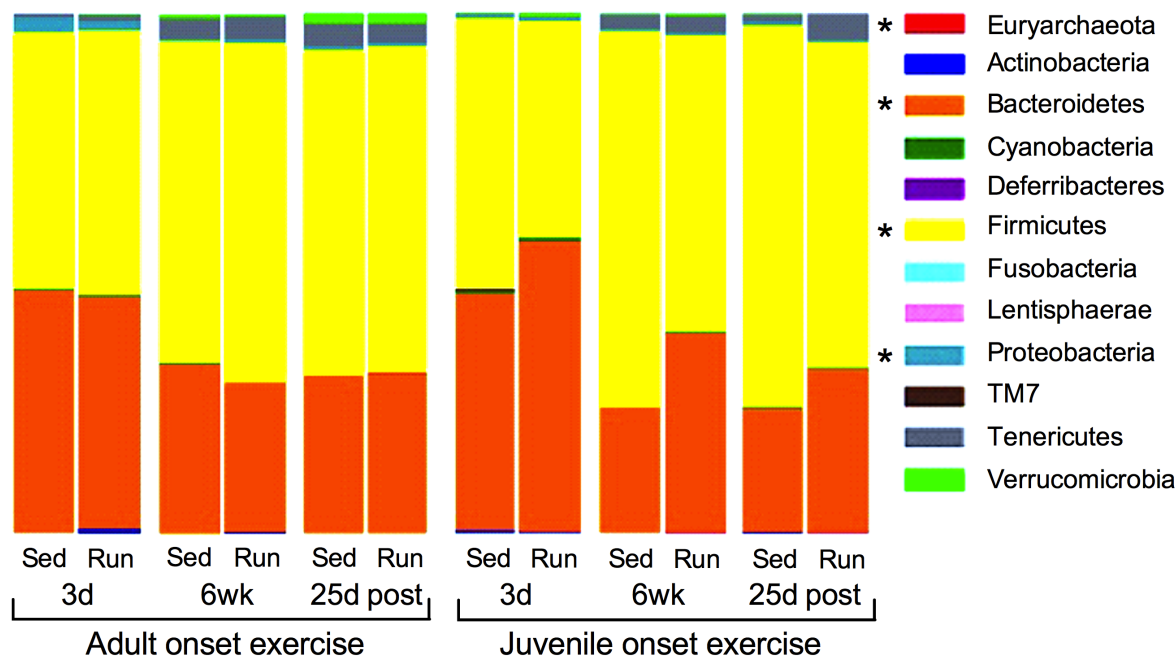


Figure 8 depicts the relative abundance of 12 phyla, calculated for adult and juvenile run and sed rats across all three time points. Run by age interactions revealed that significant differences due to exercise were only observed in juvenile runners. Specifically, juvenile onset exercise increased relative abundance of Euryarchaeota and Bacteroidetes and decreased relative abundance of Firmicutes and Proteobacteria. These patterns were observed overall as well as after six weeks of exercise. * $p < 0.05$. Additionally, juvenile onset runners show a trend toward a persistent decrease in Firmicutes 25 days following exercise cessation ($p = 0.061$).

vi. Supervised Learning

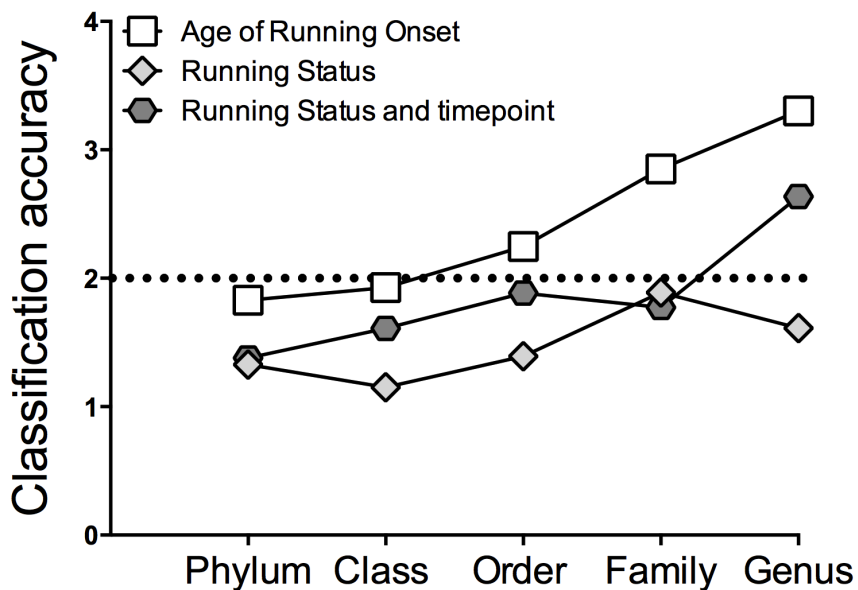
a. Classification accuracy

Supervised learning is a type of machine learning approach that splits data into training and test sets to build predictive models of class (sample) labels given the features (OTUs) in those samples. Here, we employ the popular random forests (RF) algorithm (See detailed description in statistical analyses section).

Figure 9A depicts classification accuracy as a function of the taxonomic level of the features. When collapsing samples into the following three categories: age of running onset, running status, and running status and time point, supervised learning reveals that the highest classification accuracy is observed for age of running onset. Interestingly, classification accuracy increases across levels of taxonomy, and is highest for age of running onset at level 6 (the genus level). This indicates that a *specific* subset of genera can be used to distinguish between samples belonging to juvenile versus adult runners, signifying pronounced differences in microbial composition between these two groups.

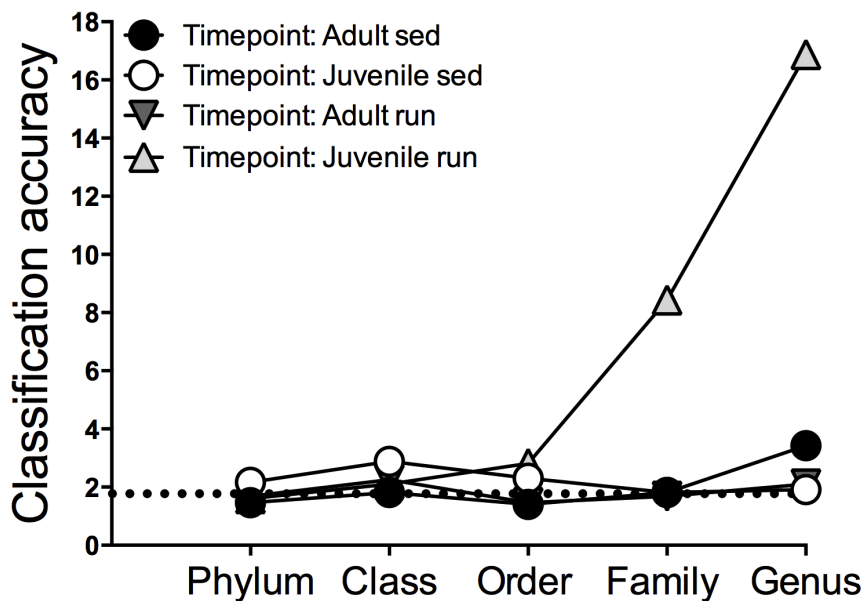
Next, each experimental group was considered separately, and the supervised learning algorithm attempted to classify which time point the sample came from (Figure 9B). When considering the juvenile runners only, the classification accuracy of predicting time of sample collection (i.e., three days after start of exercise, six weeks after start of exercise, or 25 days post exercise) increased to 16.875 times better than random guessing, indicating that specific genera may be serving as extremely accurate predictors of sample time point. This strongly suggests that specific genera are being altered in the juvenile runners, more so than in the other groups, and that this change is persistent even after the cessation of running.

