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# Exercise prevents high fat diet-induced bone loss, marrow adiposity and dysbiosis in male mice

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ABSTRACT

High fat diets can have detrimental effects on the skeleton as well as cause intestinal dysbiosis. Exercise prevents high fat (HF) diet-induced obesity and also improves bone density and prevents the intestinal dysbiosis that promotes energy storage. Previous studies indicate a link between intestinal microbial balance and bone health. Therefore, we examined whether exercise could prevent HF-induced bone pathology in male mice and determined whether benefits correlate to changes in host intestinal microbiota. Male C57Bl/6 mice were fed either a low fat diet (LF; 10 kcal% fat) or a HF diet (60 kcal% fat) and put under sedentary or voluntary exercise conditions for 14 weeks. Our results indicated that HF diet reduced trabecular bone volume, when corrected for differences in body weight, of both the tibia (40% reduction) and vertebrae (25% reduction) as well and increased marrow adiposity (44% increase). More importantly, these effects were prevented by exercise. Exercise also had a significant effect on several cortical bone parameters and enhanced bone mechanical properties in LF but not HF fed mice. Microbiome analyses indicated that exercise altered the HF induced changes in microbial composition by reducing the *Firmicutes/Bacteriodetes* ratio. This ratio negatively correlated with bone volume as did levels of *Clostridia* and *Lachnospiraceae*. In contrast, the abundance of several *Actinobacteria* phylum members (i.e., *Bifidobacteriaceae*) were positively correlated with bone volume. Taken together, exercise can prevent many of the negative effects of a high fat diet on male skeletal health. Exercise induced changes in microbiota composition could represent a novel mechanism that contributes to exercise induced benefits to bone health.

## 1. Introduction

Osteoporosis affects > 10 million people in the U.S. and annually accounts for over 2 million bone breaks [1]. Many factors including obesity contribute to low bone mass [2]. Obesity in the U.S. is increasing and currently accounts for > 2 billion people [3,4]. While the etiology of obesity is complex and involves both genetic and environmental factors [3], the rise in obesity is especially attributed to the western diet, which is high in fat, simple carbohydrates, and processed foods [5]. While a healthy increase in body mass can benefit bone health, due to the elevated weight bearing properties, these benefits can be lost within the context of a high fat (HF) diet-induced weight gain [2,6].

The impact of obesity and HF diets on bone health is an area of significant concern [7–11]. Rodent models provide a means to examine the direct effects of HF diets on bone health and obesity-related pathologies [12]. Many reports demonstrate that a high fat diet (i.e.: 60 kcal% fat) given to young (5 to 6-week-old) rodents for 12 weeks results in reduced bone density, formation and stiffness as well as increased marrow adiposity [13–16]. High fat diets (60 kcal% fat) also promoted alveolar bone loss, with greater bone loss occurring in young compared to adult mice [17,18]. In fact, adult mice display variable responses to HF consumption, including increased bone density initially (possibly due to the increased weight-bearing load) followed by bone loss at later time points [19,20]. Interestingly, not all fats have negative

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effects on bone; unsaturated fat and diacylglycerol are reported to have beneficial bone effects [21–23].

Recent studies demonstrate that HF diets significantly alter the intestinal microbiota which may in turn lead to systemic pathologies such as metabolic syndrome and cardiovascular disease [24–28] The human gut harbors at least $10^{14}$ bacteria with unique metabolic functions that cannot be performed by the host [29,30]. Therefore, healthy composition of gut microbiota (eubiosis) plays an important role in physiological homeostasis. In contrast, intestinal dysbiosis, characterized by an imbalance of host microbes or a predominance of harmful bacteria in the gut can result in the pathogenesis of disease [31]. Environmental factors such as diet can modify microbiota composition. For example, mice fed a HF have reduced levels of *Bacteroidetes* in fecal samples, while a HF and high sugar diet increased the amount of *Clostridium innocuum*, *Catenibacterium mitsuokai* and *Enterococcus* spp. in the feces [27,32]. In humans, phyla level shifts have been reported with HF containing Western diets that appear to impact host metabolism and contribute to the development of obesity [25,26]. Obese individuals have an imbalance in primary bacterial phyla comprising the gastrointestinal microbiota, characterized by a decreased abundance of *Bacteroidetes* and a greater *Firmicutes:Bacteriodetes* ratio [26,29,33]. How intestinal dysbiosis can affect the skeleton is an area of active investigation [34–38]. The gut-bone signaling axis is thus receiving increasing attention as a therapeutic target to treat osteoporosis [36,39–45].

Regular exercise is a well-accepted means to protect against many chronic diseases, including obesity [46]. Exercise can normalize body weight, reduce body fat, improve glucose metabolism as well as reduce markers of systemic inflammation in mouse models of diet-induced obesity [47–50]. Voluntary exercise can prevent HF-induced intestinal dysbiosis [51] as well as reduce the incidence of colon cancer [46,52]. Numerous studies demonstrate that exercise increases bone density, consistent with Wolf's law of bone adaption to loading [53]. Although exercise and diet can influence bone health separately, it is not well known if exercise can prevent HF diet-induced changes in skeletal physiology. Thus, the current study tested the hypothesis that voluntary exercise can prevent HF-induced changes in bone structure and strength in male mice. Similar to published studies in female mice [54,55], we find that exercise prevents the effects of a HF diet on bone (osteoporosis and marrow adiposity). We further show that microbiota changes correlate with changes in bone health parameters and may represent a novel mechanism by which exercise may prevent the bone compromise reported with HF consumption.

