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Gut microbiota diversity associates with cardiorespiratory fitness in post-primary treatment breast cancer survivors

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Abstract

Previous studies have not adequately considered the influence of adiposity and activity-related energy expenditure (AEE) on gut microbe diversity. We determined associations of gut microbiota diversity with measures of cardiorespiratory fitness while accounting for the potential effects of %body fat and free-living AEE. Cancer treatment uniquely triggers multiple physiologic shifts detrimental to overall health. Though previous research indicates a link between gut microbiota and cardiorespiratory fitness, it is unclear whether these findings are due to potential underlying effects %body fat or free-living activity energy expenditure (AEE). Microbe composition of fecal specimens from 37 breast cancer survivors were determined using 16S microbiome analyses. Individual-sample microbiota diversity (α -diversity) and between-sample community differences (β -diversity) were examined. Peak oxygen uptake ($\dot{V}O_{2peak}$) was estimated from a graded exercise test (GXT) consistent with the modified-Naughton protocol, in which, exercise terminates at 85% age-predicted heart rate max (HR_{max}). AEE was measured over 10-days using doubly-labeled water wherein %body fat was calculated from total body water. Pearson correlations revealed α -

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Competing Interests
None declared.

diversity indices (Chao1, Observed Species, PD Whole Tree, Shannon) positively associated with $\dot{V}O_{2\text{peak}}$ ($r = 0.34$ to 0.51 ; $p < 0.05$) whereas %HR_{max} during Stages 1–4 of the GXT ($r = -0.34$ to -0.50 ; $p < 0.05$) and %body fat ($r = -0.32$ to -0.41 ; $p < 0.05$) were negatively associated with the same α -diversity indices. Multiple linear regression models showed $\dot{V}O_{2\text{peak}}$ accounted for 22% and 26% of the variance in taxonomic richness (Observed Species) and phylogenetic diversity after adjustment for %body fat and menopausal status. Unweighted UniFrac (β -diversity) was significant for several outcomes involving cardiorespiratory fitness and significant taxa comparisons were found. Associations between gut microbiota and free-living AEE were not found. Results from the present work suggest cardiorespiratory fitness, not physical activity, is a superior correlate of gut microbiota diversity.

Keywords

cardiovascular; doubly-labeled water; energy expenditure; gut microbiome; maximal oxygen uptake

INTRODUCTION

Early detection and targeted therapies have improved 5-year relative survival rates (89%), such that, the number of breast cancer survivors (BCS) in United States is projected to soon exceed 4 million (Miller et al., 2016). Regrettably, among these women, the combined influence of cancer treatment and advancing age frequently coincides with several physiological shifts detrimental to cardio-metabolic health including increased adiposity (Vance et al., 2011), dysbiosis (Sheflin Whitney and Weir., 2014), and reduced cardiorespiratory fitness (Jones et al., 2012).

Compelling evidence indicates an active cross-talk exists between the gut and brain wherein endocrine cell-mediated interactions from gut microbiota and enterochromaffin cells perform neuro-immune response modulation and regulatory functions (Furness and Clerc, 2000; Rhee, Pothoulakis and Mayer, 2009). While approximately two-thirds of gut microbes are reflective of diet and lifestyle (Qin et al., 2010), species diversity is generally considered representative of a healthy commensal gut microbiota. Dysregulation of this relative balance (i.e., dysbiosis) may play an underlying role as previous research in murine models has shown that an increased ratio of Firmicutes (p) to Bacteroidetes (p) corresponds with obesity (Turnbaugh et al., 2006). In recent human studies, uneven clustering of gut microbes have been associated with cardiovascular disease (Kelly et al., 2016) and type II diabetes (Larsen et al., 2010). Thus, gut microbiota diversity has been proposed as an emerging biomarker of health status (Bian et al., 2017).

Since habitual exercise represents a cornerstone to the prevention of cardio-metabolic disease, several studies have explored the connection between cardiorespiratory fitness and gut microbiota diversity (Estaki et al., 2016; Yang et al., 2017). Of the few available human investigations, Estaki et al. (2016) reported peak oxygen uptake ($\dot{V}O_2$) explained $\approx 20\%$ of the variance in gut microbiota diversity (i.e., species richness) among young, healthy men and women. Alternatively, others have reported that the association between cardiorespiratory fitness and gut microbiota diversity may be mediated by %body fat (Yang

et al., 2017). Still, it is unknown if underlying differences in activity-related energy expenditure (AEE) are contributing to the relationship between cardiorespiratory fitness and gut microbiota, which is particularly important given the prevalence of obesity and insufficient physical activity among BCS. Given the myriad of direct/indirect consequences of cancer treatment, as well as, the propensity for obesity and insufficient physical activity, it remains unclear if cardiorespiratory fitness associates with gut microbiota diversity in BCS.

Gut microbes have long been theorized to modify estrogen metabolism (Adlercreutz et al., 1984). Consistent with this principle, Flores and colleagues (2012) reported taxonomic richness (e.g., Shannon index, Observed Species) positively associated with circulating, non-ovarian estrogens in men and postmenopausal women. While previous research has shown a positive association between body mass index (BMI) and estrogen (Boyapati et al., 2004), it is understandable that the risk of incident estrogen-related diseases, like breast cancer, are linked with obesity in postmenopausal women. Though habitual physical activity is thought to modulate this relationship (McTiernan et al., 2006), it is unclear whether the benefits of physical activity are driven by changes in adiposity or gut microbiota, as both associate with systemic estrogens. Recent comparative analyses of microbiota from urine and local breast tissue (Wang et al., 2017), as well as gut microbes (Goedert et al., 2015) have revealed compositionally distinct microbiomic profiles between women with and without breast cancer. Thus, a complex interaction exists between gut microbiota diversity, adiposity, menopausal status, and free-living physical activity.

Therefore, the current study sought to examine the associations between gut microbiota diversity with multiple measures of cardiorespiratory fitness while accounting for the potential influence of %body fat, menopausal status, and AEE among a cohort of post-primary treatment, non-metastatic BCS. Based on previous findings, gut microbiota diversity may be a correlate of health status, and thus, closely linked with substrate delivery and utilization as evidenced by peak $\dot{V}O_2$. Consequently, we hypothesized peak $\dot{V}O_2$ and percent of age-predicted maximal heart rate during exercise, would associate with species richness and evenness (α -diversity). Similar to previous work (Paulsen et al., 2017), it was hypothesized that measures of cardiorespiratory fitness (e.g., peak $\dot{V}O_2$) would associate with between-sample community differences (β -diversity) with the potential for significant taxa comparison differences.

METHODS

Ethical approval

All study procedures were conducted in accordance with the ethical guidelines set forth by the local institutional review board (IRB-121114008) at the University of Alabama at Birmingham and the Declaration of Helsinki ([ClinicalTrials.gov](https://clinicaltrials.gov) NCT00929617). Written informed consent was obtained from all participants prior to study involvement.