FIGURE 9A: CLASSIFICATION ERROR FOR AGE, RUNNING STATUS AND TIME



Classification accuracy generated from supervised learning is depicted across each level of taxonomy in Figure 9A. When samples were collapsed into age of running onset, running status, and running status and time point categories, supervised learning revealed that the highest classification accuracy was observed for age of running onset.

FIGURE 9B: CLASSIFICATION ERROR FOR TIMEPOINT ACROSS EACH GROUP



When each experimental group was considered separately, the classification accuracy of predicting time point increased to 16.875 times greater than random guessing for juvenile runners only. This shows that certain genera were significantly altered across time in juvenile runners only relative to all other groups.

b. Feature Importance scores

Supervised learning can distinguish the particular microbial taxa that are acting as extremely accurate predictors of sample timepoint. The algorithm accomplished this by assigning an importance score to each taxa based upon the decrease in classification accuracy observed when that particular taxa was removed as a predictor. Here, a particular taxa was considered to be highly predictive if the mean decrease in accuracy was 1.0% or more. We examined importance scores for juvenile runners at the genus level only, since prior analyses revealed that classification accuracy was highest at this level. Seven genera were identified as having an importance score of 1.0% or higher; this indicates that these genera were significantly altered by juvenile onset exercise, and served as key predictors for discerning time point in samples obtained from juvenile runners. These seven genera described in Table 1.

TABLE 1: SUPERVISED LEARNING FEATURE IMPORTANCE SCORES

PHYLUM	GENUS	FEATURE IMPORTANCE SCORE
Bacteroidetes	Rikenellaceae g_	2.98%
	Parabacteroides spp.	2.97%
	Bacteroides spp.	1.40%
Firmicutes	Ruminococcus spp	1.43%
	Christensenellaceae g_	1.22%
Actinobacteria	Bifidobacterium spp.	1.20%
Euryarchaeota	Methanosphaera spp.	1.00%

These taxa were important for predicting which time point a sample obtained from a juvenile onset runner came from. The feature importance score of each taxa depicts the decrease in classification accuracy observed when that particular feature is removed as a predictor. Here, a feature was considered to be important if the mean decrease in accuracy was 1.0% or more.

vii. Relative Abundance of Microbial Taxa at the Genus level

a. ANOVA main effects and interactions summary

Given that supervised learning analyses revealed high classification accuracy at the genus level, additional analyses at this level of taxonomy were performed. After correcting for false discovery rate, mixed design ANOVA revealed that several bacterial genera were significantly modulated by age, exercise status, and time. Detailed main effects and interactions are summarized in Table 2.

TABLE 2: SUMMARY OF ANOVA AT THE GENUS LEVEL

EFFECT	PHYLUM	GENUS	FDR P VALUE	DIRECTION
Time	Bacteroides	Bacteroides spp.	p=0.027	Decreased; 3d, 6wk > 25d post
		Prevotella spp.	p=0.046	Decreased; 3d, 6wk > 25d post
	Deferribacteres	Mucispirillum spp.	p=0.012	Decreased; 3d > 6wk, 25d post
		Lactobacillus spp.	p=0.032	Decreased; 3d, 6wk > 25d post
	Firmicutes	Anaerofustis spp.	p=0.048	Increased at 6wk
		Coprococcus spp.	p=0.048	Decreased; 3d > 6wk, 25d post
		Peptococcaceae g	p=0.05	Decreased; 3d > 6wk, 25d post
		Eubacterium spp.	p=0.037	Decreased; 3d > 25d post
		Allobaculum spp.	p=0.012	Decreased; 3d > 6wk, 25d post
		Christensenellaceae g	p=0.02	Decreased; 3d > 25d post
		Clostridiales f g	p=0.02	Increased; 3d < 6wk, 25d post
	Proteobacteria	Sutterella spp	p=0.046	Decreased; 3d, 6wk > 25d post
		Desulfovibrio spp.	p=0.003	Decreased; 3d, 6wk > 25d post
Age	Actinobacteria	Bifidobacteria spp.	p=0.048	Increased in juveniles
		Adlercreutzia spp.	p=0.012	Increased in adults
	Bacteroidetes	Prevotella spp.	p=0.012	Increased in juveniles
		Rikenellaceae g_	p=0.048	Increased in juveniles
	Firmicutes	Coprococcus	p=0.046	Increased in juveniles
		Bacillaceae g	p=0.012	Increased in adults
		Streptococcus spp.	p=0.046	Increased in adults
		Clostridium spp.	p=0.032	Increased in adults
		Erysipelotrichaceae g	p=.012	Increased in adults
	Exercise	Euryarchaeota	Methanosphaera spp	p=0.042
Turicibacter spp.			p=0.02	Increased by exercise
Firmicutes		Dehalobacteriaceae g	p=0.048	Increased by exercise
		Lactococcus spp.	p=0.020	Increased by exercise
		Oscillospira spp.	p=0.037	Decreased by exercise
Time x Age	Bacteroidetes	Prevotella spp.	P=0.01	Increased in juveniles at 3d
		Rikenellaceae g	P=0.03	Increased in juveniles at 3d
	Firmicutes	Peptococcaceae g	P=0.01	Increased in adults at 3d
		Ruminococcaceae g_	P=0.02	Increased in juveniles at 3d, 6wk
		Oscillospira spp.	P=0.05	Increased in adults at 3d
Time x exercise	Proteobacteria	Sutterella spp	P=0.01	Increased by exercise at 6 wk

Summary of ANOVA main effects and interactions at the genus level of taxonomy.

b. Differential impact of juvenile vs. adult onset exercise on bacterial genera

Notably, after correcting for false discovery rate, a run by age interaction was identified in seven genera; seven of these genera were significantly modulated by juvenile onset exercise, while three were modulated by adult onset exercise. Additionally, given the previous findings (Queipo-Ortuno et al., 2013) showing that exercise elevated *Lactobacillus spp.*, this genus was excluded from FDR correction and analyzed separately.

