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Effects of Broad-spectrum Antibiotic Treatment or Germ-free Status on Endurance Performance and Exercise Adaptations in Mice

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Abstract

Purpose: Endurance exercise alters the gut microbiome independently of diet. The extent to which gut microbes are responsible for physiologic adaptations to exercise training is unknown. The purpose of these experiments was to determine the role of gut microbes in performance and muscle adaptation to six weeks of voluntary wheel running (VWR) in mice.

Methods: We depleted microbes with broad-spectrum antibiotic treatment (ABX) and used germ-free (GF) mice to determine effects on adaptations to VWR. Male and female C57Bl/6 mice (n=56) were assigned to daily VWR or sedentary conditions. After the intervention, treadmill endurance and glucose tolerance were assessed, and gastrocnemius and soleus tissues were harvested and analyzed for citrate synthase (CS) enzyme activity and expression of exercise training-sensitive genes.

Results: ABX treatment and GF status resulted in VWR volumes ~22% and 26% lower than controls, respectively. Analysis of variance revealed that while VWR increased treadmill endurance, ABX had no effect. GF status significantly reduced treadmill performance in trained GF mice post-training. VWR increased gastrocnemius CS enzyme activity in all groups, and ABX

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and GF status did not reduce the VWR effect. VWR also increased muscle expression of PGC1a, but this was not affected by ABX treatment.

Conclusions: ABX treatment and GF status reduced VWR behavior but did not affect VWR-induced adaptations in endurance capacity, CS activity, or expression of muscle metabolic genes. However, GF status reduced endurance capacity. These data indicated that reducing microbes in adulthood does not inhibit endurance-training adaptations in C57Bl/6 mice, but that GF mice possess a reduced responsiveness to endurance exercise training, perhaps due to a developmental defect associated with lack of microbes from birth.

Keywords

ENDURANCE; EXERCISE; MICROBES; MICROBIOME; ANTIBIOTIC; GERM-FREE; ADAPTATION; PERFORMANCE

INTRODUCTION

Made up of a diverse consortium of microorganisms, the gut microbiota continually modulates numerous immune, metabolic, and nervous system functions of its mammalian host. Accordingly, disturbances to the gut microbiota (i.e. dysbiosis) have been associated with a wide variety of chronic health conditions (1–7). These findings highlight a critical need to expand our understanding of the gut microbiota and to identify interventions that may influence these microbes to improve human health.

Recent data have indicated the existence of a ‘gut-muscle axis’ whereby products of gut microbes affect muscle structure and function and, vice-versa, products of exercising muscle affect gut microbe physiology (8–15). Fecal microbial transplant from ‘high functioning’ older adults into germ-free (GF) mice increased grip strength in comparison to those receiving transplants from ‘low functioning’ individuals (8). Other studies have found exercise performance benefits utilizing various probiotic microbe strains (9–12). Scheiman et al. discovered that a strain of *Veillonella atypica,* which functions via lactate catabolism, increased around competition time in the feces of marathoners, and whose colonization into mice was capable of increasing treadmill running performance (12). Also supporting a ‘gut-muscle axis’, Lahiri et al. demonstrated that muscles from GF mice were atrophic, with impaired expression of genes associated with hypertrophy, mitochondrial function, and neuromuscular junctions, and that transplantation of an intact microbiota was able to mitigate many of these deficits (13). Moreover, various antibiotic treatment regimens have been shown to reduce muscle mass, endurance running performance, and *ex vivo* contractile capacity while increasing atrogene expression (14, 15). Taken together, current literature supports the notion that gut microbial products significantly impact muscular physiology and performance outcomes.

Participation in regular, moderate endurance exercise increases lean mass, reduces body fat, improves glucose uptake, and reduces the incidence of metabolic, inflammatory, and intestinal diseases by altering the biochemistry of a wide range of tissues including muscle, adipose, liver, brain, and gut (16–18). Human and animal studies from our laboratory indicate that endurance exercise alters the gut microbiome and increases the capacity of gut

microbes to produce short chain fatty acids (SCFA) such as acetate, propionate, and butyrate (19–21). SCFA are largely microbially-derived and exhibit numerous beneficial effects on metabolism (including as a source of energy), inflammation, and gut health, and while they have been shown to improve endurance performance, whether or not they contribute to exercise adaptation is unknown (12,22).

In this study, we aimed to determine the contribution of host microbiota to endurance exercise adaptations by comparing how young GF mice and mice treated with long-term broad-spectrum antibiotics (ABX) responded to 6 weeks of voluntary wheel running compared to control mice with an intact microbiota (CON). We hypothesized that GF and antibiotic-treated mice would exhibit reduced wheel running behavior and adaptations to wheel running compared to their conventionally raised counterparts with an intact gut microbiota.

METHODS

Animals and treatments

Adult six-week-old male (n = 20) and female (n = 20) specific pathogen free (SPF) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility (23). C57BL/6 GF male (n = 10) and female (n = 10) mice were reared in the Rodent Gnotobiotic Facility at the University of Illinois at Urbana-Champaign (UIUC) and were received for study at 6 weeks of age. After a 2-wk acclimation period followed by a baseline treadmill fatigue test, SPF and GF mice were randomized into either sedentary (CON) or voluntary wheel running (VWR) groups with equal numbers of males and females. All mice were housed individually and VWR mice had free access to telemetered running wheels (Med Associates, Fairfax, VT) for 6 weeks. Running data is represented through Day 31 (and not 42) because manipulations (i.e. IPGTT, treadmill testing) were performed after this time that reduced running behavior. There was no treatment bias to this effect. Before beginning the intervention, the SPF groups were further randomized into control or broad-spectrum antibiotic treatment (ABX) three days before and throughout the intervention. Broad-spectrum ABX treatment (vancomycin hydrochloride [4.5mg/kg/day], kanamycin sulfate [40mg/kg/day], ampicillin [33mg/kg/day], and metronidazole [21.5mg/kg/day] was delivered in the drinking water. Similar mixtures have been shown to significantly deplete microbes, especially within the gut and not cause overt sickness, weight loss, or systemic inflammation (24–26). Altogether, treatment groups consisted of four groups of SPF mice (CON, VWR, ABX, VWR/ABX) and 2 groups of GF mice (GF, GF/VWR). All mice were fed a commercial diet (Teklad 8640; Harlan Laboratories, Indianapolis, IN) with food and water intake tracked via disappearance ~weekly throughout the study. All mice were maintained on a 12:12 reverse (lights off at ~9am) light/dark cycle. Body weight was measured periodically during the SPF experiment, but not in GF mice due to risk for contamination. One week before post-testing, an intraperitoneal glucose tolerance test (IPGTT) was conducted as described below. All experiments were approved by the UIUC IACUC and adhered to American College of Sports Medicine animal care standards.

Euthanasia and sample collection

To minimize the influence of possible acute exercise-induced changes in the gut microbiome and muscle gene expression, SPF mice were euthanized ~48 hours after the post-intervention treadmill test via rapid CO_2 asphyxiation followed by cervical dislocation. Due to the risk of microbial contamination because the treadmill could not be introduced into the germ-free vivarium, GF mice were euthanized immediately (and not 48 h) after treadmill performance testing and the acute effects of exercise (versus training effects) on gene expression measures could not be separated making it hard to interpret the training effect. Upon euthanasia, gastrocnemius and soleus muscle, perigonadal fat, heart, and spleens were collected and/or weighed to gauge endurance training responses. Muscles were snap frozen in liquid N_2 and stored at −80°C.

Fecal DNA quantification and microbiome analysis

Fecal samples were collected from all mice at the end of the intervention period before post testing, and DNA was isolated from 60 mg of sample using a DNeasy powersoil pro kit (Qiagen, Germantown, Maryland), yielding 50 μl of DNA solution. Concentrations of DNA using 1 μl of resulting solutions were then quantified using a Qubit fluorometer with high sensitivity dsDNA reagents. The V4 region of the 16s rRNA gene was sequenced from fecal DNA obtained before (pre) and after (post) experimental treatments on a Miseq Nano. Using DADA2 for R, demultiplexed reads were truncated at 240 base pairs from each end and filtered at minimum quality score of 20. Identical sequences were then dereplicated, sequences were merged, and chimeras were removed. Taxonomy was then assigned as amplicon sequence variants (ASVs) using Silva 138.1 taxonomic database. Phyloseq Version 1.37 for R was used in all downstream analyses, and the GGplot package was used to make figures. In bacterial abundance measures, ASVs not present at least 3 times in 20% of the samples were filtered out. Alpha diversity was calculated via the Shannon index.