Participants and design

Thirty-seven ($n = 37$) post-primary treatment BCS volunteered for the present cross-sectional, proof-of-concept investigation. Several recruitment strategies were used including

periodical advertisement, institutional cancer registry, and contact with local cancer support groups. Preliminary phone screenings were conducted with interested respondents. Inclusion criteria were: 1) English-speaking females between the ages of 18–70 with a previous diagnosis of ductal carcinoma *in situ* or stage I-III breast cancer, 2) physician's clearance for study participation, 3) ambulatory, 4) not currently receiving or planning to receive radiation/chemotherapy, and 5) participating in < 30 minutes of vigorous-intensity physical activity *or* < 60 minutes of moderate-intensity physical activity per week for at least 6 months. Exclusion criteria were: 1) contraindications to exercise, 2) recurrent or metastatic breast cancer, 3) antibiotic use within the previous week, 4) dementia/psychological disorders preventing study participation, 5) non-ambulatory, and 6) current participation in an exercise study. Eligible participants were scheduled for an orientation visit, during which, study expectations and testing procedures were explained. Testing procedures were performed over three visits within 10 days. With the exception of prescription medications, participants were asked to abstain from alcohol, caffeine, and smoking at least 12 hours before each visit. Vigorous-intensity physical activity was restricted at least 24 hours prior to testing. Following an overnight fast, participants reported to the Physical Activity Core Laboratory between 0730 and 0900.

Instrumentation and Measurements

A self-reported questionnaire was administered to collect descriptive data concerning age, race, medical history (i.e., breast cancer status, months since diagnosis), menopausal status, antibiotic use history, and comorbidities. Dietary components (grams per day of carbohydrate, sugar, and fiber) were quantified using a 3-day food record that included two weekdays and one weekend. Weight (kg) and standing height (m) were measured to calculate BMI. Following exhalation, a tape measure with a 4 oz. tension indicator was used to determine waist circumference. Physical activity patterns over a 10-day period were measured by a hip-worn triaxial accelerometer (GT3X, Pensacola, FL). In agreement with accepted physical activity guidelines (Physical Activity Guidelines Advisory Committee, 2009), average minutes of vigorous-intensity ($5725 \text{ counts}\cdot\text{min}^{-1}$) were doubled then added to minutes of moderate-intensity ($1952\text{--}5724 \text{ counts}\cdot\text{min}^{-1}$) to quantitate moderate-to-vigorous physical activity (MVPA; $\text{min}\cdot\text{week}^{-1}$). Average MVPA was calculated from daily wear-time and used for analyses.

Resting Energy Expenditure

Resting energy expenditure (REE) was determined while participants laid quietly awake in a softly lit, well-ventilated room. Ambient temperature was maintained between 22–23 °C. Oxygen uptake ($\dot{V}O_2$) and carbon dioxide ($\dot{V}CO_2$) production were continuously measured for 30 minutes using indirect calorimetry (Vmax Encore, Loma Linda, CA) coupled with a ventilated canopy. Energy expenditure (kcal) and respiratory quotient ($\dot{V}CO_2:\dot{V}O_2$) were averaged in 60 second intervals by Vmax software using a modified-Weir equation. The concluding 20 minutes were used for analyses.

Standardized Treadmill Task

A two-stage, standardized treadmill task was used to evaluate physiological responses at submaximal intensities in a subset of participants ($n = 18$). Following the equilibration

period, breath-by-breath $\dot{V}O_2$ and $\dot{V}CO_2$ were measured via indirect calorimetry (MAX II, Pittsburgh, PA). Participants performed two 4 minute stages of 0.89 m/s at 0% and 2.5% grade. Steady-state $\dot{V}O_2$, defined as the highest 30 second average during each stage was used for analyses. Oxygen-pulse, representing the $\dot{V}O_2$ used by peripheral tissues per heartbeat, was calculated from steady-state $\dot{V}O_2$ ($mLO_2 \cdot kg^{-1} \cdot min^{-1}$) divided by the corresponding heart rate (bpm). Units are expressed in $mLO_2 \cdot kg^{-1} \cdot beat^{-1}$ to account for weight-bearing nature of walking and variance in body mass. Higher values of oxygen-pulse indicate greater $\dot{V}O_2$ per heartbeat (index of stroke volume) (Oliveira, Myers, & de Araujo, 2011).

Graded Exercise Test

Within 5–6 days of completing the standardized treadmill task, a graded exercise test (GXT), consistent with the modified-Naughton protocol, was used to estimate peak $\dot{V}O_2$ from ACSM regression equations (American College of Sports Medicine, 2010). Treadmill workload was increased at two minute intervals until participants reached 85% of their age-predicted ($220 - \text{age}$) maximum heart rate (HR_{max}). Heart rate was measured continuously (Polar Electro, Kempele, Finland) and used to calculate the percent of age-predicted HR_{max} ($\%HR_{max}$) during each stage. Since Stage 4 (0.89 m/s at 7% grade) estimates a peak $\dot{V}O_2$ of $15.6 mLO_2 \cdot kg^{-1} \cdot min^{-1}$, closely resembling the minimum peak $\dot{V}O_2$ needed for functional independence in older women (Shephard, 2009), Stages 1–4 were of interest and included for analyses. Heart rate recovery was measured at minutes one and two upon the termination of the GXT as indices of parasympathetic reactivation and sympathetic withdrawal, respectively.

Doubly-Labeled Water

Total energy expenditure (TEE) was calculated over a 10-day period under free-living conditions using doubly-labeled water (DLW) (Hunter et al., 2015). The DLW technique makes use of the inherent difference in elimination kinetics between deuterium (2H) and oxygen-18 (^{18}O) in the body. Deuterium is lost solely through body water whereas ^{18}O is lost from both water and carbon dioxide. Briefly, a baseline urine specimen was provided in the morning, after which, participants were administered an oral dose of 2H_2O and $H_2^{18}O$ based on their measured body weight (54 g if ≤ 60 kg; 63 g if 60.1 to 75 kg; 74 g for 75.1 to 95 kg; and 89 g for > 95 kg). Post-dose urine samples were collected at +3 hours and +4 hours to permit isotopic equilibrium. Ten days later, two additional urine samples were collected in the morning. Carbon dioxide production rates were determined using a fixed constant for the dilution space ratio (1.0427) provided by Speakman et al. (Speakman, Nair and Goran., 1993) and converted to energy expenditure using the equation by de Weir (Weir, 1949): $TEE (kcal \cdot d^{-1}) = 3.9 (rCO_2 / RQ) + 1.1$, where rCO_2 is the rate of carbon dioxide production in liters per day and RQ is the respiratory quotient (0.88) used for all participants. Urine samples were stored at $-20^\circ C$ until measured in duplicate by isotope ratio mass spectrometry. Coefficient of variation (CV) for repeated-measures in our laboratory is 4.3%. Free-living AEE was estimated by initially reducing TEE by 10% to account for the thermogenic effects of digestion. AEE was then calculated by subtracting REE from the adjusted-TEE. Percent body fat was derived from total body water (Hunter et al., 2015).