## 2. Methods

### 2.1. Mouse model

Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine) as littermates at 5 weeks of age. Mice were allowed to acclimate to their environment for 1 week with a standard chow (18 kcal% fat, 58 kcal% carbohydrate, 24 kcal% protein) before diets were altered (at 6 weeks of age) and running wheels were mounted in cages for the corresponding voluntary exercise groups. After the acclimation period, mice were given either low fat diet chow (LF: 10 kcal% fat, 20 kcal% protein, 70 kcal% carbohydrate for a total of 3.85 kcal/g; catalog number D12450B) or high fat diet chow (HF: 60 kcal% fat, 20 kcal% protein, 20 kcal% carbohydrate for a total of 5.24 kcal/g; catalog number 12492) for 14 weeks (Research Diets Inc., New Brunswick, New Jersey). The source of fat is lard (cholesterol content is 72 mg/100 g). Major differences between the diets are that the low fat diet contains 20 g% lard compared to 245 g% lard in the high fat diet, whereas the high fat diet contains 0 g% corn starch compared to 315 g% cornstarch in the low fat diet. For the groups allowed voluntary exercise, a non-load bearing 14.6 cm hamster wheel (PetSmart, Phoenix, AZ) was suspended from a metal rod in the cages and revolutions were recorded using a bike odometer. Running distances were calculated from the product of wheel circumference and revolutions, as previously described [51]. All mice were kept on a light/dark (12 h/12 h) cycle at 23 °C. Final body weight was determined at the end of week 12 on the protocol. At week 14 of the protocol mice were euthanized and tissues were harvested. The right and left soleus weights, heart and epididymal fat pad weights, and right and left tibia lengths were recorded. The protocol was approved by the Midwestern University Institutional Animal Care and Use Committee and studies were conducted in accordance with safe animal care and use following National Institutes of Health guidelines for humane animal care.

### 2.2. Fasting blood glucose measurements

Fasting blood glucose was measured after 13 weeks of the diet and exercise protocols. Mice were fasted overnight in cages without bedding prior to measuring tail vein blood glucose levels using a OneTouch UltraMini® monitor (LifeScan, Inc. Milpitas, CA).

### 2.3. Micro-computed tomography (μCT) analysis

One tibia was fixed in formalin at harvest and placed in 70% ethanol 24 h later for scanning using the GE Explore μCT system at a voxel size of 20 μm from 720 views with a beam strength of 80 kV and 450 μA. Thresholds were chosen based on the average of the system autothresholds obtained from the low fat diet sedentary mouse group. An average isosurface threshold of 700 for trabecular bone and 1200 for cortical bone was used. Each scan included tibias from each experimental group and a phantom control. Trabecular bone analyses were made in a region of trabecular bone immediately distal to the growth plate and extending to 10% of the tibia length toward the diaphysis and excluding the outer cortical shell. The trabecular region was oriented so that the region analyzed was perpendicular to the growth plate. Trabecular isosurface images were taken from the same region as the trabecular bone was analyzed. Trabecular bone mineral density (BMD), bone volume fraction (BV/TV), thickness (Tb.Th), spacing (Tb.Sp), and number (Tb.N) were determined using GE Healthcare MicroView software. Cortical measurements were performed in a 2 × 2 × 2 mm cube aligned distally where the external diaphyseal shape transitions from pointed to nearly circular. Cortical bone parameters measured include thickness, cross sectional surface area, inner perimeter, outer perimeter, moment of inertia (MOI), marrow area, and bone mineral density values (measured using a 0.1 × 0.1 × 0.1 mm cube in the same region) were determined using MicroView software.

### 2.4. Histomorphometry

Tibias were fixed in 10% formalin for 24 h then changed to 70% Ethanol. Fixed samples were processed on an automated Thermo Electron Excesior tissue processor for dehydration, clearing, and infiltration using a routine overnight processing schedule. Samples were embedded in Surgipath-embedding paraffin on a Sakura Tissue Teck II-embedding center. Paraffin blocks were sectioned at 5 μm on a Reichert Jung 2030 rotary microtome. Slides were stained for TRAP activity and counterstained with hematoxylin according to manufacturer's protocol (387A-1KT, Sigma, St. Louis, MO). Slides were photographed in 5 sections per slide at 25× magnification for osteoblast and osteoclast counts and at 10× magnification for adipocytes. Image Pro-plus software was used in analysis of slide images. In the tibia trabecular region, ranging from the growth plate to 2 mm toward the diaphysis, osteoblast and osteoclast surface area was measured and expressed as a percentage of total bone surface. Similarly, adipocytes > 30 μm in size were counted in the same area and expressed as the number per μm of marrow area. Histological analyses and measurements were performed in a blinded manner to treatment groups.