Intraperitoneal Glucose Tolerance Test (IPGTT)

After a 12h fast, mice were weighed, and baseline blood glucose was measured with a OneTouch Ultra Mini glucometer (Lifescan, Malvern, PA) using the tail-nick method to obtain blood. After baseline measurements, mice were injected intraperitoneally with a 20% glucose solution (2g of glucose/kg body mass) as follows: volume of glucose injection (μl) = 10 x body weight (g). Blood glucose was then measured 30, 60 and 90 min after injection.

Treadmill endurance performance test

A gold standard measure of endurance exercise adaptation is an improvement in endurance performance. On testing days, mice were placed on the treadmill and put through a protocol in which the speed started at 15m/min and increased 1m/min until 25m/min was reached (10 min), then held at 25m/min for 20 min until minute 30. Thereafter, speed was increased 1m/min every 5 min until exhaustion was reached. Sponges were placed at the back of the treadmill lane for tactile sensation and mice were occasionally prodded by researchers to ensure continued running. Exhaustion was defined as the point at which a mouse refused to run for 5 continuous seconds, or the point at which a mouse couldn’t keep pace with the

treadmill speed for 5 continuous seconds, even when prodded. All tests were done during the first 3 hours of the dark cycle.

Muscle Gene Expression

Total RNA was isolated from soleus (SPF only) and half of gastrocnemius muscle using the TRIzol and chloroform method, then reverse transcribed to cDNA using the Applied Biosystems High-Capacity Reverse Transcription Kit, according to the manufacturer's instructions. Primers were designed using ThermoFisher's Oligoperfect primer design program (Waltham, MA). Primers were validated using a 1:3 serial dilution made from pooled cDNA (5 standards, 1 blank), then run in duplicate on QuantStudio 5. Primers with an overall single peak in the melt curve and no primer dimers in the blank samples were further analyzed. Resulting cycle threshold (Ct) values per primer were plotted against the serial dilution to generate a standard curve, and primers that showed a linear trend line with an R^2 value greater than 0.95 were considered validated. For quantitative PCR (qPCR), cDNA samples were run in duplicate on QuantStudio 5 using validated SYBR Green primers, according to the manufacturer's instructions. Resulting qPCR data was analyzed using the ΔΔ Ct method, with the average of beta-actin and RPLP0 (ribosomal protein, large, P0) used as housekeeping genes.

Muscle Citrate Synthase (CS) Activity

Gastrocnemius muscles from all mice were cut in half, weighed and homogenized with a mortar and pestle on ice in ice cold lysis buffer (250 mM sucrose, 10 mM Tris base, and 1 mM EGTA, pH 7.4). Homogenates were then incubated on a shaker at 4°C for 1 hr. Protein quantification was performed using a Pierce BCA Protein Assay Kit according to manufacturer instructions (Thermo Scientific, Waltham, MA). Two microliters of homogenate were used to determine CS activity with an assay kit according to manufacturer instructions (#701040, Cayman Chemical). Raw CS activity values were then relativized to protein concentration in the homogenate.

Statistical Analyses

Separate analyses were done to compare the effects of antibiotic treatment (CON, VWR, ABX, VWR/ABX) or germ-free status (CON, VWR, GF, GF VWR) on all dependent variables. Primary outcomes for endurance adaptations included distance and time to fatigue on the treadmill endurance test and gastrocnemius citrate synthase activity. Univariate analysis of variance (ANOVA) with repeated measures was used to determine between and within-group differences in serial measures of body weight, food and fluid disappearance, running wheel distance, treadmill performance, blood glucose, and response to I.P. glucose challenge. General Linear Model univariate ANOVA's were performed on all other data. Post hoc comparisons were performed with Bonferroni correction for multiple comparisons. When the Shapiro-Wilk test revealed data did not meet the normality assumption, a Wilcoxon rank sum test was used. If data were normally distributed but Bartlett's test revealed them to be heteroscedastic, they were compared with Welch's T test. In instances where significant effects of sex (main effect or interaction) were observed, data are presented separately for males and females in Table 1 and supplemental figures 1 and 3 (see Supplemental Figure 1, Supplemental Digital Content, Food and water disappearance in

SPF mice, and Supplemental Figure 3, Supplemental Digital Content, Body weight changes over time for male and female SPF mice). Males and females are included together in all other figures and tables. Alpha was set at $p \leq 0.05$. All analyses were done with SPSS v27 (SPSS, Chicago, IL) and Rstudio v2022.02.3. Data are presented as mean ± SE.

RESULTS

Animal Health

Of the 60 mice that began the study, three mice died during experimentation (one GF VWR mouse had dermatitis and was euthanized, one CON and one ABX mouse died of unknown cause). Data from another mouse in the GF VWR group was excluded from the analyses because an orthopedic problem prevented it from using the running wheel during the intervention. Both VWR groups consumed more food over time than the CON group (see Supplemental Figure 1a, Supplemental Digital Content, Food and water disappearance in SPF mice). ABX treatment did not affect food intake in any group. While VWR and ABX both increased water disappearance, there was no difference in water disappearance between ABX and VWR/ABX indicating that these groups received a similar cumulative dose of antibiotics (see Supplemental Figure 1b, Supplemental Digital Content, Food and water disappearance in SPF mice).

Fecal DNA concentrations and microbiome

Fecal DNA concentration was measured as an indicator of the effectiveness of ABX treatment and GF status. Collapsed across VWR groups, both ABX and GF mice exhibited lower bacterial load as measured by fecal DNA concentrations when compared to CON ($p<0.05$, Figure 1a). Of note, broad spectrum ABX mice still contained a significant amount of fecal DNA, but previous studies have obtained similar results, and demonstrated significantly attenuated microbial community structure and activity (24–26). VWR did not significantly affect fecal DNA concentrations in SPF ($p=0.22$) or GF ($p=0.23$) mice. The small amount of DNA detected in the GF group is attributable to host, and not bacterial DNA, as all facility sterility of sentinel mice remained negative for microbes during this study. ABX treatment resulted in a highly significant decrease in alpha diversity in the Shannon Index ($p<0.001$, Figure 1b), but was not significantly affected by VWR ($p\sim=0.5$). ABX treatment resulted in a significant depletion of the top 40 taxa in many samples (Figure 1c).

Treadmill endurance performance

Treadmill performance was tested before and after the experiment in SPF mice, but pre-measures were not conducted in GF mice due to limitations of the germ-free environment and the potential for contamination using the motorized treadmill (Figure 2). In all groups of SPF mice, VWR increased time run, distance run, and maximal running speed ($F_{1,34}$ = 169; $F_{1,34}$ = 285; $F_{1,34}$ = 180, all $p < 0.001$ for time run, distance run, and maximal running speed, respectively), indicating that ABX did not hinder performance improvements induced by endurance training (Figure 2a–c). While ABX-treated mice performed numerically lower (~5–13% reduction dependent on measure) on the treadmill test post-training, this difference was not statistically significant (time x ABX group: estimated p's ~ 0.33 – 0.38) and was

consistent with the lower volume of training done in the ABX group (Figure 5a). Thus, ABX treated sedentary and endurance trained mice performed similarly to control mice before and after training.

For the GF mice, there was a technical error with the calibration of the motorized treadmill discovered after testing such that only 7 mice (4 GF, 3 GF/WVR) tested at the same speeds as the SPF mice. The 12 other GF mice were tested with a higher intensity protocol, where speed at every timepoint was doubled. While these mice did exhibit significant performance improvements because of VWR in this faster test (not shown), the error made direct comparisons between results from these mice and the SPF mice inappropriate. In the 7 mice tested at the same speed as SPF mice, GF VWR exhibited significant improvements in time to fatigue and maximal running speed when compared to GF SED. There were improvements in distance to fatigue, though not significant (p=0.06). When comparing results to SPF mice, the magnitude of improvement was lower in GF mice when compared to SPF mice (Figure 2a–b).