Gut Microbiota Diversity

Gut microbiota were examined from fecal wipes processed according to the protocol described by Kumar et al. (2014) and Paulsen et al. (2017). In short, samples were collected and sent via overnight mail to our local Microbiome Resource Laboratory where they were stored at -80°C until analyses were performed. Individual samples were dissolved in a buffer solution then processed with a DNA Miniprep Kit to acquire isolated fecal DNA. Polymerase chain reaction (PCR) was used to amplify the V4 region of the 16S rRNA gene then analyzed with Illumina MiSeq DNA sequencing. Two hundred fifty base paired-end sequences were obtained, and following filtering of low quality reads, the paired-end sequences were merged and analyzed. Quality control, operational taxonomic unit (OTU) picking, and taxonomic assignment were performed as previously described (Kumar et al., 2014). Within-sample microbiota diversity (α -diversity) was evaluated using Chao1, Shannon, and Simpson indices which measure gut microbiota richness and evenness. Additional α -diversity assessments were performed with Observed Species and PD Whole Tree which measure species richness and phylogeny, respectively. Between-sample microbiota community differences (β -diversity) were analyzed using weighted (quantitative) and unweighted (qualitative) UniFrac distance metrics (Lozupone and Knight., 2005). For β -diversity, the following continuous outcomes were divided into quartiles (i.e., the difference in the minimum and maximum values were divided by four): age, number of comorbidities, BMI, waist circumference, %body fat, months since cancer diagnosis, peak $\dot{V}\text{O}_2$, %HR_{max} Stages 1–4 of GXT, AEE, MVPA, sugar intake, carbohydrate intake, and fiber intake. The following outcomes were dichotomized: ethnicity (African-American vs. other), menopausal status (yes vs. no), history of chemotherapy (yes vs. no), and history of radiation (yes vs. no) with the original categories maintained for breast cancer stage (0 vs. 1 vs. 2 vs. 3 vs. 4) and hormonal therapy (none vs. 1 year vs. > 1 year).

Statistical Analyses

Participant characteristics are presented as means and standard deviations unless noted otherwise (Table 1). The Shapiro-Wilk test was used to confirm the normality of distributions. Two-tailed parametric (Pearson) correlations were performed on variables of interest. Based on consequent results, multiple linear regression was used to examine the associations for peak $\dot{V}\text{O}_2$ and %HR_{max} (Stage 3, GXT) on α -diversity while adjusting for %body fat and menopausal status. Of note, adjusting variables were included following significant bivariate association and/or plausible physiologic link to gut microbiota. Additionally, Stage 3 of the GXT was used as surrogate for cardiorespiratory fitness since all 37 participants completed the stage and workload matched a vigorous-intensity effort as evidenced by an average $70 \pm 10\%$ %HR_{max}. Collinearity of diagnostics for all variables were within acceptable limits as variable inflation factors for each model were less than 2.22. Separate univariate analyses were used to evaluate differences in α -diversity between participants in the highest quartile for peak $\dot{V}\text{O}_2$ ($22.4 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) versus those below. all others. Substantive differences were determined with Hedges' g as a measure of effect size: 0.2 as small; 0.5 as medium; and 0.8 as large. Principal coordinate analysis (PCoA) was used to visualize β -diversity differences among outcome categories or quartiles. Statistical significance of the β -diversity/outcomes relationships were determined using permutational multivariate analysis of variance (PERMANOVA). Kruskal-Wallis testing

with false discovery rate (FDR) correction procedures compared relative taxa level frequencies using QIIME for variables with statistically significant β -diversity. The threshold of statistical significance for all tests was set *a priori* and defined as two-sided p -value 0.05 (or q -value 0.05 for analyses using FDR correction).

RESULTS

Overview

Descriptive data are shown in Table 1. Coinciding with established BMI stratifications, 21 of 37 (57%) of participants were obese ($> 30 \text{ kg/m}^2$) while 19 of 37 (51%) had an increased risk of cardio-metabolic disease as evidenced by a waist circumference $> 88 \text{ cm}$ (Janssen, Katzmarzyk & Ross., 2004). Accelerometry-based measurement showed 20 of 37 (54%) participants appeared to be meeting the minimum recommendation of accumulating at least 150 minutes of MVPA per week. Mean cohort data indicated peak $\dot{V}O_2$ was $20 \pm 5 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ or approximately 33% greater than the minimum cardiorespiratory fitness needed for independent-living among older women (Shephard, 2009). Food record data showed that just 6 of 36 (17%) met the American Dietary Guidelines (22.4 g/day) for daily fiber intake in women aged 51 years and older (Department of Health and Human Services, 2015).

α -Diversity

Consistent with our hypotheses, zero-order correlation coefficients revealed several α -diversity indices significantly associated with markers of cardiorespiratory fitness including peak $\dot{V}O_2$ and $\%HR_{\text{max}}$ during the GXT (Table 2). Surprisingly, free-living AEE and TEE were not associated with α -diversity. Percent body fat, but not waist circumference, negatively associated ($r = -0.32$ to -0.41 ; $p < 0.05$) with Chao1, Observed Species, and PD Whole Tree. Menopausal status trended toward significance for Chao1 ($r = -0.32$; $p = 0.057$), PD Whole Tree ($p = 0.10$) and Observed Species ($p = 0.11$) suggesting premenopausal participants tended to have greater species richness. However, no other demographic or diet variable associated with α -diversity.

In Table 3, multivariate regression modeling showed that peak $\dot{V}O_2$ was shown to account accounted for 22% and 26% of the variance in richness and phylogenetic diversity as represented by Observed Species and PD Whole Tree, respectively. Participants were stratified into quartiles based on peak $\dot{V}O_2$ wherein analyses revealed participants ($n = 13$) in the highest quartile ($> 22.4 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) exhibited significantly higher α -diversity scores compared to participants ($n = 24$) occupying the lower quartiles (Figure 1). Unadjusted scatterplots shown in Figure 2, illustrate the distribution of alpha diversity with respect to cardiorespiratory fitness. Given the resultant negative associations, it appears participants with greater cardiorespiratory fitness, as evidenced by a *lower* percentage of age-adjusted $\%HR_{\text{max}}$, had higher gut microbiota diversity.