Here, juvenile onset exercise overall increased relative abundance of *Methanosphaera spp.* within the Euryarchaeota phylum ($p < 0.05$; 10A) and *Anaerostipes spp.* ($p < 0.05$; 10B), as well as *Blautia spp.* within the Firmicutes phylum ($p < 0.05$; 10C). In contrast, juvenile onset exercise overall decreased *Desulfovibrio spp.* within the Proteobacteria phylum ($p < 0.05$; 10D), an unidentified spp. within the Rikenellaceae family ($p < 0.05$; 10E) as well as Rikenellaceae_AF12 ($p < 0.05$; 10F) within the Bacteroidetes phylum. Both of these species within the Rikenellaceae family were increased in the adults, along with *Turicibacter spp.* within the Firmicutes phylum ($p < 0.05$; 10G). See graphs for post-hoc comparisons at each time point.

Figure 10H depicts relative abundance of *Lactobacillus spp.* within the Firmicutes phylum. *Lactobacillus spp.* decreased across time ($F(2,36)=6.431$; $p < 0.005$). Juveniles, regardless of running status, exhibited more *Lactobacillus spp.* overall ($F(1,18)=2.957$; $p < 0.001$), and post hoc analyses revealed that *Lactobacillus spp.* were significantly increased three days following exercise onset ($p < 0.05$).

FIGURE 10: RELATIVE ABUNDANCE OF MICROBIAL GENERA

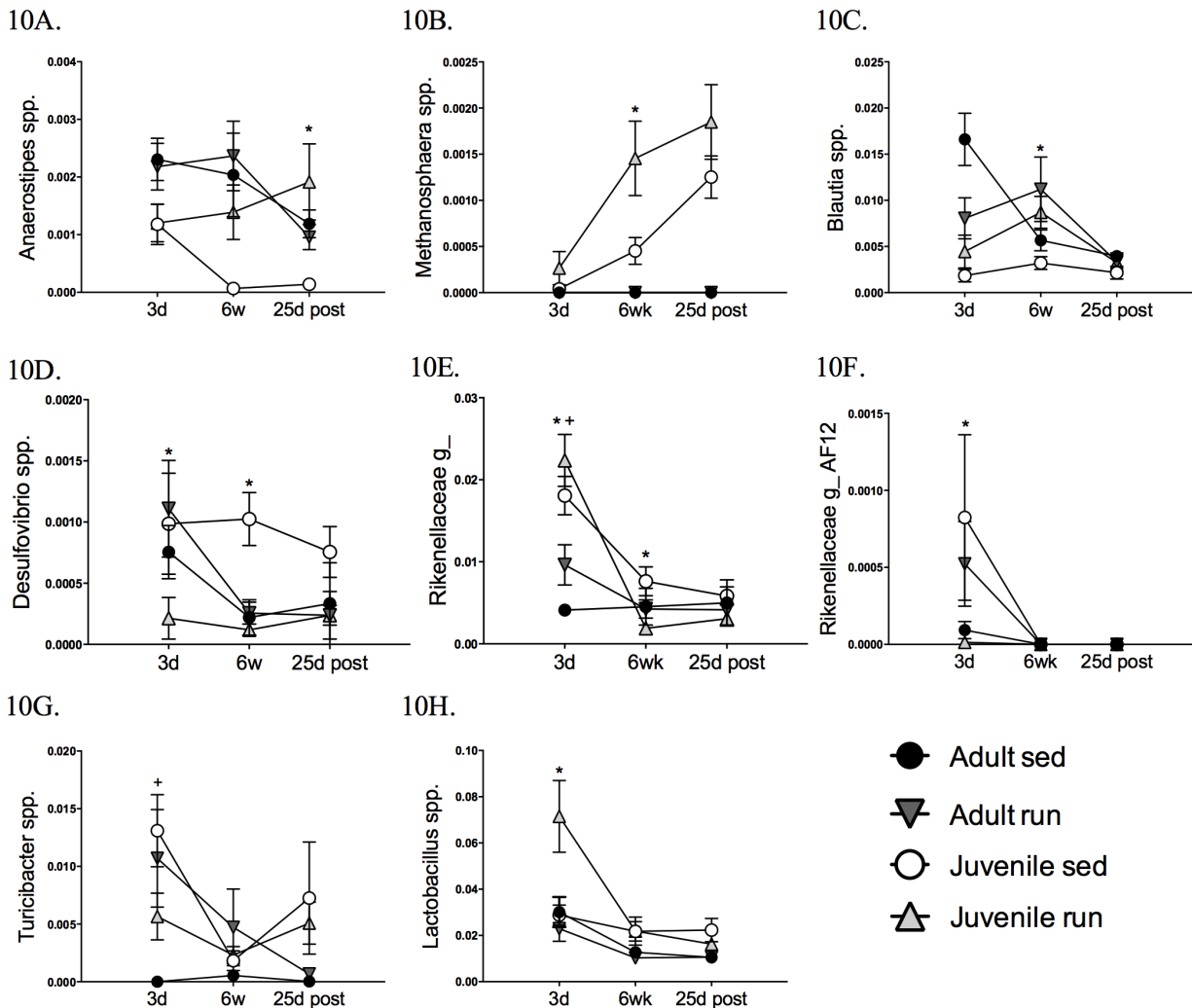
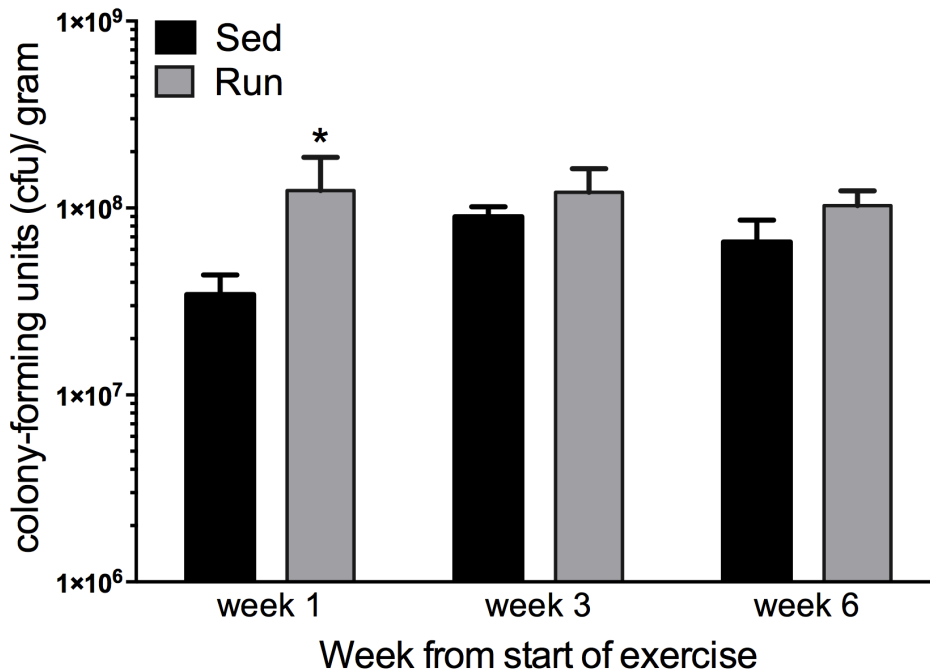