Citrate Synthase Activity

Gastrocnemius CS enzyme activity (Figure 3) increased in both SPF and GF mice in response to VWR (VWR main effect $F_{1,29} = 4.4$, $p = 0.04$ and $F_{1,32} = 7.6$, $p = 0.01$, for ABX and GF comparisons, respectively). There were no ABX, GF, or interaction effects noted indicating that ABX or GF status did not affect the VWR-induced increase in CS activity. There was, however, a tendency for GF mice to have lower gastrocnemius CS activity, driven mainly by the sedentary GF group.

Body and organ weights

VWR reduced body weight gain in male SPF mice when compared to SPF control and ABX groups, but this did not occur in VWR/ABX mice (see Supplemental Figure 2a, Supplemental Digital Content, Effects of wheel running and broad-spectrum antibiotic treatment on body weight over time in males and females). Both GF groups gained less weight than all SPF groups and this was not affected by wheel running. In females (see Supplemental Figure 2b, Supplemental Digital Content, Effects of wheel running and broad-spectrum antibiotic treatment on body weight over time in males and females), wheel running did not affect body weight change in either SPF or GF mice. Changes in absolute body weight by sex over time in SPF mice can be found in Supplemental Figure 3 (Supplemental Digital Content). ABX treatment reduced perigonadal fat weight (ABX $F_{1,30} = 34.2$; $p = 0.000$) and this effect was greater in males than females (ABX x sex $F_{1,30} = 6.0$; $p = 0.02$). Perigonadal fat pads were smaller in females when compared to males across all groups except for VWR/ABX (sex $F_{1,30} = 16.1$; $p = 0.000$, Table 1). SPF females had lower heart weights than males (sex $F_{1,30} = 14.8$; $p = 0.0001$) and realized an (VWR x sex $F_{1,30} = 6.4$; $p = 0.02$) increase in heart weight in response to VWR, while males did not (Table 1). ABX had no effect on heart weight. GF mice had lower absolute (GF $F_{1,30} = 56.7$; $p = 0.000$) and relative (GF $F_{1,30} = 72.5$; $p < 0.000$) heart weights when compared to SPF mice with the exception of female GF. VWR increased (GF x VWR x Sex $F_{1,30} = 7.1$; $p = 0.01$) relative heart weight in GF males, whereas VWR increased it in SPF females.

Muscle gene expression

Gene expression analysis in the gastrocnemius of SPF mice (Table 2a) revealed training-induced increases in *PGC1α*, while expression of *GLUT*4 and *MCT*4 decreased. ABX treatment had no effect on these changes. MCT1, LC3, SDH and PPARγ mRNA was similar between groups. In the soleus (Table 2b), citrate synthase and SDH were increased by VWR and there was no effect of ABX on this increase. No treatment effects were seen in *PGC1α*, *MCT1*, *GLUT4*, *PPARγ*, and *FBXO32* mRNA expression. In GF mice, gene expression data was confounded by the fact that muscle was taken immediately after a treadmill test to exhaustion (due to potential microbial contamination of waiting 24–48h after the last running session) and VWR training effects cannot be interpreted. We present the data (see Supplemental Table 1, Supplemental Digital Content, Gene expression in gastroc muscles of germ-free mice when compared to sedentary SPF control values), to demonstrate the GF mice are capable of altered gene expression of key genes in response to acute exercise; *PGC1 α* (~3-fold), *PPARγ* (~50–60-fold), and LC3 (~1.5-fold) mRNA increased, while *MCT1* and *GLUT4* decreased.

Intraperitoneal Glucose Tolerance Test and Fasting Glucose

ABX treated mice had lower (~20%) fasting blood glucose ($F_{1,34} = 9.8$; $p = 0.004$) than non-ABX groups (Figure 4a). Blood glucose increased at the 30 and 60-minute time points in response to the I.P. challenge in SPF mice and VWR nor ABX treatment affected this response. Additionally, ABX had no effect on glucose clearance when calculated as AUC (subtracting pre) (see Supplemental Figure 4, Supplemental Digital Content, Total AUC for 30, 60, and 90 minute timepoints during intraperitoneal glucose tolerance test). In GF mice (Figure 5b and Supplemental Figure 4, Supplemental Digital Content), VWR had no effect on either fasting glucose or glucose tolerance. However, GF mice had lower glucose tolerance to the I.P. challenge (main effect: $F_{1,33} = 10.7$; $p = 0.003$. Time: $F_{3,99} = 7.7$; $p < 0.001$) when compared to SPF mice. These measures were unaffected by sex.

Voluntary wheel running volume and circadian running

Analysis of running wheel distance (Km/day) indicated that, while treatment group alone did not affect running distance ($F_{1,24} = 2.56$, $p = 0.10$), daily running distance increased over time ($F_{30,720} = 14.5$; $p < 0.001$) and this effect differed based on treatment group (interaction $F_{30,720} = 1.65$; $p = 0.002$; Figure 5). Throughout the intervention, VWR mice ran more than VWR/ABX and GF VWR mice. There were no differences in running behavior between the latter two groups. Cumulative running distance across the training was ~78% and ~74% that of VWR (for VWR/ABX and GF VWR mice, respectively). As there have been reports of circadian rhythm disruption in ABX-treated (27) and GF (28) mice, we analyzed circadian running patterns expressed as percent running in the light (see Supplemental Figure 5, Supplemental Digital Content, Effects of ABX or germ-free status on diurnal wheel running behavior). We observed altered diurnal running patterns in GF mice exhibited by increased time spent running during the light cycle on 6 out of the first 17 days of running, but none thereafter (see Supplemental Figure 5, Supplemental Digital Content). Diurnal running decreased over time in all groups.

DISCUSSION

Emerging ‘gut-muscle axis’ literature implicates ingestion of antibiotics and germ-free status as mediators of skeletal muscle physiology (13,15). The goal of this study was to determine the role of the gut microbiota in mediating endurance exercise performance and skeletal muscle adaptations to exercise training in mice. Contrary to our hypothesis, chronic treatment with broad-spectrum antibiotics during a six-week endurance exercise intervention did not significantly attenuate performance or skeletal muscle adaptations to endurance exercise. Further, sedentary GF mice performed similarly to untrained control mice, but did not perform as well as SPF mice after training. These results indicate that, while lack of microbes may affect development of optimal muscular adaptive capacity from birth, their reduction or absence in adult mice during a training period, did not reduce it enough for there to be comprehensively significant reductions in adaptation to exercise training. However, the significant reduction in time to fatigue observed in wheel-trained GF mice in comparison to SPF controls indicates that the developmental deficits induced by the absence of microbes from birth inhibit endurance training-induced performance adaptations.

Results from previous studies that have investigated the role of gut microbiota in modifying exercise performance may be conflicting due to heterogeneous performance testing protocols. While Lahiri et al saw significant reductions in grip strength, a treadmill test to exhaustion at 15m/min did not reveal any significant differences between untrained SPF, GF, or conventionalized (GF transplanted with microbes) animals (13). In contrast, Huang et al (29) and Nay et al (15) found significant reductions in comparatively shorter duration, higher intensity exercise performance tests in GF mice on a forced swim test, and antibiotic-treated mice on a treadmill test, respectively. The ‘fast twitch’ nature of these higher intensity protocols relative to our treadmill performance test may indicate greater dependence on utilization of muscular glycogen stores, as they become the body’s primary fuel source during high intensity exercise (30). Also, of note, a study by Okamoto et al found that antibiotics were able to significantly reduce treadmill performance on a low intensity test of much greater duration than all aforementioned studies (31). However, this test was conducted after a 6 h fast, which likely reduced available energy stores and resulted in all mice reaching exhaustion in similar time periods to our study while running at considerably lower speeds.