Shown in Table 4, $\%HR_{\text{max}}$ (Stage 3, GXT), adjusted for %body fat and menopausal status, accounted for 28% to 35% of the variance in three of the five models examining α -diversity. Oxygen-pulse during Stage 1 ($r = 0.52$ to 0.70) and 2 ($r = 0.62$ to 0.75) of the standardized treadmill task positively associated with several α -diversity indices. Increased aerobic

capacity (represented by a greater $\dot{V}O_2$ per heartbeat) positively associated ($r = 0.75$; $p = 0.001$; $n = 17$) with PD Whole Tree. Interestingly, the correlation coefficients between oxygen-pulse and α -diversity were reliably stronger during the graded walking task. Additionally, heart rate recovery during minutes 1 and 2 were found to be negatively associated ($r = -0.47$ to -0.60 ; $p < 0.05$) with indices of α -diversity. As shown in Figure 3, a greater reduction in heart rate 1-minute post-exercise, indicative of greater parasympathetic reactivation, and thus, increased aerobic fitness associated with the Shannon index.

β -Diversity

With unweighted UniFrac clustering used to detect differences in specific taxa present, β -diversity differences were found for BMI ($p = 0.043$), waist circumference ($p = 0.017$), %HR_{max} during Stage 2 ($p = 0.046$) and Stage 3 ($p = 0.016$) of the GXT, along with peak $\dot{V}O_2$ ($p = 0.04$). Similar to our previous work (Paulsen, et al, 2017), quartiles for continuous variables were dichotomized (1 and 2 vs. 3 and 4) to test taxa differences responsible for the β -diversity findings. Quartiles 1 and 2 vs. 3 and 4 for %HR_{max} during Stage 2 and 3 of the GXT, exhibited statistically significant taxa differences following FDR correction for organisms within the phyla of Actinobacteria (p), Bacteroidetes (p), and Proteobacteria (p). Because visualization of the 3D PCoA plot showed distinct differences between quartile 1 and 4 of peak $\dot{V}O_2$, taxa comparisons were performed wherein significant taxa differences for Verrucomicrobia (p) and Verrucomicrobiae (c) were found after FDR correction (0.002737 vs. 0.047163 for quartile 1 vs. quartile 4; FDR corrected q -value = 0.024 and 0.002737 vs. 0.047163, $q = 0.048$, respectively).

Unweighted UniFrac clustering revealed significant differences for heart rate recovery at minute one ($p = 0.047$) and minute two ($p = 0.018$) post-exercise. However, following FDR correction significant taxa differences in heart rate recovery remained for minute one (parasympathetic reactivation) but not minute two (sympathetic withdrawal). Given that 30 different taxa were different, we report those OTUs at the genus level with differences noted for organisms within the phyla of Actinobacteria (p), Firmicutes (p), and Proteobacteria (p). Among the four aerobic fitness measures with taxa differences, Since several taxa were detected at a very low mean relative abundance suggesting these taxa make up a very minor population of microbes in the sample; hence, To aid reader interpretation, we provided the top 10 genera for peak $\dot{V}O_2$ (Figure 4). As indicated greater aerobic capacity is associated with higher relative abundance of *Bacteroides* (g) and *Prevotella* (g), lower relative abundance of *Escherichia* (g), and variable differences in multiple organisms within Firmicutes (p).

DISCUSSION

Though previous work (Estaki et al., 2016; Yang et al., 2017) has shown a link between cardiorespiratory fitness and gut microbiota diversity, the underlying effects of %body fat and free-living AEE have not been previously examined. To this end, the hypotheses of the present investigation were based on the widely-accepted premise that %body and AEE both associate with cardiorespiratory fitness. Hence, we sought to explore the inter-associations between gut microbiota diversity and measures representative of cardiorespiratory fitness

(e.g., peak $\dot{V}O_2$, %HR_{max} during exercise) while accounting for the potential influence of %body fat, menopausal status, and AEE. Consistent with our hypotheses, our findings revealed that peak $\dot{V}O_2$ accounted for 22% and 26% of the variance in taxonomic richness and phylogenetic diversity, respectively, after adjustment for %body fat and menopausal status. Age-adjusted %HR_{max} during Stage 3 of the GXT accounted for 28% to 35% of the variance in 3 of 5 α -diversity regression models. Remarkably similar findings, linking higher aerobic fitness and greater gut microbiota diversity, were also found for oxygen-pulse during exercise and heart rate recovery. After FDR correction, significant taxa differences were observed for four aerobic fitness measures (peak $\dot{V}O_2$, %HR_{max} during Stages 2 and 3 of the GXT, and heart rate recovery at 1-minute post-exercise). Collectively, the present findings suggest aerobic fitness associates with gut microbiota diversity among post-primary treatment, non-metastatic BCS.

Whereas a high cardiorespiratory fitness is known to attenuate cardiovascular disease risk/progression, the contributing features are generally thought to involve favorable arterial compliance, autonomic tone, and endothelial function. However, our data adds further support to the probability that a high peak $\dot{V}O_2$ may also offset the risk of various chronic disease states through interactions with the gut microbiota. Similar to previous work (Estaki et al., 2016, Yang et al., 2017), our results demonstrate that peak $\dot{V}O_2$ associates with microbiota species richness and phylogenetic diversity after adjustment for %body fat and menopausal status, while participants with peak $\dot{V}O_2 \geq 22.4 \text{ mL}O_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ had greater α -diversity. Although our cross-sectional design restricts causal inferences we can make concerning peak $\dot{V}O_2$ and gut microbiota diversity, Allen and colleagues (2018) recently showed six weeks of aerobic training (in the absence of dietary modifications), altered gut microbiota and microbial-derived short chain fatty acids in a group of lean and obese participants. In their study, participants exhibited compositionally distinct microbiomic profiles at baseline (lean vs. obese) that disappeared following exercise training. Interestingly, among the obese participants, the changes in gut microbiota diversity corresponded with an improved maximal $\dot{V}O_2$. Certainly, these data highlight the responsive nature of gut microbiota to exercise training and strong link to cardiorespiratory fitness.

Due the systemic, cardio-metabolic benefits of physical activity, it is logical that insufficient quantities contribute to poorer health outcomes including dysbiosis (Nehra et al., 2016). However, in humans, the underlying mechanisms whereby physical activity and gut microbiota interact to effect host physiology remains largely unexplored. Data from animal models offers intriguing evidence supporting voluntary physical activity (i.e., wheel running) as an effective modulator of gut microbes (Allen et al., 2015; Matsumoto et al., 2008). While compelling, the translational implications to humans are less straight-forward, as it is difficult to disentangle the true physical activity/exercise-mediated effects on gut microbiota (*or vice versa*) under free-living conditions. Since variation in non-exercise activity thermogenesis is a key feature to overall health and weight maintenance, our group was surprised to discover weekly minutes of MVPA and AEE ($\text{kcal} \cdot \text{day}^{-1}$) were unrelated to indices of α -diversity and β -diversity. Nevertheless, our data appears suggestive that cardiorespiratory fitness, not physical activity, is a better correlate of gut microbiota diversity. It is worth noting, our relatively small sample size may have restricted our ability to identify possible relationships between AEE and gut microbiota diversity, as the precision

to measure free-living AEE via DLW is less than that to measure heart rate and $\dot{V}O_2$ (e.g., CV < 3% in our laboratory). It is intriguing to consider how genetic endowment for high cardiorespiratory fitness may interact with gut microbiota in defense against cardio-metabolic disease through the lifecycle.