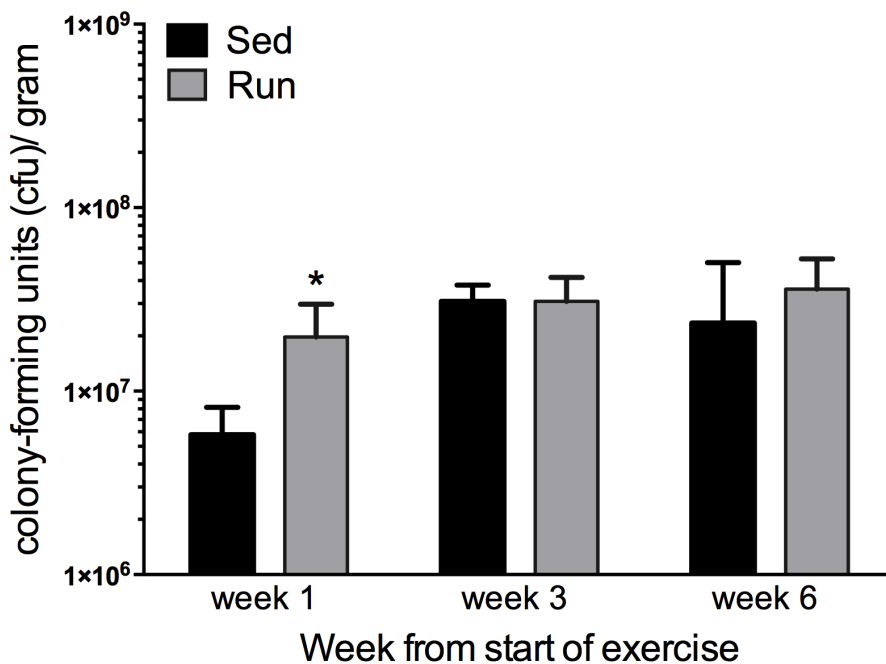
Figure 10 depicts patterns due to exercise and age at the genus level. Here, juvenile onset exercise increased relative abundance of *Anaerostipes spp.*, *Methanosphaera spp.* and *Blautia spp.* overall (10A-10C). In contrast, juvenile onset exercise overall decreased *Desulfovibrio spp.*, an unidentified spp. within the Rikenellaceae family, and Rikenellaceae_AF12 overall (10D-9F). Both of these species within the Rikenellaceae family were increased overall in the adult runners, along with *Turicibacter spp.* (10E-10G). Relative abundance of *Lactobacillus spp.* was significantly increased three days following exercise onset only (10H). Data are represented as mean \pm SEM, + $p < 0.05$ for comparisons between adult seds vs. runners at particular time points, * $p < 0.05$ for comparisons between juvenile seds vs. runners at particular time points.

viii. *Selective Lactobacillus spp. Analysis*

As depicted in Figure 11A, *Lactobacillus spp.* were increased in juvenile runners one week following the start of exercise ($t(8) = 3.143$; $p=0.013$), but not three weeks ($t(10)=1.8313$; $p=0.09$) or six weeks ($t(10)= 3.154$; $p=0.01$). Similarly, *L. rhamnosus* was increased one week following the start of exercise ($t(9)= 3.003$; $p=0.015$), but not three weeks ($t(10)= 0.0055$; $p=0.99$) or six weeks ($t(10)=0.963$; $p=0.35$).

FIGURE 11A: ALL *LACTOBACILLUS SPP.*

As depicted in Figure 11A, *Lactobacillus spp.* were increased in juvenile runners one week following the start of exercise, but not three weeks or six weeks.

FIGURE 11B: *LACTOBACILLUS RHAMNOSUS*

As depicted in Figure 11B, *L. rhamnosus* increased in juvenile runners one week following the start of exercise, but not three weeks or six weeks.

ix. Body composition

a. Running distance

Given the potential of early life exercise-induced microbial changes to contribute to a lean phenotype, we examined body composition in juvenile versus adult runners. Figure 12 depicts the mean total weekly running distance for chemical carcass and MRI cohorts, estimated per rat. Since no significant differences in running distance were found between cohorts, running distances were collapsed across the two cohorts. Running distance increased for both adult and juveniles across six weeks ($F(5,180)=39.572$; $p<0.0001$), and age did not impact overall running distance ($F(1,36)=1.788$; $p=0.1895$). A time by age interaction was observed ($F(5,180)=23.499$; $p<0.0001$). Similar to the running pattern observed in the 16S cohort, adult onset runners ran significantly more than juvenile onset runners in the beginning of the exercise regimen. However, during the second half of the exercise, juveniles ran significantly more than adults (see graph for detailed post-hoc comparisons).

FIGURE 12: BODY COMPOSITION RUNNING DISTANCE

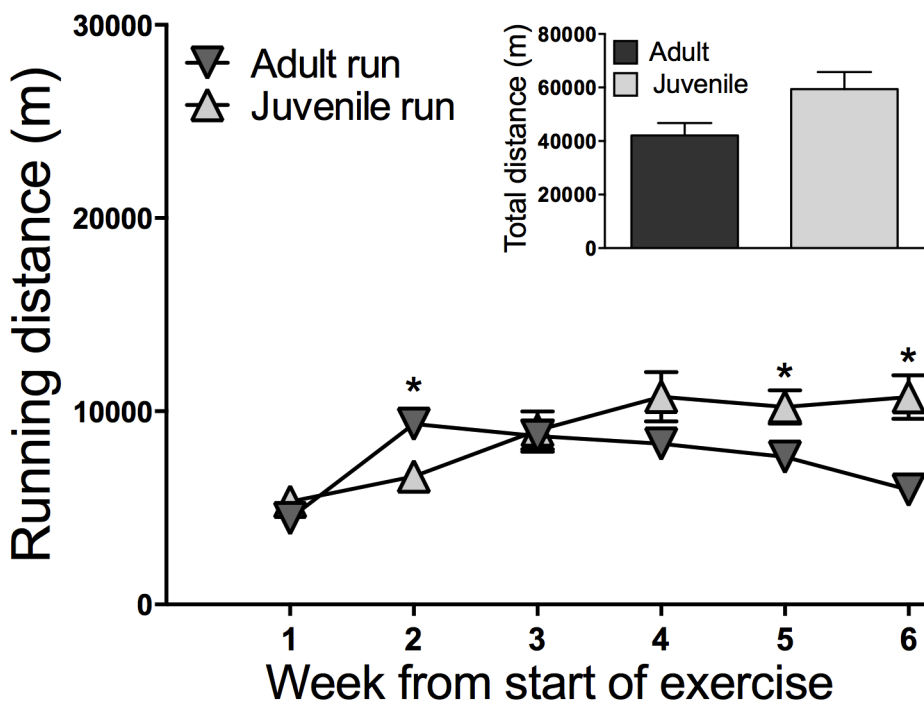


Figure 12 depicts total weekly running distance across time, collapsed across both chemical carcass and MRI cohorts, and estimated per rat. Adults ran more in the first half of exercise, whereas juveniles ran more during the second half of exercise, although average total weekly distance was not different between age groups. Data are represented as mean \pm SEM; * $p < 0.05$.