While Lahiri et al found significant reductions in markers of mitochondrial functional capacity in germ free mice, these deficits did not translate to significant detriments in treadmill performance or abnormalities in expression of genes associated with fatty acid oxidation (13). Likewise, in our study, while tendencies for reduced treadmill endurance and CS activity in untrained GF mice suggests reduced muscular oxidative capacity, our data on CS activity and gene expression in VWR trained animals indicate they seem capable of responding to endurance exercise by increasing muscle oxidative capacity. While glucose metabolism was not directly measured in the present study, our data showing failure in the later, higher intensity stages of the treadmill testing protocol, indicate that detriments in endurance performance due to antibiotic-induced microbial depletion and/or germ free status may only be pronounced when the required exercise workload is great enough such that oxidative pathways of energy production cannot sufficiently compensate for impaired

utilization of glucose and glycogen (13,15,32). This conclusion is further supported by our findings that GF mice exhibit reduced capacity to take up and utilize glucose from circulation in the IPGTT, and the work of Houghton et al (32), who found that microbially-derived phenolic compounds were capable of potently inducing adaptations in muscular glucose metabolism *in vitro.*

While Lahiri et al found that low-dose penicillin treatment down-regulated electron transport chain gene expression in SPF mice compared to controls, endurance performance was not measured, nor was an effect at the protein level seen (13). In addition, our data on CS activity suggests the effect may not result in a functional change. An important caveat in comparing our study with studies like those of Manickam et al that show that muscle expression of oxidative genes of SPF and GF mice are impacted by ABX, is that they used doses roughly 10x those used in our study, which could confer differential effects on the recipient (14). It may be that none of the mitochondrial biogenesis cascades such as PGC1α or NRF require metabolites from the gut microbiota to adequately respond to intramuscular stimuli such as high concentrations of calcium, AMP/ATP, nitric oxide, or peroxide (33).

A recent study by Valentino et al. (26) demonstrated that antibiotic treatment prior to and during a weighted wheel running intervention blunted hypertrophic responses, indicating a potential role for gut microbes in muscle anabolic processes. An important caveat in this study is that differences in the strength of the animals was not measured, thus conclusions on adaptations in functional performance capacity cannot be made (26). Work of this nature suggests the possibility that the contribution of gut microbial metabolites may also be of greater influence in interventions targeting muscular atrophy and hypertrophy.

Our data does not eliminate the possibility that targeted manipulation of specific microbial strains might potentially affect endurance exercise capacity or adaptation to exercise interventions. Multiple preclinical studies have demonstrated the capacity of probiotic administration to induce improvements in high intensity endurance performance, grip strength, and body composition in the absence of an exercise intervention in SPF mice (9, 10, 12). The major byproducts of successful probiotic strains have been lactate and propionate, which, in addition to acetate and butyrate have been identified as viable fuel sources for multiple tissues which may aid in endurance exercise performance or adaptation (31, 34–39). Such findings demonstrate a potential role for gut microbes in supporting optimal exercise performance and adaptation, and represent limitations in the current work. Microbial metabolite production was not directly measured or investigated. Investigation of gut microbial composition was limited by sample size, meaning adequate sensitivity was not present to conduct adequate comparisons and/or correlations between the gut microbiome and study outcomes.

The microbiota may also affect the central nervous system through the gut-brain axis, including areas involved in voluntary exercise (40–42). Antibiotic treatment initiated amid a VWR period has previously been shown to reduce wheel running in mice bred for their high wheel running activity, but this effect was not replicated in a control group of conventional mice (43). Another significant aspect and potential limitation of the present study is that, using this volitional wheel-running model, we found that antibiotic treatment reduced

cumulative wheel running behavior by 22% and that GF mice ran cumulatively 26% less than SPF. However, both ABX-treated and GF mice ran over 10Km per day and these mice still exhibited significant improvements in forced exercise performance and biochemical changes in muscle. Taken together, these data support the notion that gut microbes may influence motivation for voluntary exercise via an undefined gut-brain circuit, presenting important targets for future studies.

CONCLUSIONS

We hypothesized that antibiotic-treated and germ-free mice would display attenuated adaptations to endurance exercise in comparison to controls. Our data leads us to disprove our hypothesis, with the caveat that microbes may be important in exercise motivation and in certain aspects of performance/adaptation dependent on the developmental state of the organism. Practically speaking, if an athlete must go on antibiotics during a period approaching a competition, our pre-clinical data suggest that it may not severely impede exercise-induced adaptations or performance. With that said, the long duration of broad-spectrum antibiotic treatment given to mice in this study does not mimic typical human antibiotic treatment regimens which last only a week or two. Future studies investigating shorter duration, more targeted antibiotic treatment in human athletes could provide more practically relevant results. In addition, studies assessing adaptations in capacity for short duration, high intensity exercise performance (weight training, sprint or HIIT), which would rely on different patterns of muscle recruitment and energy production and be influenced by other patterns of substrate partitioning, could help to inform a greater population of athletes. Lastly, our data in GF mice indicate that exposure to microbes at critical times of muscular development may be important in adaptations to exercise in adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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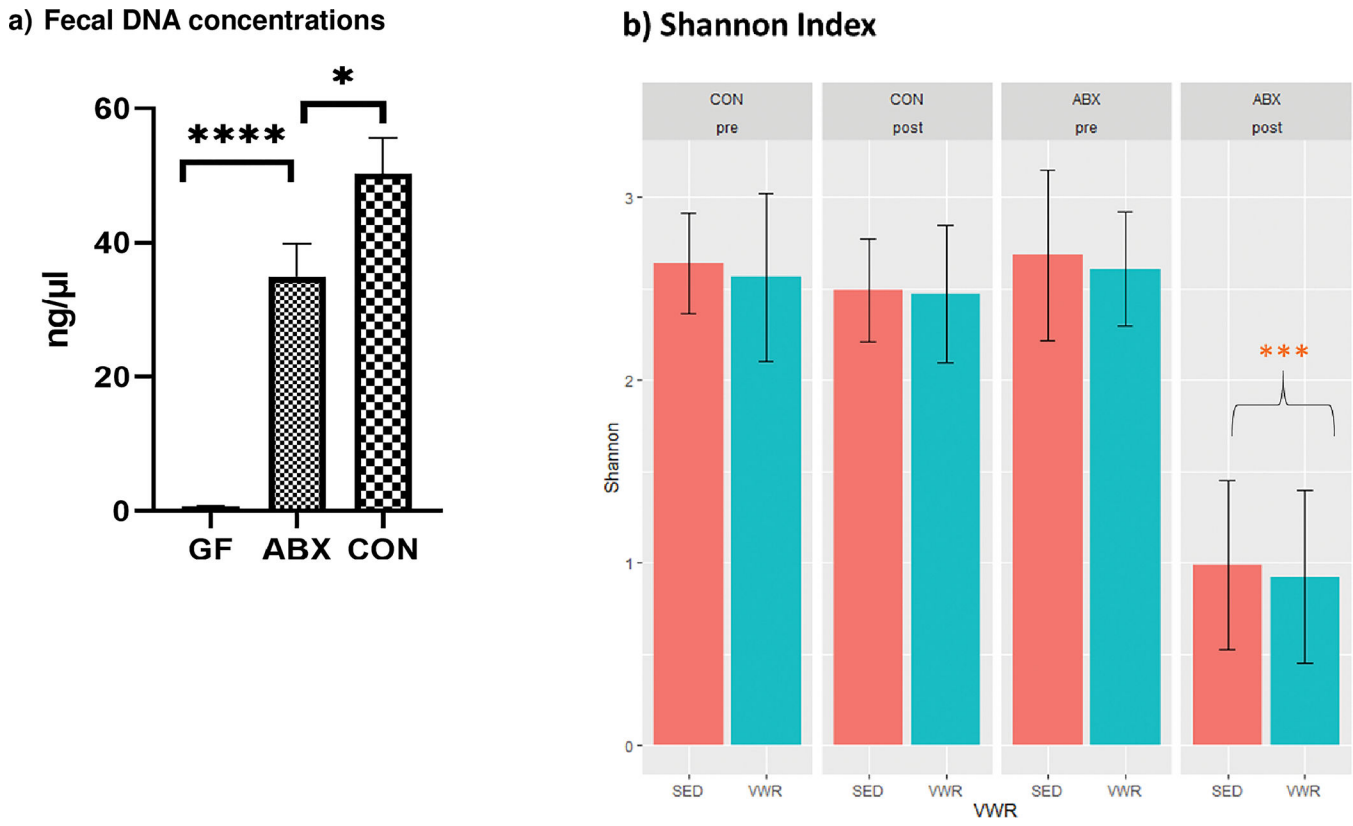
REFERENCES