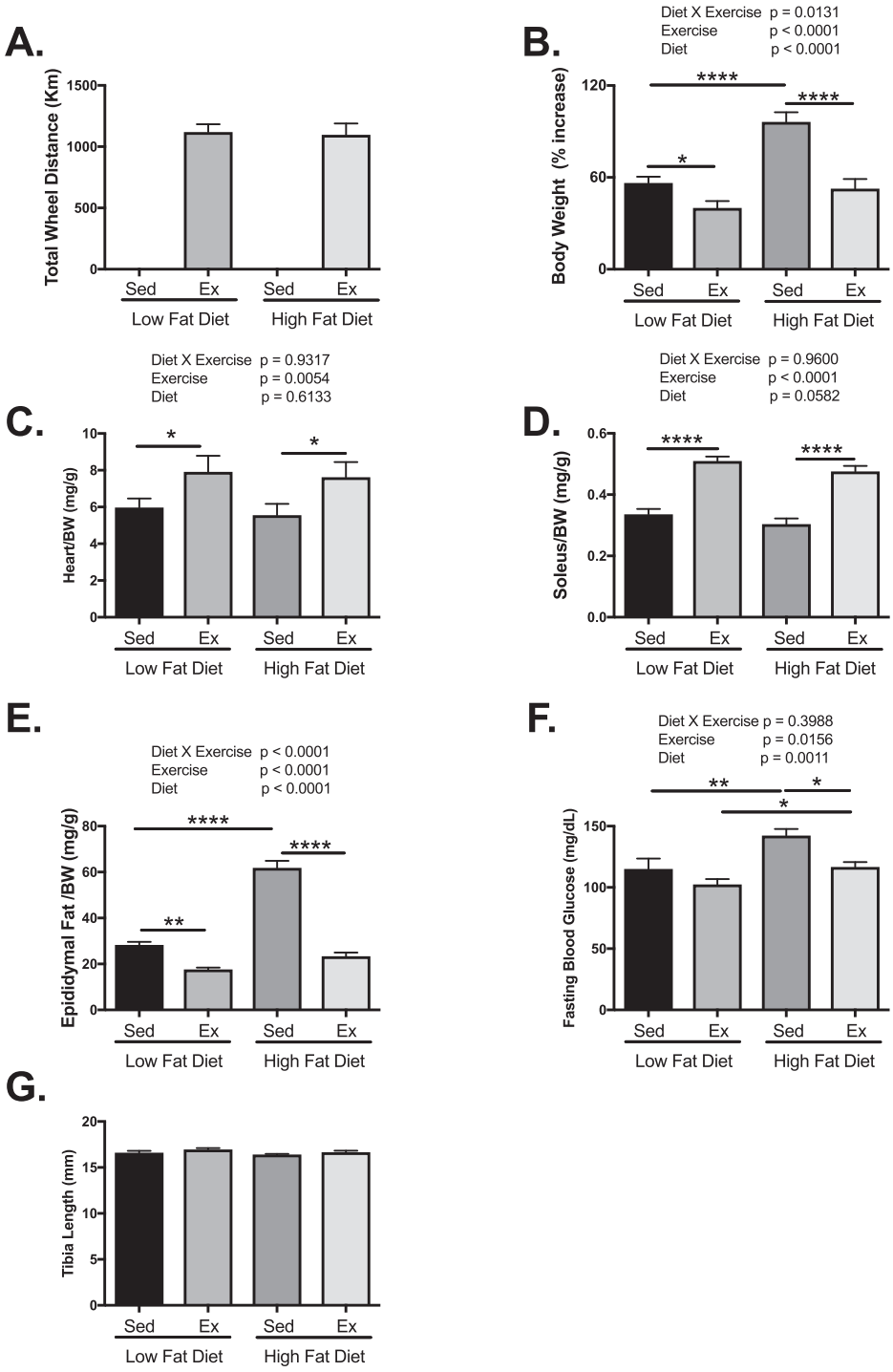


**Fig. 1.** General mouse body parameters confirm exercise (Ex) reduces fat mass and increases muscle mass. Male mice (6 weeks of age) were fed a low fat or high fat diet for 14 weeks. Exercise groups (Ex) had running wheels in their cage, sedentary groups (Sed) did not. At 20 weeks of age, the experiment was ended and mouse weight, tissue mass and bone length were determined. A) Total distance traveled in running wheel during the course of the experiment (14 weeks) as determined by wheel monitors (n = 6/group). B) Body weight expressed as a percent increase relative to baseline weights. C–E) Final heart, soleus muscle and epididymal fat pad mass corrected to final body weight, respectively ($n \geq 16$ per group). F) Fasting blood glucose levels. G) Tibia length at harvest ($n \geq 16$ per group). Values are average ± SE. For B-F statistical analyses were performed by 2-way ANOVA with Fisher post-test. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$.

### 2.5. Serum measurements

Blood was collected at the time of harvest via cardiac puncture, allowed to clot at room temperature for 5 min, and then centrifuged at 4000 rpm for 10 min. Serum was removed and stored at −80 °C. Tartrate-resistant acid phosphatase (TRAP5b) and Osteocalcin (OC) were measured using Mouse assay kits (SB-TR103, Immunodiagnostic Systems Inc., Fountain Hills, AZ and BT-470 Biomedical Technologies Inc., Stoughton, MA, respectively) according to the manufacturer's protocol.

### 2.6. Mechanical testing

Mouse tibial diaphyses were wrapped in gauze which was soaked in 1× phosphate buffered saline and stored at −20 °C until ready for analysis. Tibia diaphysis were then thawed at room temperature and subjected to three-point bending. The bones were placed on the support of the apparatus with the flat lateral side facing down and loaded using an MTS Insight at 0.05 mm/s until failure. The stiffness, elastic modulus and ultimate stress were calculated as previously described [56]. Measures were done blinded.

**Table 1**
Tibia trabecular parameters.

| | Low fat diet | | High fat diet | |
|---|---|---|---|---|
| | Sedentary (n = 21) | Exercise (n = 17) | Sedentary (n = 19) | Exercise (n = 18) |
| BVF (%) | 31.6 ± 2.9 | 24.9 ± 2.0* | 22.3 ± 1.4** | 26.6 ± 2.2 |
| BMD (mg/cm$^3$) | 149.6 ± 12.0 | 135.8 ± 10.3 | 133.6 ± 4.6 | 150.9 ± 10.3 |
| BMC (mg) | 0.36 ± 0.03 | 0.33 ± 0.02 | 0.31 ± 0.01 | 0.31 ± 0.01 |
| Tb.Th (μm) | 43.7 ± 1.4 | 43.6 ± 1.1 | 40.1 ± 0.6 | 45.8 ± 1.5^ |
| Tb.N (1/mm) | 6.80 ± 0.43 | 5.66 ± 0.39* | 5.66 ± 0.30 | 5.90 ± 0.40* |
| Tb.Sp (μm) | 110.9 ± 13.0 | 151.1 ± 16.8* | 145.4 ± 9.7 | 137.8 ± 15.0 |

BVF, bone volume fraction; BMD, bone mineral density; BMC, bone mineral content; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing. * $p \leq 0.05$, ** $p \leq 0.01$, compared to low fat sedentary, ^$p \leq 0.01$, compared to high fat sedentary. Statistics 2-way ANOVA with Fisher's post-test.

**Table 2**
Vertebral trabecular parameters.

| | Low fat diet | | High fat diet | |
|---|---|---|---|---|
| | Sedentary (n = 13) | Exercise (n = 9) | Sedentary (n = 13) | Exercise (n = 13) |
| BVF (%) | 82.5 ± 1.4 | 76.7 ± 2.0 | 77.2 ± 2.2 | 79.7 ± 2.3 |
| BMD (mg/cm$^3$) | 311 ± 7 | 286 ± 6* | 282 ± 8** | 289 ± 7 |
| BMC (mg) | 0.56 ± 0.03 | 0.48 ± 0.03 | 0.51 ± 0.03 | 0.50 ± 0.05 |
| Tb.Th (μm) | 99.2 ± 4.8 | 85.2 ± 4.3 | 85.4 ± 6.7 | 88.5 ± 5.2 |
| Tb.N (1/mm) | 8.5 ± 0.3 | 9.1 ± 0.2 | 9.4 ± 0.4 | 9.0 ± 0.3 |
| Tb.Sp (μm) | 20.2 ± 1.1 | 25.4 ± 1.5 | 23.7 ± 1.7 | 22.4 ± 1.8 |

BVF, bone volume; BMC, bone mineral content; BMD, bone mineral density; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing. Values are mean ± SE. * $p \leq 0.05$, ** $p \leq 0.01$ to sedentary low-fat mice.