b. chemical carcass body weight

Figure 13 depicts the body weights of sacrificed adult and juvenile onset runners immediately before chemical carcass procedures. Overall, the adults weighed more than the juveniles ($F(1,38)=270.104$; $p<0.001$). ANOVA also revealed significant interactions between age and exercise status ($F(1,38)=24.757$; $p<0.001$). Post hoc comparisons revealed that adult onset runners weighed significantly less than their sedentary counterparts immediately after exercise cessation ($p<0.01$) and returned to sedentary levels 25 days following exercise cessation. In contrast to the pattern observed in the adults, juvenile onset runners weighed more immediately after exercise cessation ($p<0.002$), and continued to weigh more than their sedentary counterparts 25 days following exercise cessation ($p<0.007$).

FIGURE 13: CHEMICAL CARCASS BODY WEIGHT

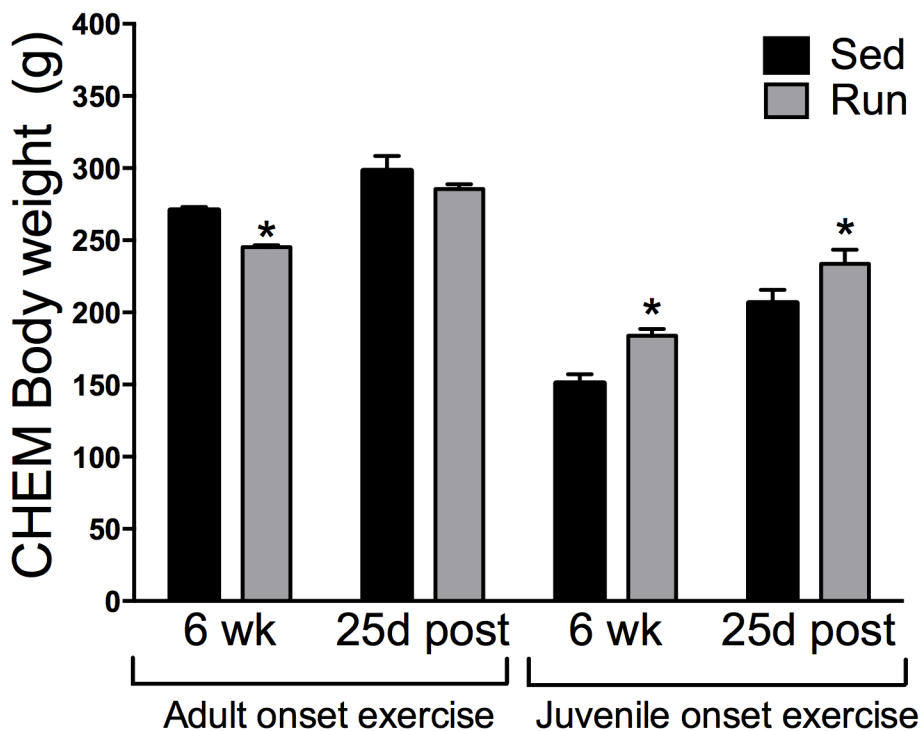


Figure 13 depicts body weight for adult versus juvenile runners and their sedentary counterparts after 6 wks of exercise, and 25 days following exercise cessation for rats used for chemical carcass analyses. Adult runners weighed less than adult seds immediately after exercise, however they returned to sedentary levels 25 days following exercise cessation. On the other hand, juvenile onset runners consistently weighed more than their sedentary counterparts. Data are represented as mean \pm SEM; * $p < 0.05$.

c. chemical carcass lean mass

Carcass analysis revealed that lean mass increased across time ($F(1,38)=56.779$; $p<0.0001$), and was higher in adults ($F(1,38)=274.400$; $p<0.0001$) and runners ($F(1,38)=7.189$; $p<0.0108$, Figure 14A). ANOVA further revealed an age by exercise interaction ($F(1,38)=20.492$; $p<0.0001$). Follow up analyses showed that lean mass was significantly increased in juvenile runners only, following six weeks of exercise ($p<0.0003$) and 25 days following exercise cessation ($p<0.0001$), indicating that early life exercise can cause lasting increases in lean mass. No such patterns were observed in the adult runners.

d. chemical carcass fat mass

Carcass analysis also revealed that fat mass increased across time ($F(1,38)=54.942$; $p<0.0001$), and was higher in adults ($F(1,38)=444.092$; $p<0.0001$), and sedentary rats ($F(1,38)=17.073$, $p<0.0002$, Figure 14B). ANOVA further revealed a run by age interaction ($F(1,38)=14.286$; $p<0.0005$), in that fat mass was greater in adult sedentary rats at both time points ($p<0.0012$, $p<0.0001$, respectively), indicating that the impact of being sedentary on fat mass was greater for adults than juveniles.

FIGURE 14A: CHEMICAL CARCASS LEAN MASS

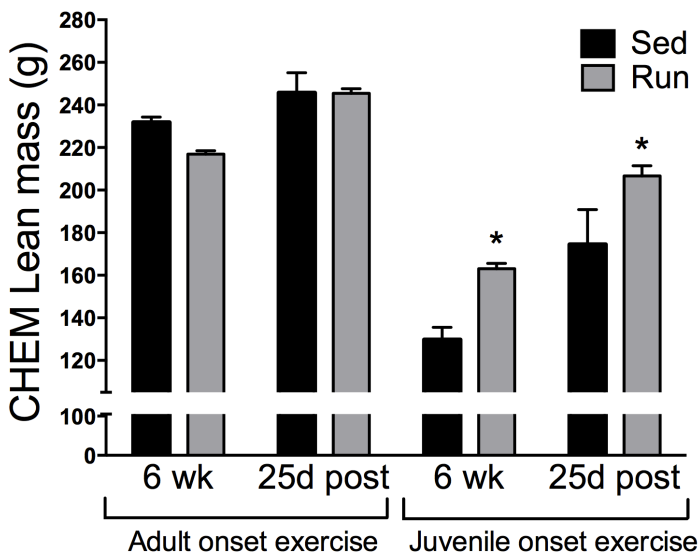


Figure 14A depicts chemical carcass performed after 6 weeks of exercise and 25 days following exercise cessation. Sustained increases in lean mass were found in juvenile onset runners only, following 6 wks of exercise and 25 d following exercise cessation. Data are represented as mean \pm SEM; * p <0.05.