1. Liu BN, Liu XT, Wang JH. Gut microbiota in obesity. World J Gastroenterol. 2021;27(25):3837–50. [PubMed: 34321848]
2. O'Keefe SJ, Ou J, Aufreiter S, et al. Products of the colonic microbiota mediate the effects of diet on colon cancer risk. J Nutr. 2009;139(11):2044–8. [PubMed: 19741203]
3. Lamas B, Richard ML, Leducq V, et al. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. Nat Med. 2016;22(6):598–605. [PubMed: 27158904]
4. Cait A, Hughes MR, Antignano F, et al. Microbiome-driven allergic lung inflammation is ameliorated by short-chain fatty acids. Mucosal Immunol. 2018;11(3):785–95. [PubMed: 29067994]

5. Dopkins N, Nagarkatti PS, Nagarkatti M. The role of gut microbiome and associated metabolome in the regulation of neuroinflammation in multiple sclerosis and its implications in attenuating chronic inflammation in other inflammatory and autoimmune disorders. Immunology. 2018;154(2):178–85. [PubMed: 29392733]
6. Gerhardt S, Mohajeri MH. Changes of colonic bacterial composition in Parkinson's disease and other neurodegenerative diseases. Nutrients. 2018;10(6):708. [PubMed: 29857583]
7. Lucas S, Omata Y, Hofmann J, et al. Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss. Nat Commun. 2018;9(1):55. [PubMed: 29302038]
8. Fielding RA, Reeves AR, Jasuja R, Liu C, Barrett BB, Lustgarten MS. Muscle strength is increased in mice that are colonized with microbiota from high-functioning older adults. Exp Gerontol. 2019:127:110722. [PubMed: 31493521]
9. Chen YM, Wei L, Chiu YS, et al. Lactobacillus plantarum TWK10 supplementation improves exercise performance and increases muscle mass in mice. Nutrients. 2016;8(4):205. [PubMed: 27070637]
10. Lee MC, Hsu YJ, Ho HH, et al. Lactobacillus salivarus subspecies salicinius SA-03 is a new probiotic capable of enhancing exercise performance and decreasing fatigue. Microorganisms. 2020;8(4):545. [PubMed: 32283729]
11. Munukka E, Rinatala A, Toivonen R, et al. Faecalibacterium prausnitzii treatment improves hepatic health and reduces adipose tissue inflammation in high fat fed mice ISME J. 2017;11(7):1667–79. [PubMed: 28375212]
12. Scheiman J, Luber JM, Chavkin TA, et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. Nat Med. 2019;25(7):1104–9. [PubMed: 31235964]
13. Lahiri S, Kim H, Garcia-Perez I, et al. The gut microbiota influences skeletal muscle mass and function in mice. Sci Transl Med. 2019;11(502):eaan5662. [PubMed: 31341063]
14. Manickam R, Oh HYP, Tan CK, Paramilangam E, Wahli W. Metronidazole causes skeletal muscle atrophy and modulates muscle chronometabolism. Int J Mol Sci. 2018;19(8):2418. [PubMed: 30115857]
15. Nay K, Jollet M, Goustard B, et al. Gut bacteria are critical for optimal muscle function: a potential link with glucose homeostasis. Am J Physiol Endocrinol Metab. 2019;317(1):E158–E171. [PubMed: 31039010]
16. Neufer PD, Bamman MM, Muoio DM, et al. Understanding the cellular and molecular mechanisms of physical activity-induced health benefits. Cell Metab. 2015:22(1):4–11. [PubMed: 26073496]
17. Packer N, Hoffman-Goetz L, Ward G. Does physical activity affect quality of life, disease symptoms and immune measures in patients with inflammatory bowel disease? A systematic review. J Sports Med Phys Fitness. 2010;50(1):1–18. [PubMed: 20308966]
18. Boyle T, Heyworth J, Bull F, et al. Timing and intensity of recreational physical activity and the risk of subsite-specific colorectal cancer. Cancer Causes Control. 2011;22(12):1647–58. [PubMed: 21922204]
19. Allen JM, Mailing LJ, Cohrs J, et al. Exercise training-induced modification of the gut microbiota persists after microbiota colonization and attenuates the response to chemically-induced colitis in gnotobiotic mice. Gut Microbes. 2018;9(2):115–30. [PubMed: 28862530]
20. Allen JM, Berg Miller ME, Pence BD, et al. Voluntary and forced exercise differentially alters the gut microbiome in C57BL/6J mice. J Appl Physiol (1985). 2015;118(8):1059–66. [PubMed: 25678701]
21. Allen JM, Mailing LJ, Niemiro GM, et al. Exercise alters gut microbiota composition and function in lean and obese humans. Med Sci Sports Exerc. 2018;50(4):747–57. [PubMed: 29166320]
22. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short chain fatty acids in health and disease. Adv Immunol. 2014;121:91–119. [PubMed: 24388214]
23. AAALAC International Web site [Internet]. Frederick (MD): AAALAC; [cited 2022 Jul 6]. Available from: www.aaalac.org.

24. Hernandez-Chirlaque C, Aranda CJ, Ocon B, et al. Germ-free and antibiotic-treated mice are highly susceptible to epithelial injury in DSS colitis. J Crohns Colitis. 2016;10(11):1324–35. [PubMed: 27117829]
25. Koh A, Molinaro A, Stahlman M, et al. Microbially produced imidazole propionate impairs insulin signaling through mTORC1. Cell. 2018;175(4):947–61.e17 [PubMed: 30401435]
26. Valentino TR, Vechetti IJ, Mobley CB, et al. Dysbiosis of the gut microbiome impairs mouse skeletal muscle adaptation to exercise. J Physiol. 2021:599(21):4845–63. [PubMed: 34569067]
27. Kuang Z, Wang Y, Li Y, et al. The intestinal microbiota programs diurnal rhythms in host metabolism through histone deacetylase 3. Science. 2019; 365(6460):1428–34. [PubMed: 31604271]
28. Leone V, Gibbons SM, Martinez K, et al. Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. Cell Host Microbe. 2015;17(5):681–89. [PubMed: 25891358]
29. Huang WC, Chen YH, Chuang HL, Chiu CC, Huang CC. Investigation of the effects of microbiota on exercise physiological adaption, performance, and energy utilization using a gnotobiotic animal model Front Microbiol. 2019;10:1906. [PubMed: 31551939]
30. Murray B, Rosenbloom C. Fundamentals of glycogen metabolism for coaches and athletes. Nutr Rev. 2018;76(4):243–59. [PubMed: 29444266]
31. Okamoto T, Morino K, Ugi S, et al. Microbiome potentiates endurance exercise through intestinal acetate production. Am J Physiol Endocrinol Metab. 2019;316(5):E956–E966. [PubMed: 30860879]
32. Houghton MJ, Kerimi A, Mouly V, et al. Gut microbiome catabolites as novel modulators of muscle cell glucose metabolism. FASEB J. 2018;33(2):1887–98. [PubMed: 30183376]
33. Jornayvaz FR, Shulman GL. Regulation of mitochondrial biogenesis. Essays Biochem. 2010;47:69–84. [PubMed: 20533901]
34. Hui S, Ghergurovich JM, Morscher RJ, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017;551(7678):115–8. [PubMed: 29045397]
35. Rabinowitz JD, Enerbäck S Lactate: the ugly duckling of energy metabolism. Nat Metab. 2020;2(7):566–71. [PubMed: 32694798]
36. Weidemann MJ, Hems R, Williams DL, Spray GH, Krebs A. Gluconeogenesis from propionate in kidney and liver of the vitamin B12-deficient rat. Biochem J. 1970;117(1):177–81. [PubMed: 5420952]
37. Stanley WC, Wisneski JA, Gertz EW, Neese RA, Brooks GA. Glucose and lactate interrelations during moderate-intensity exercise in humans. Metabolism. 1988;37(9):850–8. [PubMed: 3138512]
38. Wongkittichote P, Cunningham G, Summar ML, et al. Tricarboxylic acid cycle enzyme activities in a mouse model of methylmalonic aciduria. Mol Genet Metab. 2019;128(4):444–51. [PubMed: 31648943]
39. Donohoe DR, Garge N, Zhang X, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011;13(5):517–526. [PubMed: 21531334]
40. Jadhav KS, Peterson VL, Halfon O, et al. Gut microbiome correlates with altered striatal dopamine receptor expression in a model of compulsive alcohol seeking. Neuropharmacology. 2018;141:249–59. [PubMed: 30172845]
41. Garcia-Cabrerizo R, Carbia C, O'Riordan KJ, Schellekens H, Cryan JF. Microbiota-gut-brain axis as a regulator of reward processes. J Neurochem. 2021;157(5):1495–524. [PubMed: 33368280]
42. Hamamah S, Aghazarian A, Nazaryan A, Hajnal A, Covasa M. Role of Microbiota-Gut-Brain Axis in Regulating Dopaminergic Signaling. Biomedicines. 2022;10(2):436. [PubMed: 35203645]
43. McNamara MP, Cadney MD, Castro AA, et al. Oral antibiotics reduce voluntary exercise behavior in athletic mice. Behav Processes. 2022;199:104650. [PubMed: 35504410]