### 2.7. Fecal pellet collection and DNA extraction, sequencing and analyses

Extraction of DNA from fecal bacteria followed the protocol of Wang et al. [57]. Fecal pellets were collected directly from the mice at week 12 of the diet and exercise protocol, and stored at −80 °C prior to lysis in 1 ml extraction buffer [50 mM Tris (pH 7.4), 100 mM EDTA (pH 8.0), 400 mM NaCl, 0.5% SDS] containing 20 μl proteinase K (20 mg/ml). Bacterial disruption was achieved using 0.1-mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK) and a Mini-Beadbeater-8 k Cell Disrupter (BioSpec Products, Bartlesville, OK). Bacterial total DNA was extracted using phenol:chloroform:isoamyl alcohol technique [57]. DNA concentrations and purity were assessed by using the NanoDrop® (NanoDop ND-2000 spectrophotometer; Thermo Scientific, Wilmington, DE). A minimum ratio of 1.9–2.0 was accepted for the 260:280 ratio. Microbial sequencing was performed based on bacterial 16S rRNA gene sequencing analysis on the MiSeq Illumina platform (Argonne National Laboratory, Institute for Genomics and Systems Biology, Next Generation Sequencing Core) as previously described by Caporaso et al. [58,59]. Bioinformatic analysis of sequencing data was conducted using the QIIME 1.5.0 software suite [59]. Reads from all samples were clustered at 97% sequence identity into operational taxonomic units (OTUs) then aligned to the October 12th, 2012 Greengenes bacterial reference tree [60].

### 2.8. Statistical analyses

All measurements are presented as the mean ± SE. Statistical significance was determined by 2-way ANOVA using a Fisher's LSD post-hoc test in Prism 7 (GraphPad, Inc.). Student *t*-tests were used for comparisons of exercise between LF and HF groups. Pearson's correlations were used to identify correlations to BV/TV. A p-value of $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Voluntary exercise prevents obesity

Six-week-old male C57BL/6 mice were randomly divided into HF or LF diet groups and then further divided into either a voluntary exercise group or a sedentary group for 14 weeks. Exercise mice spontaneously exercised, with no statistical difference in the total wheel distances of the LF and HF exercise groups (Fig. 1A). At 20 weeks of age general body parameters were obtained. Exercise mice had significantly lower body weights than their sedentary counterparts (16% and 43.6% for LF and HF diets, respectively; Fig. 1B). Based on 2-way ANOVA, both diet and exercise had a significant effect on final body weight. Exercise significantly prevented LF and HF weight gain (Fig. 1B). Indicators of exercise training, heart and soleus muscle mass, were weighed and confirmed a physiologic response to exercise. Heart weights normalized to final body weight were significantly higher in exercised mice of both diet groups ($p = 0.0054$, Fig. 1C). Diet alone did not influence heart weights. Exercise also significantly increased soleus muscle mass in both diet groups, by 51.7% in the LF/exercise and 63.1% in the HF/exercise mice (Fig. 1D, $p < 0.0001$). While epididymal fat pad mass was greatest in the sedentary HF diet mice, it was decreased in both diet groups by exercise (Fig. 1E, $p < 0.0001$); LF/exercise mice had 37.6% less epididymal fat weight and HF/exercise mice had 64.5% less in pad weight than their corresponding sedentary controls. Both diet and exercise significantly influenced final fat pad weights (Fig. 1E, $p < 0.0001$). Examination of metabolic status by fasting serum blood glucose levels suggests that exercise prevented metabolic dysregulation associated with a HF diet (Fig. 1F). Tibia length measurements indicated that bone growth was not influenced by diet or exercise treatments (Fig. 1G).

### 3.2. Influence of diet and exercise on bone health

To determine the impact of the HF diet and exercise on bone health, we examined two different bone sites: tibia and vertebrae (L3). When tibial bone volume fraction (BV/TV) is not corrected to weight, exercise decreased BV/TV in LF mice likely due to the weight loss (Table 1). The HF diet fed sedentary mice had significantly lower tibial BV/TV than LF sedentary mice. Exercise in HF mice modestly increased BV/TV when the values are not corrected for body weight (Table 1). Vertebral BV/TV (not corrected for body weight) did not show a difference between groups (Table 2), suggesting that vertebral BV/TV was relatively constant across groups. However, because of the significant differences in body mass, BV/TV data was also corrected to body weight and a significant protective effect of exercise was demonstrated in both tibial and vertebral BV/TV in HF mice (Fig. 2A–D). Two-way ANOVA analyses indicated that diet and exercise interact to influence bone volume at both tibia and vertebral sites. Trabecular bone structural parameters were also affected. In tibia, exercise had negative effects on trabecular number and spacing in LF mice (Table 1). In contrast, exercise increased trabecular thickness in the HF mice (Table 1).

### 3.3. Exercise prevents marrow adiposity in HF diet fed mice

Consistent with a reciprocal relationship to bone density, the HF/sendentary mice displayed an increase in bone marrow adiposity. Exercise prevented the marrow adiposity in the HF mice, and had only a modest effect on marrow adiposity in LF mice (Fig. 2E, F). Additional cortical bone analyses indicated that LF/exercise mice had lower cortical area and thickness (Fig. 3B) but greater cortical BMD (Fig. 3B) and inner perimeter (Fig. 3C), compared to LF/sedentary mice. Similar cortical responses were seen in HF/exercised mice, except for the decrease in cortical area. Two-way ANOVA revealed that exercise