While Estaki et al. (2016) did not report a significant association between cardiorespiratory fitness and β -diversity, it is possible youthful age (mean < 30 yrs.) and higher cardiorespiratory fitness may have contributed to these findings. In the present work, we identified a greater relative abundance of several organisms within the phyla of Bacteroidetes (p), Proteobacteria (p), Actinobacteria (p), and Firmicutes (p). These phyla include organisms involved in formation of short-chain fatty acids, possibly linking gut microbiota to health (Chakraborti, 2015), yet the organisms with significant taxa comparisons reported here are not among the more prevalent and known butyrate-producing bacteria (Louis and Flint, 2009). It is noteworthy that increased Bacteroidetes (p) and Verrucomicrobia (p), as found here with greater aerobic fitness, have been reported in mice after Roux-en Y (RYGB) gastric bypass surgery along with alterations in short chain fatty acid production (\uparrow propionate and \downarrow acetate) (Liou et al., 2013). This same study reported an increase in *Escherichia* (g) in mice after RYGB, which is in contrast, to our finding that cardiorespiratory fitness may be associated with a decrease in the *Escherichia* (g) supporting the need for further research (Liou et al., 2013). Although no prior human study has reported an association between greater relative abundance of Verrucomicrobia (p) and cardiorespiratory fitness, this organism has been associated with obesity in humans (Zhang et al., 2009). Furthermore, several potential taxa differences (e.g., Proteobacteria (p)) warrant further study as potential links between exercise and improvements in psychosocial outcomes like depression (Jiang et al., 2015).

Despite the rapid increase in studies linking gut microbiota to health status, gut microbes have been known to influence estrogen metabolism for many decades (Adlercreutz et al., 1984). More recently, Flores et al. (2012) reported gut microbiota diversity positively associated with systemic, non-ovarian estrogens in men and postmenopausal women but not premenopausal women. Researchers from that study indicated negligible effects when adjustments were made for age and BMI. However, they did not have a measure for aerobic fitness. Similarly, age was not associated with gut microbiota α -diversity in the present work. Interestingly, self-reported menstrual status was a significant, independent correlate in 3 of 5 α -diversity regression models. In other words, participants who specified they had not experienced menopause exhibited greater α -diversity. While our results differ from previous work (Flores et al., 2012), it is interesting that menopausal status remained significant after adjusting for %HR_{max} and %body fat. It is important to note that men tend to have higher absolute aerobic capacities than women, and as such, the sample of men ($n = 25$) and postmenopausal women ($n = 7$) reported by Flores et al. (2012) may have simply had a greater aerobic fitness compared to the premenopausal women ($n = 19$). Still, it is difficult to completely reconcile our divergent findings since we did not have a direct measure of estrogen metabolites or control for menstrual phase, as this would require a specifically designed study.

Limitations

We recognize several limitations in the present investigation. Inherent with our cross-sectional design, we lack the ability to establish cause-and-effect or direction for the reported associations. Certainly the present outcomes are correlative in nature and should therefore be considered hypothesis-generating. Our participant sample was restricted to women with a history of breast cancer which tended to exhibit low cardiorespiratory fitness and multiple comorbidities. As such, generalizability of our results to other demographics should be performed with caution. Additionally, we do not include any functional outcomes (e.g., fecal concentrations of short chain fatty acids) of gut microbiota which are likely important for gut health. Nevertheless, strengths include the use of DLW to objectively measure free-living EE, as well as, evaluating taxa comparisons with several measures representative of aerobic capacity.

Conclusions

To our knowledge, this is the first study to examine the link between cardiorespiratory fitness and gut microbiota diversity while accounting for the underlying effects of %body fat and free-living AEE. Our primary findings showed that peak $\dot{V}O_2$ associated with taxonomic richness and phylogenetic diversity while %HR_{max} during exercise accounted for 28% to 35% of the variance in α -diversity adjusted for %body fat and menopausal status. Consequently, it seems cardiorespiratory fitness, not physical activity, is a better correlate of gut microbiota diversity. Future work should include controlled-feeding (i.e., diet standardization) and exercise training to properly examine the longitudinal changes in gut microbiota. In doing so, researchers may be able to identify strategies and optimize therapeutic approaches to enhance health outcomes among clinical populations.

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New Findings: What is the central question of this study? Does the link between cardiorespiratory fitness and gut microbiota diversity persist after adjusting for the potential effects of %body fat and activity-related energy expenditure (AEE)? What is the main finding and its importance? This is the first study to examine the link between cardiorespiratory fitness and gut microbiota diversity while accounting for the underlying effects of %body fat and free-living AEE. Results from the present work suggest cardiorespiratory fitness, not physical activity, is a superior correlate of gut microbiota diversity among post-primary treatment, non-metastatic breast cancer survivors.

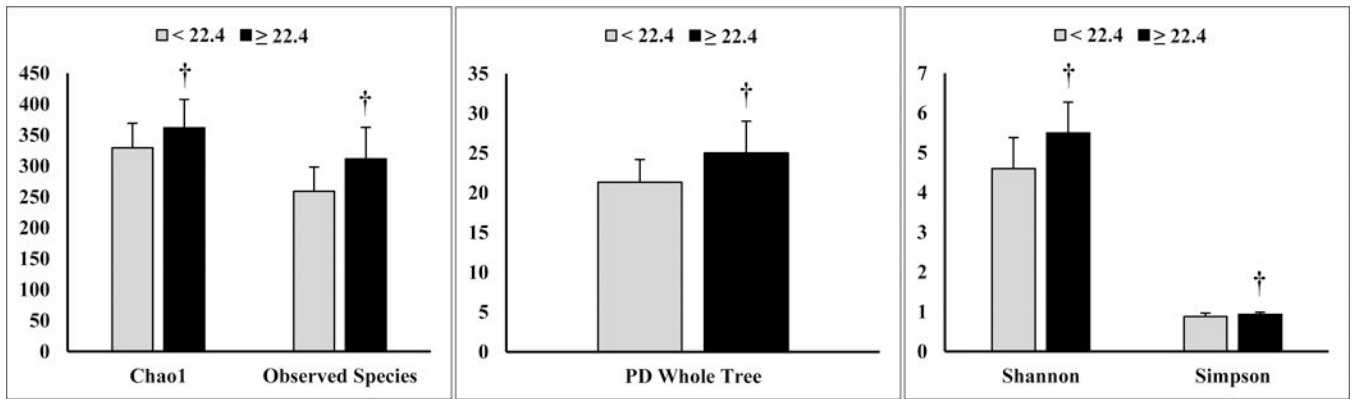


Figure 1.