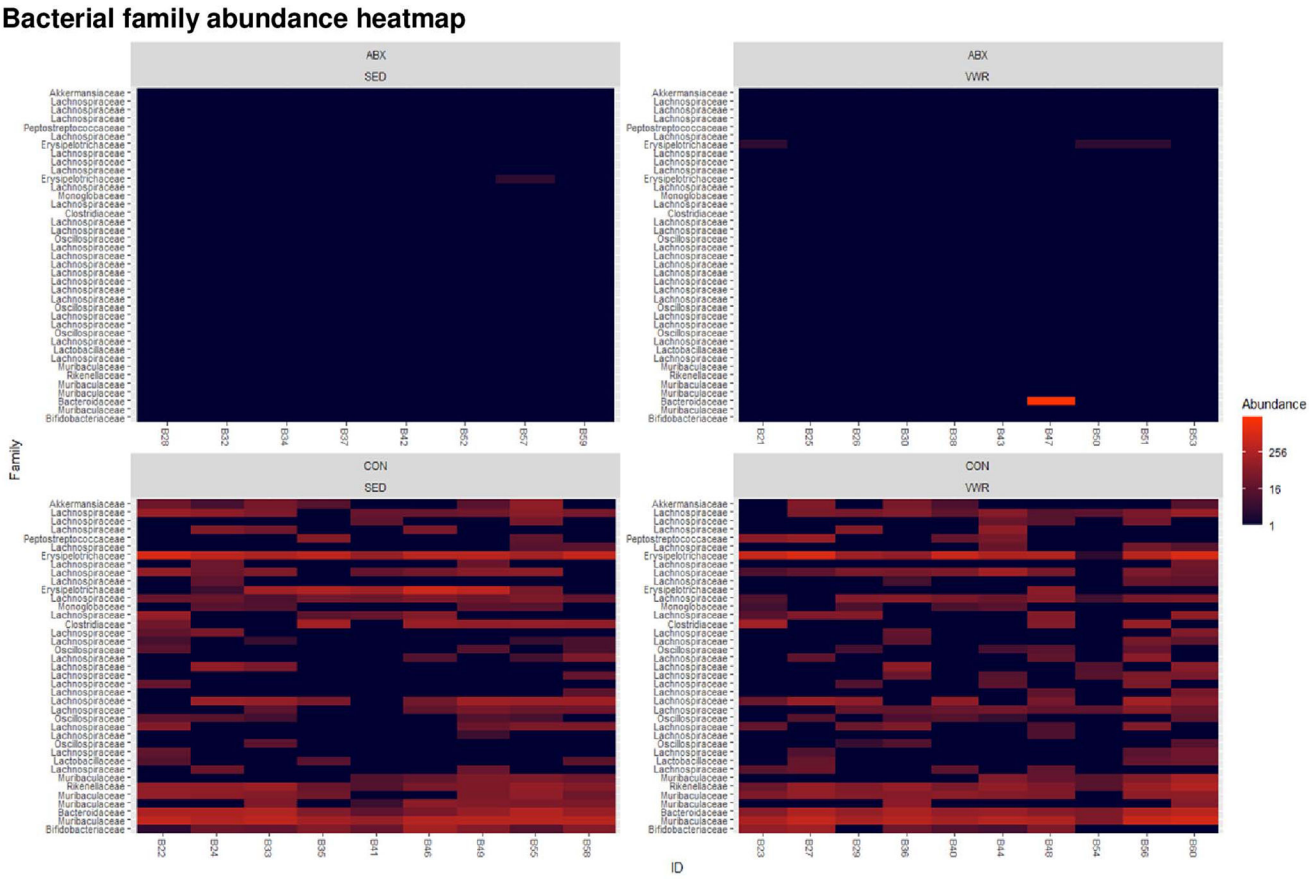


Figure 1. Effects of ABX treatment or germ-free status on fecal DNA concentrations and the fecal microbiome.

a) ABX treatment (* = p <0.05) and GF (**** = p<0.0001) status both resulted in significantly decreased fecal DNA concentrations. VWR did not significantly affect DNA concentrations in SPF (p = 0.22) or GF (p = 0.23) mice. Data are mean ± sem with n = 16, 18, and 19 for GF, ABX, and CON respectively. b) Fecal alpha diversity pre- and post-intervention. ABX resulted in a significant decrease the Shannon index (p<0.001). Neither index was significantly affected by VWR. N = 8–10/group. ***p<0.001. c) bacterial family abundance heatmap indicates potent depletion of the top 40 families in both sedentary and VWR mice.

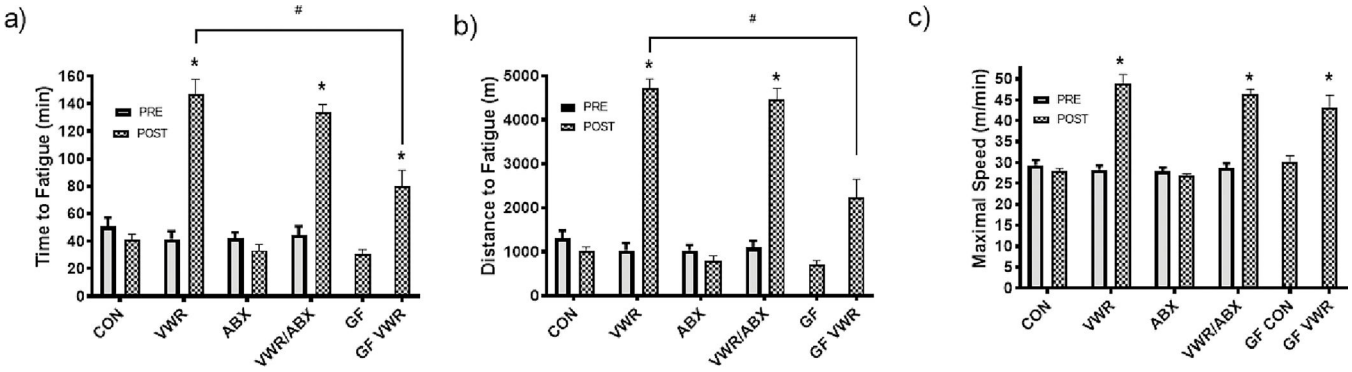


Figure 2. Effects of broad-spectrum antibiotic treatment and germ-free status on treadmill endurance performance in mice.

Endurance exercise training led to significant (*) improvements in time to fatigue (2a) and maximal running speed (1c) in both SPF and GF mice, and distance run (2b) in SPF mice. There was no effect of broad-spectrum ABX treatment on any performance measure. After VWR training, GF mice exhibited significantly reduced performance when compared to SPF mice (#). *significant difference relative to pre or GF (in the case of GF VWR); #significant difference between GF VWR and VWR. Data are mean ± sem for n = 9, 10, 9, 10, 4, and 3 for CON, VWR, ABX, VWR/ABX, GF, and GF VWR, respectively.