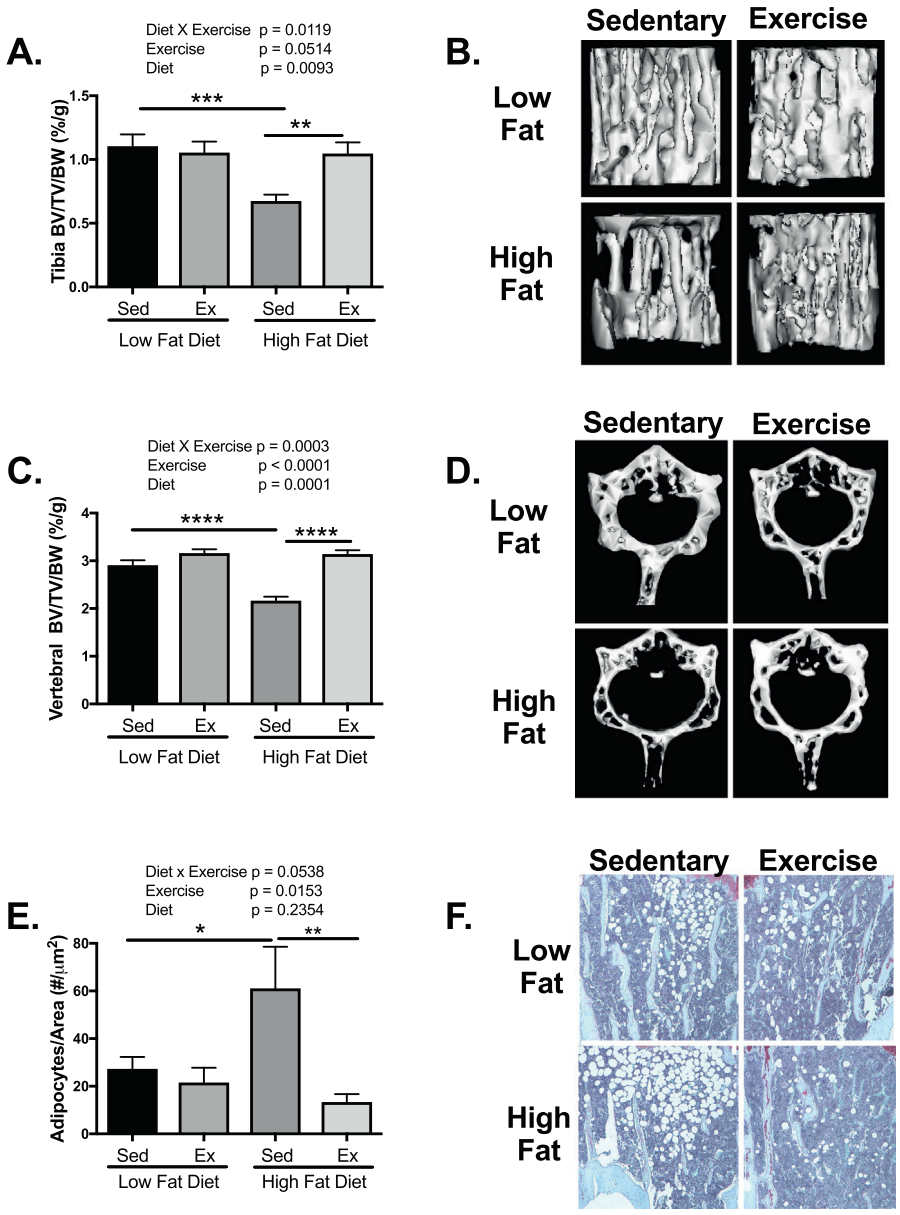


**Fig. 2.** Exercise prevents trabecular bone loss and marrow adiposity. Male mice (6 weeks of age) were fed a low fat or high fat diet for 14 weeks. Exercise groups (Ex) had running wheels in their cage, sedentary groups (Sed) did not. At 20 weeks of age, bones were scanned by microcomputed tomography and sectioned for histology. A) Proximal tibia metaphyseal bone volume fraction (BV/TV%) corrected for body weight. Isosurface images shown to right. ($n \geq 16$) B) Representative tibial isosurfaces. C) Lumbar vertebrae (L3, L4) BV/TV% corrected for body weight. Isosurface images shown to right ($n \geq 9$). D) Representative vertebral isosurfaces. E) Number of adipocytes in the marrow area of the proximal tibia. F) Representative histological images at a magnification of 10×. ($n \geq 11$). Values are averages ± SE. Statistical analysis was performed by 2-way ANOVA with Fisher post-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.01$, ****$p \leq 0.0001$.

significantly affected cortical area, thickness, inner perimeter and marrow area. When expressed relative to body weight, the cortical area, thickness and BMD were affected by both exercise and diet (Fig. 3D).

### 3.4. Effect of exercise and diet on bone strength

Calculated strength, moment of inertia, based on microcomputed tomography measures indicated there were no differences between groups, even when calculated in multiple directions (lxx, lyy) (Fig. 4). However, three-point bending analyses indicated that elastic modulus and stiffness changed in response to exercise in the LF group but not the HF group (Fig. 4).

### 3.5. Influence of diet and exercise on bone formation and resorption

Examination of osteoblast and osteoclast parameters indicated no significant differences between groups, except that serum TRAP levels were lower in the HF/exercise compared to LF/exercise groups (Fig. 5). This suggests that bone formation and resorption responses likely occurred much earlier in the response to exercise and that differences were not detectable after 14-weeks of exercise training. To identify possible associations between parameters that we measured and BV/TV we ran Pearson Correlation Analyses but general parameters such as body weight, muscle weight and marrow adipocyte number did not correlate with BV/TV or BV/TV/BW (data not shown).

### 3.6. Gut microbiome changes in response to diet and exercise

Mouse fecal microbiota were sequenced to determine if there were changes in the microbiota composition that could be associated with changes in BV/TV. We focused on the *Firmicutes* to *Bacteriodetes* ratio, since these bacterial phyla are the most abundant in the GI tract and a higher ratio is linked with dysbiosis and disease. The HF diet only modestly increased the *Firmicutes* to *Bacteriodetes* ratio in the sedentary group compared to the corresponding LF sedentary group, while exercise significantly decreased the ratio in the HF group (Fig. 6A). Two-way ANOVA analysis demonstrated that exercise had a significant effect on intestinal microbial balance, reducing the *Firmicutes:Bacteriodetes* ratio ($p = 0.0281$).