Unadjusted, between-groups comparisons of alpha diversity among participants above and below $22.4 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{ VO}_{2\text{peak}}$. Note participants ($n = 13$) above $22.4 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ had higher alpha diversity compared to those ($n = 24$) with a lower $\text{VO}_{2\text{peak}}$. Substantive differences were determined by Hedges' g as a measure of effect size: 0.77 (Chao1), 1.21 (Observed Species), 1.12 (PD Whole Tree), 1.15 (Shannon), 0.72 (Simpson). Data are represented by means and S.D. †denotes significance at $p < 0.05$.

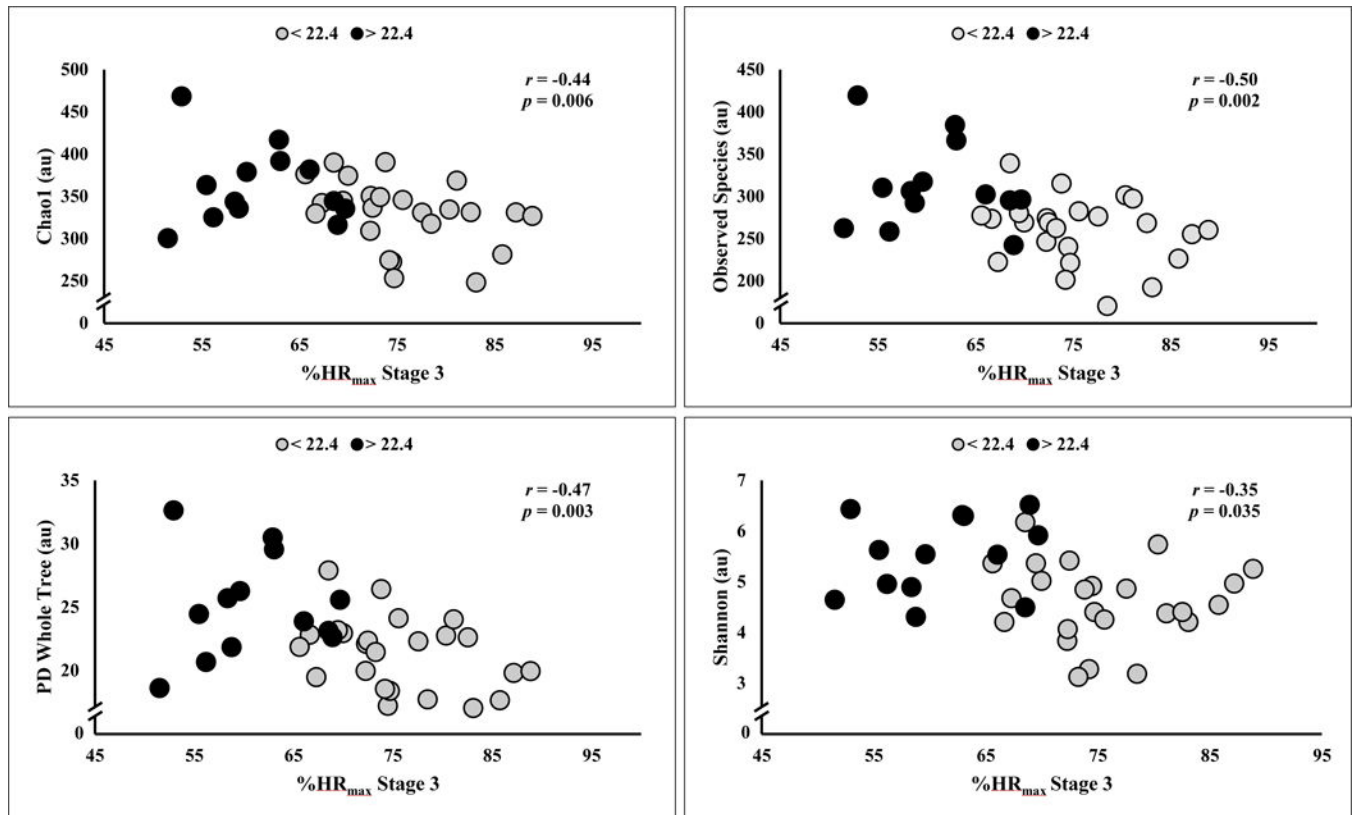


Figure 2:

Unadjusted scatterplot of alpha diversity (e.g., Chao1, Observed Species, PD Whole Tree, and Shannon) and percent of age-predicted maximal heart rate (%HR_{max}) during Stage 3 of the graded exercise test. Mean intensity corresponded to 70 \pm 10% %HR_{max}. Note the tendency for greater aerobic fitness, evidenced by lower %HR_{max} associated with higher alpha diversity. Groups are dichotomized by VO_{2peak} above and below 22.4 mL O₂·kg⁻¹·min⁻¹. $n = 37$.