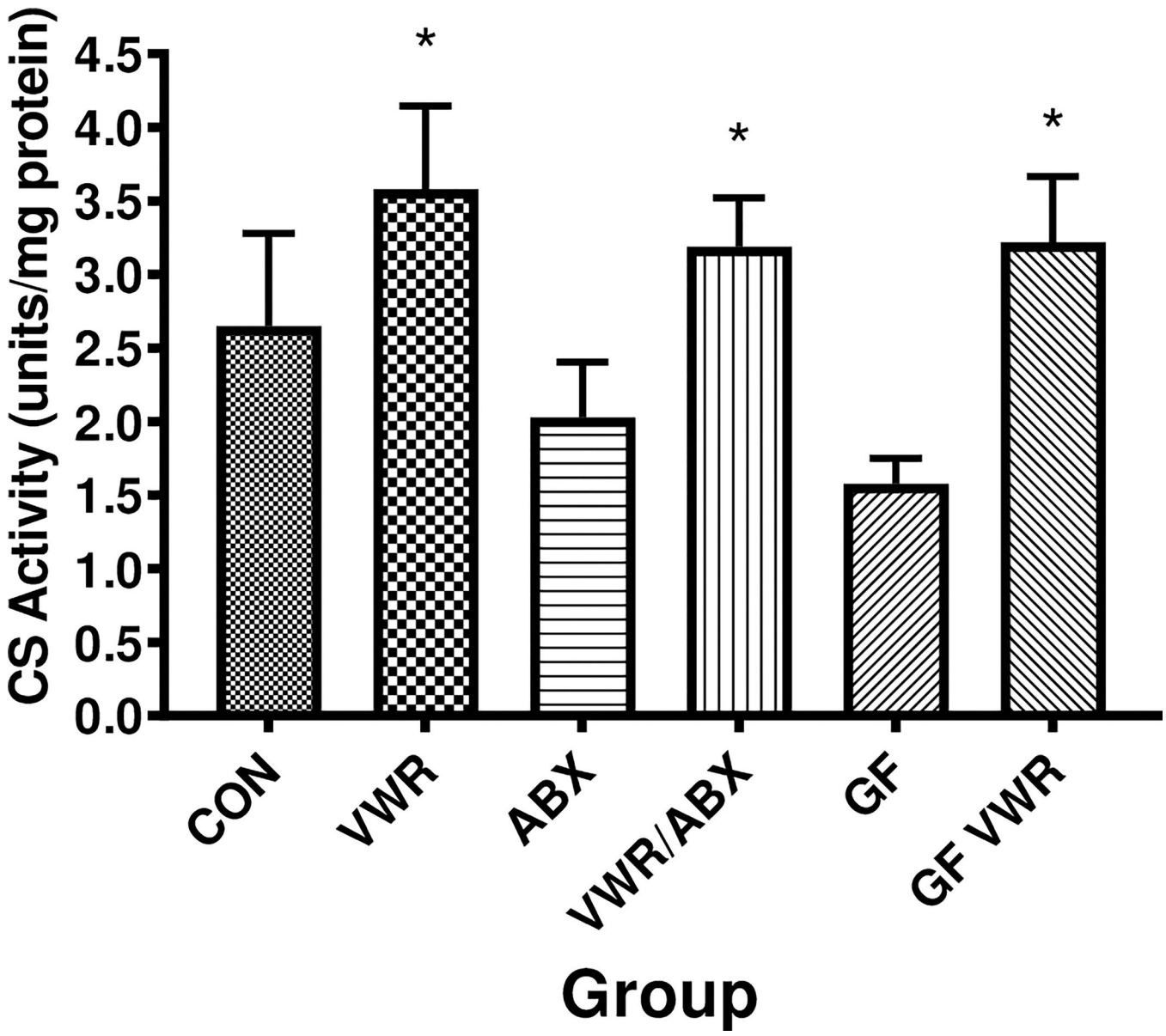


Figure 3. Effects of ABX and germ-free status on gastrocnemius citrate synthase activity.
Endurance training led to significant (*) increases in CS activity in both SPF and GF mice. However, there were no interaction effects indicating that SPF and GF mice improved similarly in response to VWR. There was a tendency ($p = 0.13$) for CS activity to be lower in GF mice, driven mainly by GF CON. Data are mean ± sem; $n = 8, 7, 9, 9, 10$, and 9 for CON, ABX, VWR, VWR/ABX, GF CON, and GF VWR, respectively. *indicates VWR groups different from respective controls.

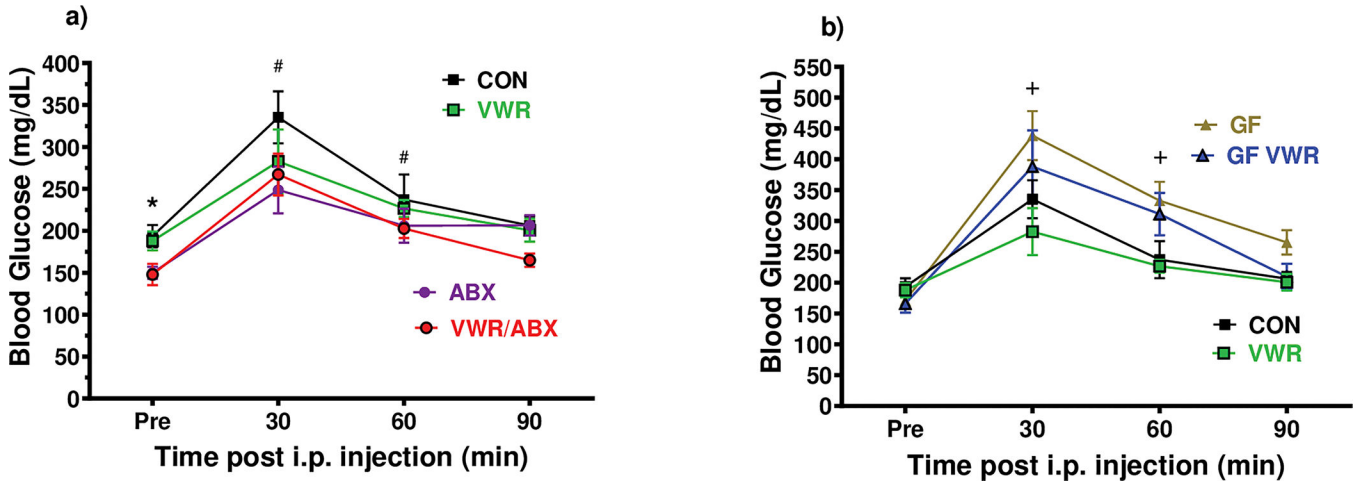


Figure 4. Antibiotic (a) and germ-free (b) effects on fasting glucose and glucose tolerance.

a) *There was a significant ABX-induced reduction in fasting glucose. #There was a significant time main effect such that glucose values were higher at 30- and 90-minute post-injection. However, ABX treatment did not affect the glucose response. b) +indicates a significantly higher glucose values in the GF mice when compared to SPF controls (*shown in* Figure 4a). Data are mean ± sem; n = 9, 10, 8, 10, 10, and 9 for CON, ABX, VWR, VWR/ABX, GF, and GF VWR, respectively.