FIGURE 14B: CHEMICAL CARCASS FAT MASS

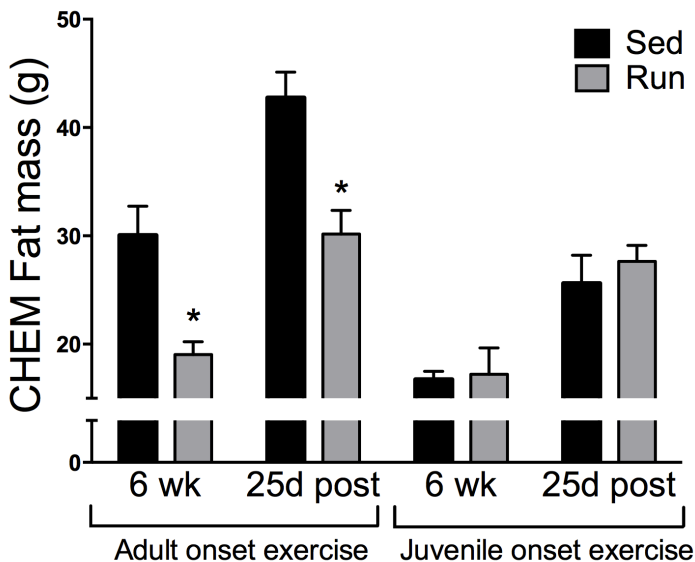


Figure 14B depicts chemical carcass performed after 6 weeks of exercise and 25 days following exercise cessation. Sustained decreases were found in fat mass in adult onset runners only at both time points. Data are represented as mean \pm SEM; * p <0.05.

e. MRI body weight

Figure 15 depicts the body weights of adult and juvenile onset runners during MRI. Overall, the adults weighed more than the juveniles ($F(1,28)=69.296$; $p<0.001$). ANOVA also revealed significant interactions between age and exercise status ($F(1,28)=6.235$; $p=0.018$). Post hoc comparisons revealed no differences in body weight between adult onset runners and their sedentary counterparts. In contrast, juvenile onset runners weighed significantly more than juvenile seds 25 days following exercise cessation ($p<0.02$).

FIGURE 15: MRI BODY WEIGHT

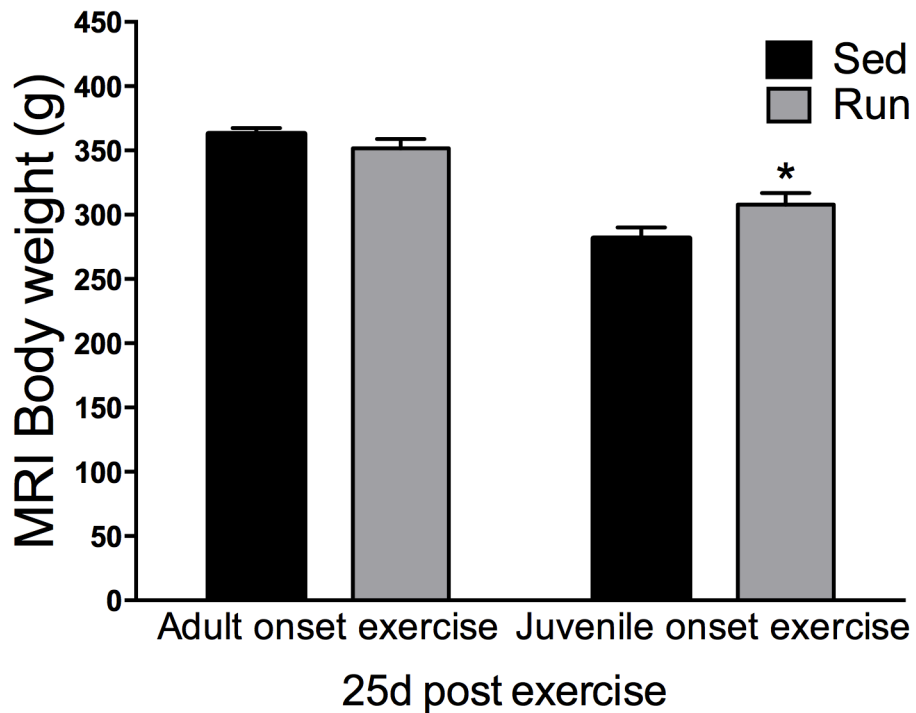


Figure 15 depicts body weight measured 25 days following exercise cessation for rats used for MRI analyses. No differences in body weight were found between adult runners versus adult seds, however juvenile runners weighed more than their sedentary counterparts. Data are represented as mean \pm SEM; * $p < 0.05$.

f. MRI lean mass

Body composition using MRI 25 days following exercise cessation largely supported results obtained from chemical carcass analysis. MRI revealed that overall, adults ($F(1,28)=69.651$; $p<0.001$) and runners ($F(1,28)=5.275$; $p<0.001$) had more lean mass compared to juvenile and sedentary rats. Importantly, ANOVA revealed an age by exercise status interaction ($F(1,28)=4.366$; $p<0.05$), indicating that the lasting increase in lean mass produced by running was more evident in the juvenile onset runners than the adult onset runners (Figure 16A).

g. MRI fat mass

Figure 16B depicts mean fat mass per group. Overall, adults had more fat mass ($F(1,28)=4.354$; $p<0.05$) than juveniles. Also, runners, regardless of age, had less fat mass than sedentary rats ($F(1,28)=17.171$; $p<0.001$). Finally, ANOVA also revealed an age by exercise status interaction ($F(1,28)=6.417$; $p<0.0172$), further supporting that sedentary status has a greater impact on fat mass in adulthood.

FIGURE 16A: MRI LEAN MASS

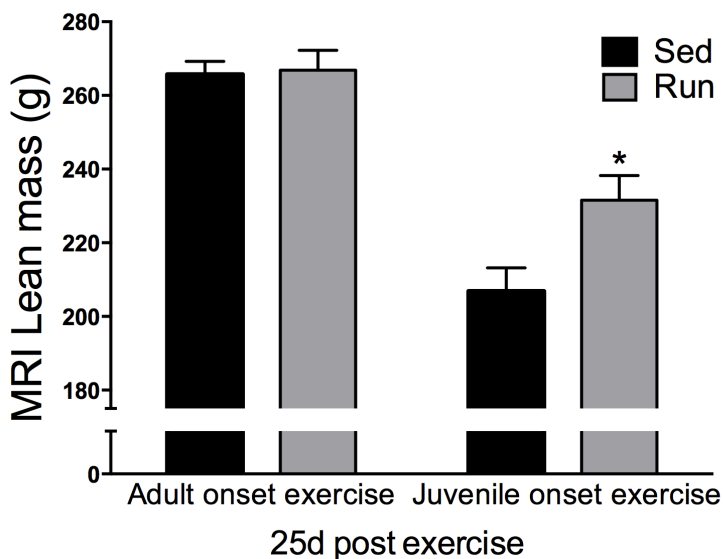


Figure 16A depicts lean mass measured 25 days following exercise cessation for rats used for MRI analyses. Juvenile onset runners show long lasting increases in lean mass 25 days following cessation of exercise. Data are represented as mean \pm SEM; * $p < 0.05$.