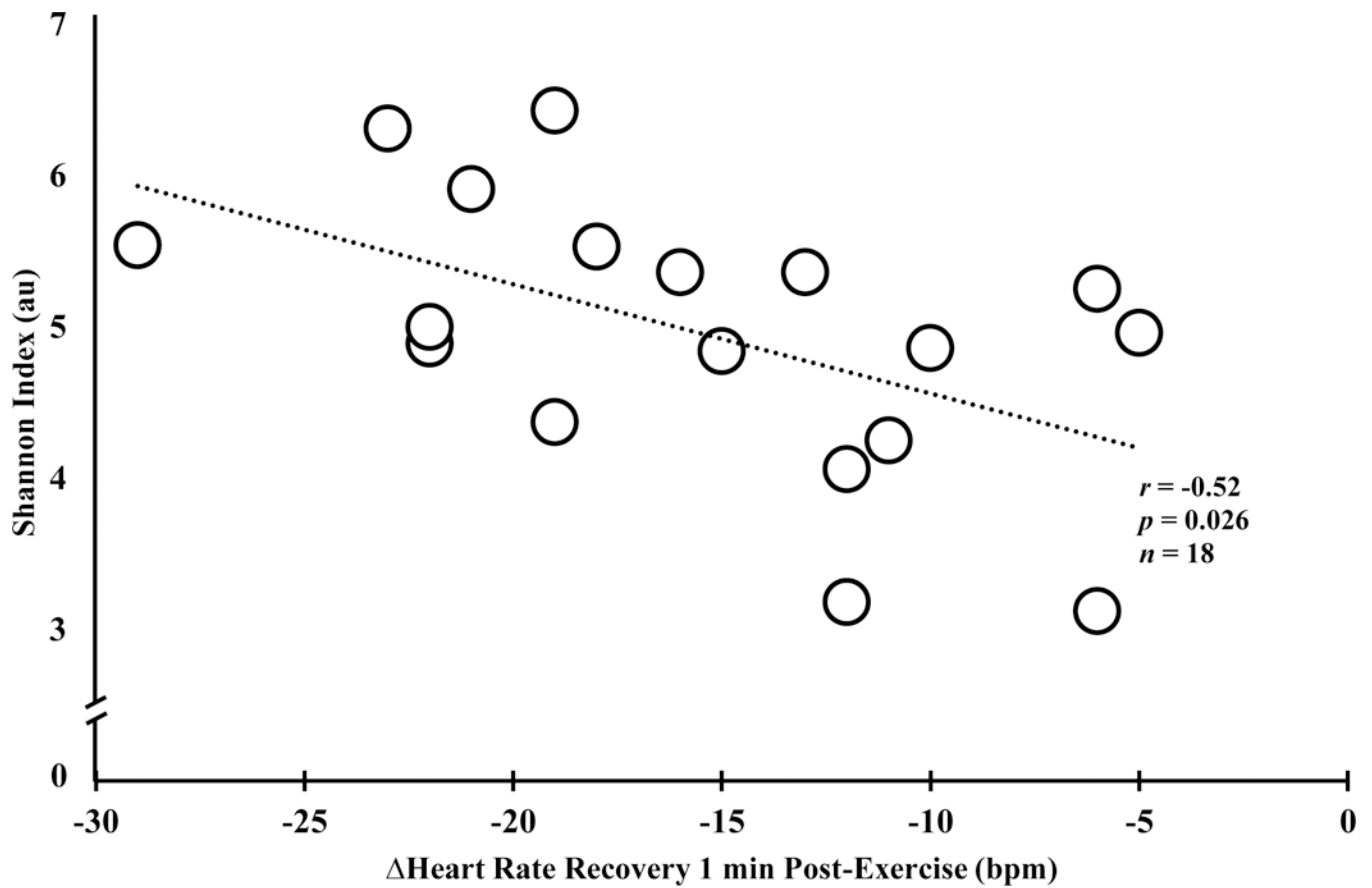


Figure 3. Unadjusted scatterplot of Shannon index and the change () in heart rate recovery 1-minute post-graded exercise test. Note the negative association depicting higher alpha diversity and greater parasympathetic reactivation, evidenced by more pronounced heart rate recovery. **significance at $p < 0.05$.

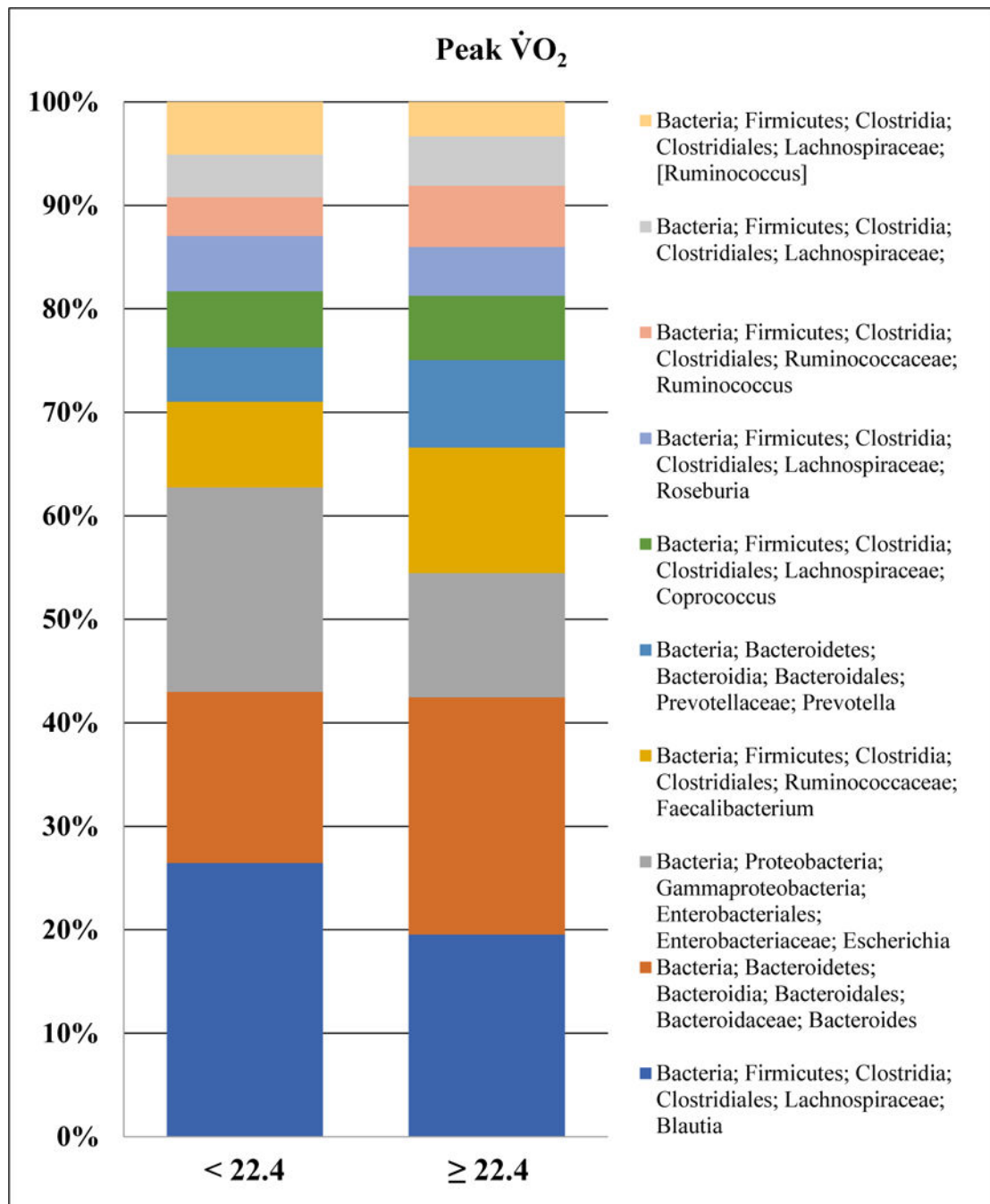


Figure 4. Top 10 genera truncated for peak $\dot{V}O_2$. Note that groups are dichotomized by VO_{2peak} above and below $22.4 \text{ mL O}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$.

Table 1.Participants characteristics ($n = 37$).