Further examination by Pearson Correlation analyses identified

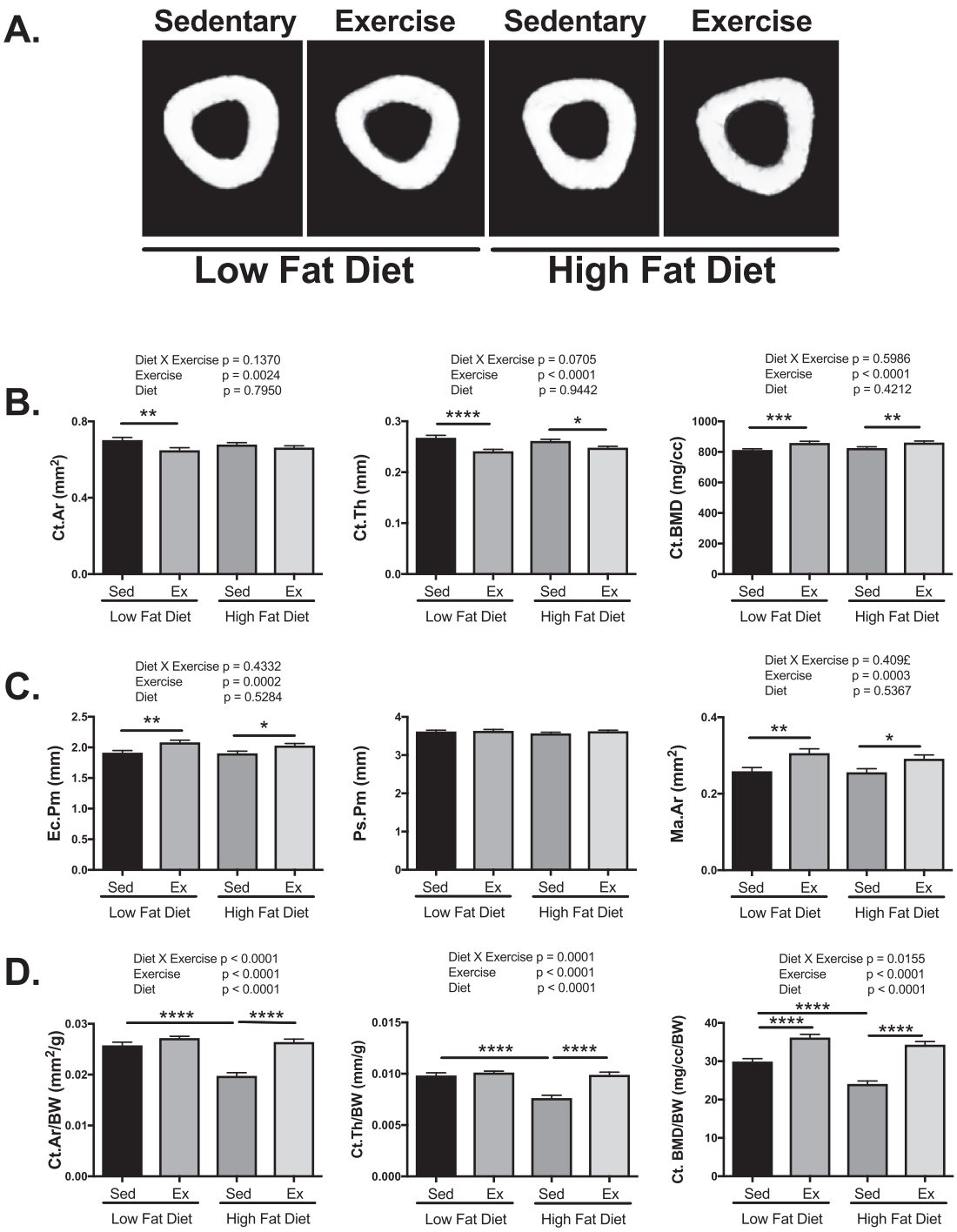


**Fig. 3.** Exercise alters cortical bone structure even in mice fed a high fat diet. Tibias obtained from low fat or high fat fed male mice, 20 weeks of age, that were sedentary (Sed) or exercised (Ex), were analyzed by microcomputed tomography for cortical bone changes. A) Representative isosurface of cortical bone cross sections of the tibia diaphysis. Cortical bone parameters measures: B) cortical area (Ct.Ar), cortical thickness (Ct.Th), cortical bone mineral density (Ct.BMD); C) endosteal perimeter (Ec.Pm), periosteal perimeter (Ps.Pm), marrow area (Ma.Ar); and D) measures expressed relative to body weight (BW). Values are averages ± SE. Statistical analysis was performed by 2-way ANOVA with Fisher post-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.01, ****p ≤ 0.0001. n = 21, 18, 20, 20.

associations between microbiota composition and BV/TV. Specifically, a significant negative correlation was found between the *Firmicutes:Bacteriodetes* ratio and BV/TV/BW ($p = 0.05$, $r = -0.4379$; Fig. 6B), consistent with dysbiosis being linked to bone loss [34]. Relationships between BV/TV and changes in specific bacteria (phylum, class, order and family) were also found. For example, *Firmicutes* subgroups, *Clostridia* and *Lachnospiraceae* (class and family subgroups, respectively) were negatively associated with BV/TV/BW (Fig. 6C). Other *Firmicutes* family members also demonstrated a negative correlation, but were not statistically significant (data not shown). A strong positive correlation was demonstrated between members of the *Actinobacteria* phylum and BV/TV/BW (Fig. 6D; $p \leq 0.0015$).

## 4. Discussion

The Western diet comprised of high fat is thought to be a key contributor to the current epidemic of obesity across the U.S. [4,5]. Diet and obesity are linked with intestinal dysbiosis [26,28–30] as well as