FIGURE 16B: MRI FAT MASS

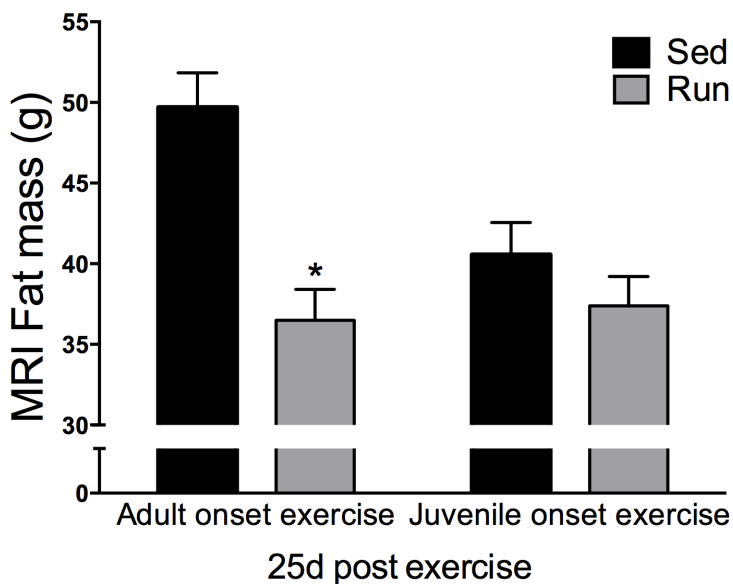


Figure 16B depicts fat mass measured 25 days following exercise cessation for rats used for MRI analyses. Adult onset runners show long lasting decreases in fat mass 25 days following exercise cessation. Data are represented as mean \pm SEM; * $p < 0.05$.

IV. DISCUSSION

Findings from measures of alpha and beta diversity, supervised learning, and traditional statistical approaches collectively support the hypothesis that exercise initiated during the juvenile period had a more robust impact on the gut microbiota than exercise initiated in adulthood. Measures of alpha diversity revealed that the microbiota of juvenile compared to adult rats was less balanced, stable, and diverse overall, an environment that perhaps allowed for exercise to have a greater impact. PCoA plots revealed an effect of exercise on the clustering of microbial communities in the juveniles only. Similarly, only juvenile onset exercise altered the relative abundance of several phyla. Specifically, the relative abundance of Euryarchaeota and Bacteroidetes was increased, and the relative abundance of Proteobacteria and Firmicutes was decreased in juvenile but not adult onset runners. Finally, supervised learning and ANOVA revealed that exercise profoundly altered specific genera within the juveniles. Such pronounced patterns of change were not present following adult onset exercise. Although several studies have demonstrated that exercise is capable to altering the gut flora, this study is the first to demonstrate that the gut microbiota may be more sensitive to exercise during early life.

Measures of alpha diversity revealed that juvenile rats had less richness (fewer species), and evenness (lower Shannon entropy) within their gut microbiota relative to adults. Similar patterns are found in the microbial diversity of humans across different ages (Yatsunenkov et al., 2012), in that the microbial composition of infants and children was also found to be less stable and diverse relative to adults. Indeed, the remarkable stability of the adult gut microbiota has been recently demonstrated in a longitudinal study by Faith et al. where the majority of bacterial strains maintained a presence within the gut over the course of five years (2013). The increased stability and complexity of the adult microbiota may make it more impervious to change (Wall et

al., 2009). Conversely, the decreased stability and diversity of the juvenile gut may be why the early microbial environment is more inclined to environmentally induced changes. In support of this premise, a recent paper demonstrated that an individual's bacterial diversity was indicative of its responsiveness to diet-induced changes; greater diversity was associated with a less responsive gut microbiota (Salonen et al., 2014). Data from the current study offer further support for these ideas, and suggest that the inherent lack of stability and complexity of the early life microbial ecosystem allow for greater exercise-induced changes.

Additionally, we demonstrated that exercise decreases species richness. This finding is novel and somewhat surprising given existing literature. Clarke et al., for example, reported an increase in several measures of microbial diversity in professional rugby players (Clarke et al., 2014). Furthermore, Petriz et al., investigated the impact of four weeks of exercise training in obese, non-obese Wistar, and spontaneously hypertensive rat strains and found that bacterial diversity increased after exercise across all strains (Petriz et al., 2014). Several factors may account for the differential impact of exercise on diversity in the present study, such as differences in diet, exercise training duration and rat strain/phenotype. Moreover, although increased microbial diversity and richness has been linked to better overall health (Bisgaard et al., 2011), a decrease in species richness needs to be interpreted in the context of our other findings. Additional analyses at the phyla and genus levels reveal that exercise selectively increases specific beneficial taxa while decreasing others. Thus, the pattern of changes, as well as the potential associated functional consequences of these changes, is important to consider when interpreting this decrease in bacterial diversity following exercise.

Measures of alpha diversity also revealed that community evenness was disrupted in juvenile but not adult onset runners. Specifically, Shannon entropy was lower after six weeks of

exercise in juvenile runners versus juvenile sedentary rats. This reduction supports our hypothesis that juvenile onset exercise had a stronger impact on the gut microbial composition than adult onset exercise. A lower Shannon value indicates an uneven community structure, suggesting that the relative abundance of specific taxa is either much higher or lower compared to other taxa. These results, along with significant differences in particular phyla and genera, collectively show that juvenile onset exercise is altering the abundance of specific taxa in a significant manner.

Early life exercise significantly modulated relative abundance of several phyla, whereas no changes at this taxonomy level were apparent in the adults. Exercise-induced changes at the phyla level in adult humans and rats have been previously reported (Clarke et al., 2014; Petriz et al., 2014). Differences in age of running onset, strain of rats, as well as length and duration of exercise regimen can account for this lack of an effect. We did observe, however, some changes at the genus level of taxonomy following adult onset exercise, as previously reported by others. Our results suggest that although adult onset exercise can modulate the abundance of a few genera, early onset exercise can alter overall phylogenetic structure of the microbial ecosystem.