Demographics	
Age (yrs.)	55 ± 9
Race (no.(%))	
African American	16(43)
European American	19(51)
Other	2(6)
Comorbidities	2.1 ± 1.9
BMI (kg/m ²)	31.8 ± 7.8
Waist circumference (cm)	92 ± 16
Body fat (%) [‡]	46 ± 7
Cancer status	
Months since diagnosis	53 ± 60
Breast cancer stage (no.(%))	
DCIS	5 (14)
I	11 (29)
II	17(46)
III	4(11)
Chemotherapy (yes)	26(70)
Radiation (yes)	20(54)
Fitness and heart rate responses	
Peak V _O ₂ (mL _O ₂ kg ⁻¹ min ⁻¹)	20 ± 5
%HR _{max} stage 1	61 ± 7
%HR _{max} stage 2	64 ± 8
%HR _{max} stage 3	70 ± 10
%HR _{max} stage 4	75 ± 11
Physical activity measures	
TEE (kcal day ⁻¹) [‡]	2012 ± 302
AEE (kcal day ⁻¹) [‡]	650 ± 229
MVPA (min week ⁻¹)	173 ± 90
Diet [‡]	
Sugar (g)	85 ± 42
Carbohydrates (g)	206 ± 66
Fiber (g)	16 ± 8
Alpha diversity (au)	
Chaol	341 ± 44
Observed species	277 ± 50
PD whole tree	23 ± 4
Shannon	5 ± 1
Simpson	0.9 ± 0.08

\dot{V} _n = 32; \dot{V} _n = 36;

BMI, body mass index; ductal incarcinoman in situ; Peak oxygen uptake (VO₂) estimated from the graded exercise test; % HR_{max} stage 1–4, percent of age-predicted heart rate (HR) max derived (from 220-age) during stage 1–4 of modified-Naughton protocol; TEE, total energy expenditure; AEE, activity-related energy expenditure; MVPA, moderate-to-vigorous physical activity. Diet represents daily averages. Values are shown as means and SD's unless noted otherwise.

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Table 2.

Bivariate correlation matrix ($n = 37$)

Variables	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1) Peak VO ₂	-															
2) %HRmax stage 1	-0.77 [†]	-														
3) %HRmax stage 2	-0.83 [†]	0.97 [†]	-													
4) %HRmax stage 3	-0.88 [†]	0.90 [†]	0.94 [†]	-												
5) %HRmax stage 4	-0.53 [†]	0.56 [†]	0.62 [†]	0.56 [†]	-											
6) Body fat (%)	-0.74 [†]	0.52 [†]	0.63 [†]	0.70 [†]	0.43*	-										
7) Waist circumference	-0.64 [†]	0.36*	0.51 [†]	0.60 [†]	0.43*	0.80 [†]	-									
8) TEE	-0.19	-0.03	0.12	0.20	0.01	0.54 [†]	0.59 [†]	-								
9) AEE	-0.07	-0.01	0.07	0.15	-0.15	0.40 [†]	0.28	0.76 [†]	-							
10) MVPA	-0.05	0.04	0.06	0.03	0.03	0.15	0.12	0.21	0.24	-						
11)Chao1	0.45 [†]	-0.38*	-0.41*	-0.44 [†]	-0.35*	-0.41*	-0.23	-0.24	-0.03	0.02	-					
12) Observed species	0.51 [†]	-0.45 [†]	-0.49 [†]	-0.50 [†]	-0.36*	-0.32*	-0.25	-0.05	0.17	0.03	0.86 [†]	-				
13) PD whole tree	0.51 [†]	-0.40 [†]	-0.45 [†]	-0.47 [†]	-0.28	-0.38*	-0.23	-0.10	0.04	0.01	0.89 [†]	0.94 [†]	-			
14)Shannon	0.34*	-0.30	-0.34*	-0.35*	-0.37*	-0.33	-0.18	-0.01	-0.04	0.01	0.54 [†]	0.68 [†]	0.66 [†]	-		
15) Simpson	0.17	-0.19	-0.24	-0.21	-0.34*	-0.27	-0.15	0.02	0.01	0.13	0.30	0.42 [†]	0.40*	0.89 [†]	-	
16) Menopausal status	-0.01	0.01	0.01	-0.10	0.18	-0.02	0.06	0.03	-0.09	-0.11	-0.32	-0.28	-0.27	-0.17	-0.15	-

Peak oxygen uptake (V O₂) estimated from the graded exercise test (mL O₂ kg⁻¹ min⁻¹); %HR_{max} stage 1–4, percent of age-predicted heart rate (HR) max derived (from 220-age) during stage 1–4 of the modified-Naughton protocol; Body fat (%) (n = 32); TEE, total energy expenditure (n = 32); AEE, activity-related energy expenditure (n = 32); MVPA, moderate-to-vigorous physical activity measured with accelerometry; Menopausal status, participants dichotomized by 0 (no) and 1 (yes) indicating whether they experienced menopause.

[†] $p < 0.01$;

* $p < 0.05$.

Note all variables were analyzed using Pearson's correlation.

Modeling alpha diversity indices adjusted for peak V O₂, percent body fat, and menopausal status ($n = 32$).

Table 3.

	R²	partial <i>r</i>	<i>p</i>-value
Model 1: Chaol	0.38		
Peak VO ₂		0.34	0.07
%Body fat		-0.09	0.63
Menopausal status		-0.39*	0.03
Model 2: Observed species	0.37		
Peak VO ₂		0.47*	0.01
%Body fat		0.11	0.55
Menopausal status		-0.34	0.07
Model 3: PD Whole Tree	0.43		
Peak VO ₂		0.51*	0.004
%Body fat		0.10	0.60
Menopausal status		-0.33	0.07
Model 4: Shannon	0.21		
Peak VO ₂		0.24	0.19
Body fat		-0.06	0.74
Menopausal status		-0.24	0.21
Model 5: Simpson	0.12		
Peak VO ₂		0.08	0.68
%Body fat		-0.14	0.47
Menopausal status		-0.21	0.27

Peak oxygen uptake (V O₂) estimated from the graded exercise test; percent (%) body fat calculated from isotopic total body water; menopausal status, participants dichotomized by 0 (no) and 1 (yes) indicating whether they experienced menopause.

* $p < 0.05$.

Modeling alpha diversity indices adjusted for heart rate max, percent body fat, and menopausal status ($n = 32$).

Table 4

	R²	partial <i>r</i>	<i>p</i>-value
Model 1: Chao1	0.49		
%HR _{max}		-0.53 *	0.003
%Body fat		0.01	0.96
Menopausal status		-0.49 *	0.006
Model 2: Observed species	0.45		
%HR _{max}		-0.55 *	0.001
%Body fat		0.15	0.43
Menopausal status		-0.43 *	0.02
Model 3: PD Whole Tree	0.50		
%HR _{max}		-0.59 *	0.001
%Body fat		0.12	0.53
Menopausal status		-0.44 *	0.02
Model 4: Shannon	0.26		
%HR _{max}		-0.34	0.07
%Body fat		-0.03	0.89
Menopausal status		-0.29	0.12
Model 5: Simpson	0.15		
%HR _{max}		-0.20	0.30
%Body fat		-0.07	0.70
Menopausal status		-0.23	0.21

%HR_{max}: percent of age-predicted heart rate (HR) max derived (from 220-age) during stage 3 of the modified-Naughton protocol;

%body fat calculated from isotopic total body water; menopausal status, participants dichotomized by 0 (no) and 1 (yes) indicating whether they experienced menopause.

* $p < 0.05$.