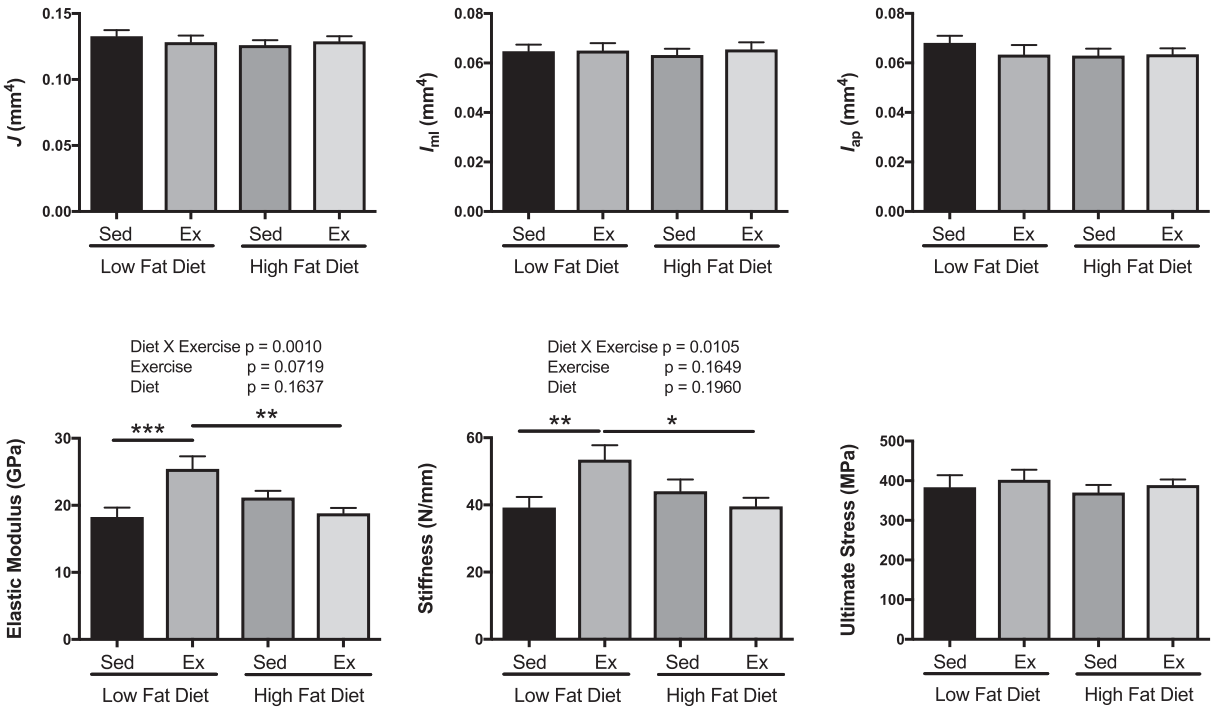


**Fig. 4.** Cortical bone strength analyses. Tibia diaphyseal cortical bone strength analyses in sedentary (Sed) and exercise (Ex) mice fed low or high fat diets for 14 weeks. *Top row*: Microcomputed tomography analyses of cortical bone strength: polar moment of inertia (z-plane) and moment of inertia in x and y planes. n = 21, 18, 20, 20. *Bottom row*: Strength testing of tibias. n = 10, 7, 9, 8. All values are averages ± SE. Statistical analysis was performed by 2-way ANOVA with Fisher post-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.01, ****p ≤ 0.0001.

bone loss [13–15,17] [61] in both human and animal studies. On the other hand, regular exercise has been shown to benefit not only overall health but also reduce obesity and benefit skeletal health [46–50,53]. In the current study, we examined the effect of voluntary exercise, during the period of 6–20 weeks of age, on HF-induced bone loss in male mice. Our findings demonstrate that voluntary wheel running prevented HF diet induced marrow adiposity as well as trabecular bone volume loss (when corrected to body weight). Interestingly, exercise had an effect on cortical bone parameters in both HF and LF mice but benefited bone strength parameters in LF mice only. Associated with these effects, our results clearly show that exercise affects microbiota composition and reduces the *Firmicutes/Bacteridetes* ratio which negatively correlated with bone volume fraction relative to body weight. Given the recent evidence in the literature supporting the role of microbiota in bone health, we propose that exercise may prevent HF-induced bone loss through changes in microbiota composition.

Numerous studies support the link between the composition of the microbiota and the regulation of bone density. Intestinal dysbiosis (microbiota imbalance favoring pathogenic over beneficial bacterial strains) as observed in inflammatory bowel disease is negatively associated with bone density [34]. Similarly, oral microbiota composition of mice promotes periodontal disease when transferred to germ-free mice [62]. On the other hand, treatment with prebiotics or probiotics, which promote a healthy microbiota can also promote skeletal health. For example, treatment of ovariectomized mice with probiotic *Lactobacillus reuteri, LGG or VSL#3* can shift the microbiota composition as well as prevent estrogen deficiency induced bone loss [37,63,64]. Probiotics are also demonstrated to benefit the bone density of healthy male mice [65], diabetic mice [66], female mice experiencing inflammation [67] and periodontal disease [68–70]. Prebiotic ingestion promotes healthy microbiota composition and bone health parameters in mice and humans [35,43,71,72]. However, specific microbiota compositions required for bone health are not known. Our study suggests that high *Firmicutes:Bacterodetes* ratios negatively correlate with bone density. This is consistent with a recent report indicating higher *Firmicutes:Bacterodetes* ratios in osteoporotic versus normal control patients [73]. Interestingly, a positive correlation was found between members of *Actinobacteria* phylum (including the *Bifidobacteriaceae* family) and bone density. Members of the *Bifidobacteriaceae* family are known gut protecting bacteria, that reduce intestinal inflammation and optimize epithelial cell health [74]. Additional studies are needed to determine if the gut-bone relationships depend upon these mechanisms.

Changes in the microbiota composition can affect bone health in a variety of ways, including by influencing intestinal barrier strength, immune cell activation, and gut-bone signaling pathways such as incretins and serotonin [37,41,45,75–77]. In addition, changes in the microbiota and diet can lead to differences in metabolites produced by the bacteria [28]. For example, short chain fatty acids (an end-product of bacterial digestion of fiber, a prebiotic) are demonstrated to enhance calcium absorption in the colon [78,79] and can also directly benefit osteoblast maturation [80]. Phytoestrogens/polyphenols can also benefit bone directly through their enhancement of estrogen receptor signaling [53] [81–83].

Only a few studies have examined the effect of exercise on HF diet induced bone pathology. Kang et al. [84] examined the bone health of 8-week-old rats fed a HF diet (45% kcal fat) for 8 weeks followed by an 8 week period of swimming. HF rats displayed reduced femur and tibia BMD, while swimming exercise raised HF rat bone density to control levels. In another study, Styner et al. [55] feed 10-week-old female C57BL/6 mice a HF (45 kcal fat %) diet and further divided the groups into sedentary and exercise (running wheel) for 6 weeks. Both regular diet and HF mice displayed increases in tibial BV/TV in response to exercise (from 10 to 14% for regular diet and from 9.2 to 13% for high-fat diet fed mice). Several cortical bone parameters were also increased in response to exercise, such as cortical area and periosteal and endosteal perimeters regardless of regular diet or high fat diet. However, HF alone did not cause bone loss. Interestingly, in our studies both HF/sendentary and the LF/exercise mice displayed a decrease bone volume