Notably at the phyla level, early life exercise increased the relative abundance of Bacteroidetes and decreased Firmicutes. An increase in Bacteroidetes and complimentary decrease in Firmicutes may be reflective of a lean phenotype, and has been associated with adaptive metabolic consequences such as increased SCFA production, increasing energy expenditure, and inhibition of fat accumulation in adipose tissue (Ridaura et al., 2013). Conversely, an increased Firmicutes to Bacteroidetes ratio has been associated with obesity (Turnbaugh et al., 2009). Interestingly, in a recent study (Evans et al., 2014), exercise prevented high-fat diet induced weight gain and produced a similar pattern of increased relative abundance

of Bacteroidetes to Firmicutes. Importantly, in the current study, the microbial pattern reflective of a lean phenotype was only observed in the juvenile but not adult onset runners, indicating that the developmental stage during which exercise is initiated may be important for establishing this adaptive change in phyla. Additionally, there is evidence for persistent decreases in Firmicutes for the juvenile runners only ($p=0.061$; 25 days following exercise cessation), suggesting that the exercise-induced changes observed within juvenile runners are both robust and long lasting.

These phyla level changes are consistent with the types of phenotypic changes in body composition found in our juvenile runners. Juvenile onset runners but not adult runners had increases in lean body mass measured using chemical carcass analyses and *in vivo* MRI that persisted after running had stopped. Collectively, these data tentatively suggest a possible connection between early life bacterial composition and body mass throughout the lifespan. In support of this idea, previous work in human has detected a relationship between early life microbial composition and body mass later in life (Kalliomaki et al., 2008). Although mechanisms for how exercise-altered microbial composition can promote stable changes in lean mass were not investigated in this paper, several positive metabolic consequences associated with a higher Bacteroidetes to Firmicutes count could play a role. For instance, an exercise-altered microbial composition could promote SCFA production and thus enhance energy availability and expenditure, as well as reduce fat storage by modulating expression of angiopoietin-like protein 4 or ANGPTL4 and decreasing lipoprotein lipase mediated triglyceride uptake (Aronsson et al., 2010; Korecka et al., 2013), leading to adaptive changes in body composition. Given that a number of previous studies have found a strong positive relationship between lean body mass and Bacteroidetes, the relationship between early life Bacteroidetes abundance and body composition across the lifespan should be further explored.

At the genus level of taxonomy, supervised learning feature importance scores together with ANOVA analyses identified several specific genera implicated in emotional behavior that were significantly modulated by juvenile onset exercise. Among the genera identified using feature important scores, *Bifidobacteria spp.* have been linked to reducing anxiety (Bercik et al., 2011) and depressive-like behavior (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008). Also, ANOVA revealed that juvenile onset exercise significantly decreased genera within the Rikenellaceae family. Genera within this family have been associated with stressor exposure in rodents (Bangsgaard Bendtsen et al., 2012) and depression in humans (Naseribafrouei et al., 2014). Furthermore, juvenile onset exercise only significantly increased *Lactobacillus spp.* following just 3 days of exercise. Bacterial culture using *Lactobacillus spp.* specific media confirmed that *Lactobacillus spp.* and specifically *L. rhamnosus* was increased in juvenile runners early following the start of exercise. *Lactobacillus spp.* have been shown to modulate anxiety-like behavior as well as alter GABA receptor expression within the brain (Bravo et al., 2011). Interestingly, multiple studies have demonstrated that microbial presence during early life can have enduring impacts on behavior (Diaz Heijtz et al., 2011) and stressor reactivity (Sudo et al., 2004). In the current study, our data suggest that early life exercise can modulate bacteria capable of altering emotional behavior and mental health. Given the potential enduring impact of these species during early life, long lasting changes in emotional behavior should be investigated following early life exercise.

Feature importance scores together with ANOVA also identified several genera that have been shown by others to be important for metabolic functions as well as body composition. For example, *Bifidobacteria spp.* have also recently been associated with leanness in humans (Million et al., 2013). Among the patterns revealed by ANOVA, *Desulfovibrio spp.* were

decreased three days after juveniles began running. Species in this genera are enriched in people suffering from inflammatory bowel diseases (Berry & Reinisch, 2013). Long lasting patterns in *Anaerostipes spp.* were observed 25 days following exercise cessation in young runners, indicating that early life exercise is capable of inducing some long-lasting changes in the microbial composition. *Anaerostipes spp.* can produce beneficial SCFAs such as butyrate (Shwiertz et al., 2002), which can enhance energy metabolism as well as inhibit fat accumulation (Gao et al., 2009). Early life exercise also increased *Blautia spp.*, a genus associated with nutrient assimilation (Eren et al., 2014). Notably, supervised learning revealed that *Methanosphaera spp.* were significantly altered by early life exercise, and ANOVA further revealed that these species were increased. These species belong to the domain Archea and their main function is to utilize hydrogen as an energy source, thus providing an important method for disposing of this gas within the intestinal environment (Wilson, 2005). Increases in hydrogen within the gut can hinder the efficiency of microbial fermentation, thus *Methanosphaera spp.* can help provide more efficient carbohydrate metabolism and increase energy production. Taken together, our results suggest that early life exercise is capable of altering specific bacterial genera in a manner that can potentially benefit host metabolism.

The mechanisms by which exercise alters microbial composition are unclear. Other groups have speculated exercise-induced change in host physiology and intestinal environment may play a role. Evans et al. (2014) for example, suggest that exercise-induced increases in AMP-Activated Kinase (AMPK) may impact gut microbial composition. AMPK is an enzyme that senses cellular as well as systemic energy states in skeletal muscle, liver, and adipose tissue and is increased by exercise (Kahn, Alquier, Carling, & Hardie, 2005). It is possible that any of these AMPK-induced physiological changes can impact gut microbial composition.

Alternatively, Choi et al. (2013) propose that exercise-induced increases in bile acid secretion (Meissner et al., 2011) could also modulate gut microbial composition (Islam et al., 2011), perhaps through various antimicrobial functions. Thus, increases in AMPK or bile acids are both potential mechanisms for how gut microbial composition is altered following exercise.

One of the limitations of this study is that microbial composition was only measured in fecal samples. Although we have previously found no differences between cecal contents and fecal samples using 16S (Maslanik et al., 2012), it is possible that samples of adherent microbes from the intestinal lumen could have yielded different results (Haange et al., 2012). Another limitation of 16S is that relative abundance of taxa at the species level is difficult to investigate. Given that our findings indicate that early life exercise increases specific microbial taxa, analyses at the species level would be worthwhile. Finally, possible physiological consequences of altered microbial composition were not directly investigated. While body composition was explored, body mass and bacterial composition were measured in separate cohorts of rats, correlations as well as causal conclusions concerning the functional consequences of gut microbial community structure are not possible. In addition, SCFA production were not investigated and should be further explored in future studies.

The present study demonstrated the novel finding that exercise initiated during the early life is capable of having a more pronounced impact on the gut microbiota than exercise initiated in adulthood. These results suggest that environmental factors such as exercise, diet, probiotics, or others geared toward altering microbial ecology may be more effective if administered early in life.

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