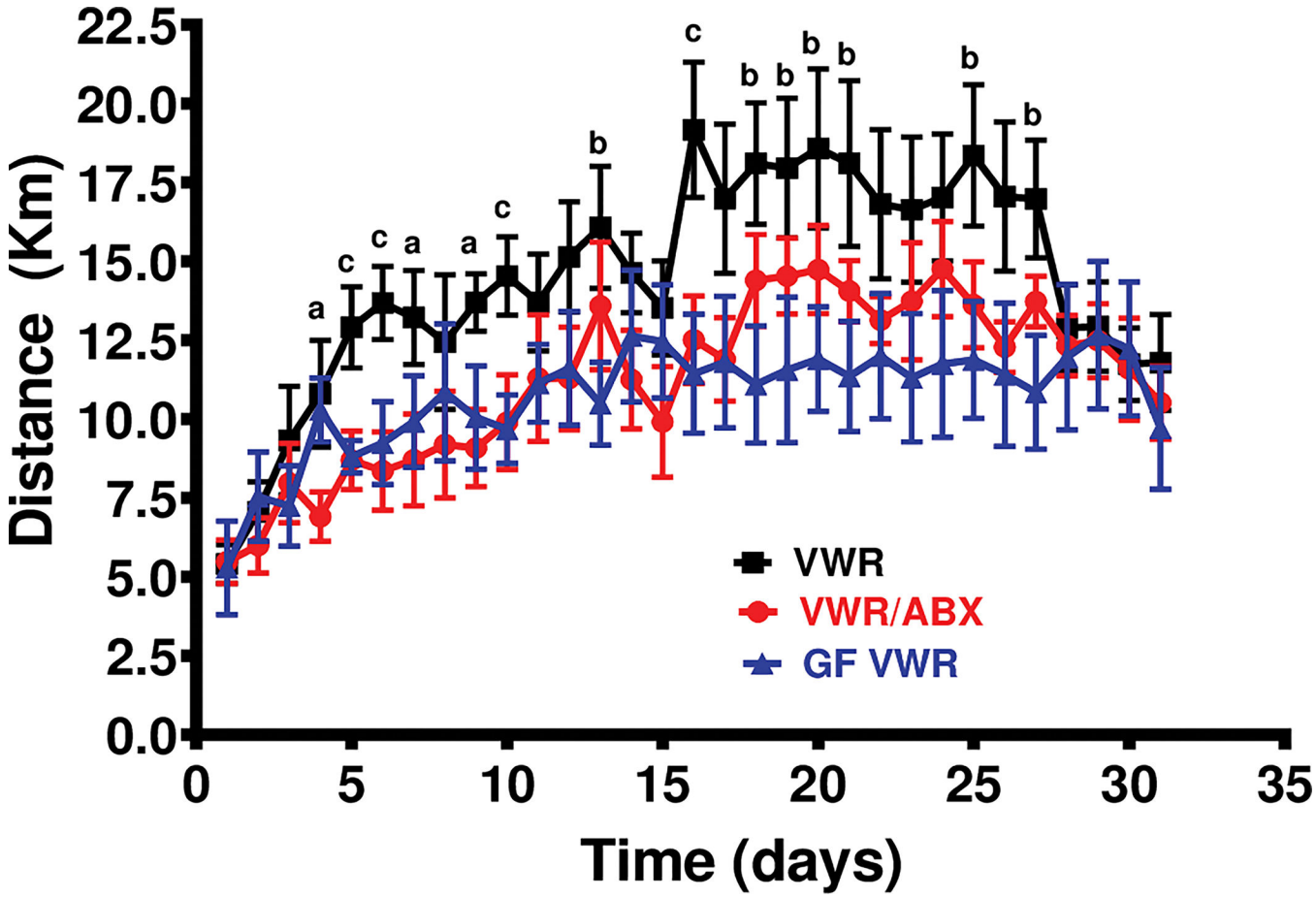


Figure 5. Effects of ABX or germ-free status on daily wheel running behavior.

There were significant ($p < 0.05$) time and time x group effects, but no main effect of group. [a]represents a significant difference between VWR and VWR/ABX; [b]a significant difference between VWR and GF VWR, and [c]a significant difference between VWR and both groups on a given day. Cumulatively, VWR/ABX and GF VWR ran ~78% and ~74% (respectively) of the volume of VWR mice. It should be noted, however, that all groups ran > 10Km/day.

Table 1.

Body and organ weights in response to exercise training.

Group	Final Body Weight		Perigonadal Fat Weight		Heart Weight		Relative Heart Weight	
	Male	Female	Male	Female	Male	Female	Male	Female
CON	29.0 ± 1.1	22.6 ± 0.2[a]	0.55 ± 0.06	0.28 ± 0.05[a]	0.17 ± 0.01	0.12 ± 0.01[a]	0.59 ± 0.03	0.53 ± 0.06
VWR	26.5 ± 0.7	22.8 ± 0.7[a]	0.29 ± 0.04[b]	0.23 ± 0.03[a]	0.16 ± 0.01	0.16 ± 0.01[b]	0.62 ± 0.03	0.71 ± 0.01[b]
ABX	28.4 ± 0.3	23.6 ± 0.2[a]	0.27 ± 0.01[c]	0.17 ± 0.02[a,c]	0.17 ± 0.02	0.13 ± 0.01[a]	0.60 ± 0.06	0.53 ± 0.03
VWR/ABX	27.3 ± 0.8	23.4 ± 0.4[a]	0.15 ± 0.02[b,c]	0.17 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.57 ± 0.04	0.60 ± 0.03[b]
GF	27.3 ± 0.26	22.7 ± 0.27[a]	0.33 ± 0.03[d]	0.15 ± 0.01[a,d]	0.11 ± 0.005	0.10 ± 0.001	0.41 ± 0.02[d]	0.46 ± 0.01
GF VWR	27.6 ± 0.94	22.8 ± 1.05[a]	0.29 ± 0.03	0.14 ± 0.03[a,d]	0.13 ± 0.005	0.11 ± 0.007	0.47 ± 0.03[d]	0.46 ± 0.01[d]

All weights in grams, relative heart weight = heart weight/body weight × 100; Mean ± sem; N=4–5/group; $p < 0.05$ with:

[a] sex effect between corresponding group

[b] VWR effect between corresponding CON group of same sex

[c] ABX effect between corresponding CON group

[d] germ free status effect when compared to corresponding SPF group.

Table 2.

Gene expression in gastroc (a) and soleus (b) muscles of mice treated with ABX when compared to sedentary SPF control values.

a) SPF gastroc muscle

Gene	SED CON Fold Change ($2^{-\Delta\Delta CT}$)	SED ABX Fold Change ($2^{-\Delta\Delta CT}$)	VWR CON Fold Change ($2^{-\Delta\Delta CT}$)	VWR ABX Fold Change ($2^{-\Delta\Delta CT}$)	VWR P-value	ABX P value	Interaction
PGC1α	1.11±0.18	0.92±0.07	2.44±0.56*	2.82±0.49*	**<0.001**	0.784	0.498
MCT1	1.02±0.1	0.92±0.08	1.19±0.11	0.99±0.074	0.180	0.093	0.548
LC3	1.03±0.1	1.10±0.10	1.02±0.14	0.82±0.10	0.197	0.570	0.215
SDH	1.02±0.1	1.02±0.08	1.16±0.13	0.94±0.07	0.743	0.257	0.260
GLUT4	1.01±0.05	0.98±0.12	0.76±0.10*	0.60±0.07*	**<0.001**	0.287	0.469
MCT4	1.04±0.1	0.94±0.14	0.61±0.09*	0.54±0.05*	**<0.001**	0.392	0.903
PPARγ	0.95±0.2	1.85±0.63	2.07±0.59	7.6±4.44	0.260	0.298	0.242

b) SPF soleus muscle

Gene	SED CON Fold Change ($2^{-\Delta\Delta CT}$)	SED ABX Fold Change ($2^{-\Delta\Delta CT}$)	VWR CON Fold Change ($2^{-\Delta\Delta CT}$)	VWR ABX Fold Change ($2^{-\Delta\Delta CT}$)	VWR P-value	ABX P value	Interaction
PGC1α	1.16±0.22	1.13±0.18	1.39±0.36	1.60±0.38	0.282	0.778	0.705
MCT1	1.04±0.13	0.97±0.13	1.15±0.16	1.20±0.17	0.287	0.952	0.687
CS	1.14±0.25	1.11±0.12	1.36±0.14	2.25±0.43*	**0.016**	0.112	0.090
SDH	1.02±0.09	1.15±0.19	1.55±0.21	2.15±0.30*	**0.002**	0.123	0.305
GLUT4	1.09±0.14	1.21±0.18	0.99±0.10	1.03±0.21	0.419	0.615	0.807
PPARγ	1.99±0.88	0.98±0.29	0.54±0.09	0.84±0.18	0.079	0.423	0.145
FBXO32	1.08±0.19	1.13±0.15	1.20±0.17	1.49±0.27	0.247	0.427	0.553

Values are means ± standard error.

* represents a significant ($p<0.05$) difference from respective sedentary group. N = 9, 9, 10 and 10 for CON, ABX, VWR, and VWR/ABX groups, respectively.