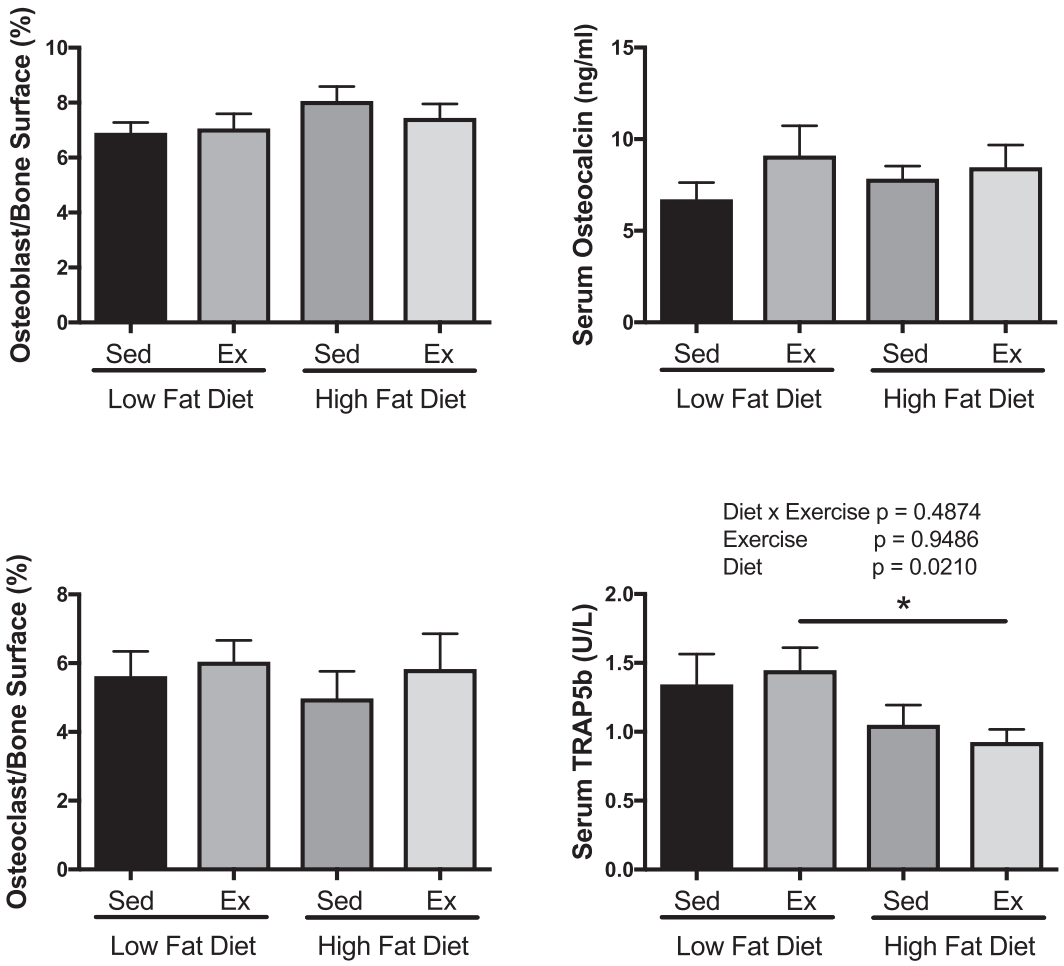


**Fig. 5.** Osteoblast and osteoclast surface and serum marker levels. Bone remodeling markers were assessed in male mice 20-weeks of age, at the end of the 14-week study on mice that were sedentary (Sed) or exercises (Ex) and fed low or high fat diets. Proximal tibia histomorphometry analyses are shown in graphs on left ($n \geq 12$). Serum osteoblast and osteoclast markers (osteocalcin and TRAP) are shown on the right ($n = 6,5,6,6$). Values are averages ± SE. Statistical analysis was performed by 2-way ANOVA with Fisher post-test. *$p \leq 0.05$.

fraction compared to LF mice. Trabecular bone structural analyses also looked worse in the LF exercise group. This suggests that LF diet may not benefit bone health under exercise conditions. The data suggests that the compromise to bone volume fraction in LF exercise mice was secondary to the reduced weight bearing load and not to a reduction in calorie consumption (data not reported). We did observe a trend to increase BV/TV in the HF exercise compared to HF sedentary group. However, when corrected to body weight, tibial and vertebral bone volume fraction did not change in LF mouse groups (suggesting the bone loss was coordinate with weight loss) but decreased in the HF sedentary mice. Similar to Styner et al. [55], cortical analyses indicated an increase in endosteal perimeter in HF mice. Previous studies support that HF can be detrimental to cortical bone parameters, especially when fed to growing mice [85]. Several variables likely contribute to the different responses between our study and the one by Styner [55] including mouse sex (male versus female), mouse age (study began at 6 versus 10 weeks), percent HF (60% rather than 45%), diet comparison (10% LF versus 18% fat), and study length.

Examination of marrow adiposity in the Styner et al. [55] study indicated that HF increases and exercise decreases marrow adiposity. Similarly, we found that exercise prevented HF induced marrow adiposity in male mice. Recently, a second study by Styner et al. (55), in 4-week-old female mice fed a 10% low fat or 45% high fat diet for 12 weeks and then allowed to exercise for 6-weeks (while still consuming the diets) indicated that HF increases bone volume while exercise had no effect on BV/TV but did decrease marrow adiposity. In our study, exercise decreased marrow adiposity only in HF mice, however bone strength measures were only benefited in LF mice, which demonstrated increased elastic modulus and stiffness. This suggests that HF may suppress beneficial bone mineral/matrix changes in response to exercise.

It is important to note that in the current study, diets were given to mice for 14 weeks, beginning at 6-weeks of age, a stage where bone growth/modeling is occurring. We were concerned that this could impact bone growth and affect our measurements. However, measures of tibial length indicated that the 60% high fat diet did not affect growth. This is consistent with studies by Bielohuby et al. [86] who found that 4-week-old male rats fed a HF and low carbohydrate diet (66% fat, 1% carbohydrate) for 4-weeks did not display any growth suppression. However, a 94% fat diet does reduce growth, likely due to reduction of protein intake [86]. Similar to our study, the HF rats exhibited bone loss and reduced markers of bone formation, while resorption was unaffected [86].

In summary, our studies support that a HF diet can modify bone density and the microbiota while exercise prevents many of these changes. Our studies further implicate a role for the microbiota as a key therapeutic target for bone health. Additional studies are needed to tease out significant relationships and identifying patterns of bacterial compositions that benefit bone health. Certainly, given the epidemic of obesity in the US, this is an important area of research that could shed

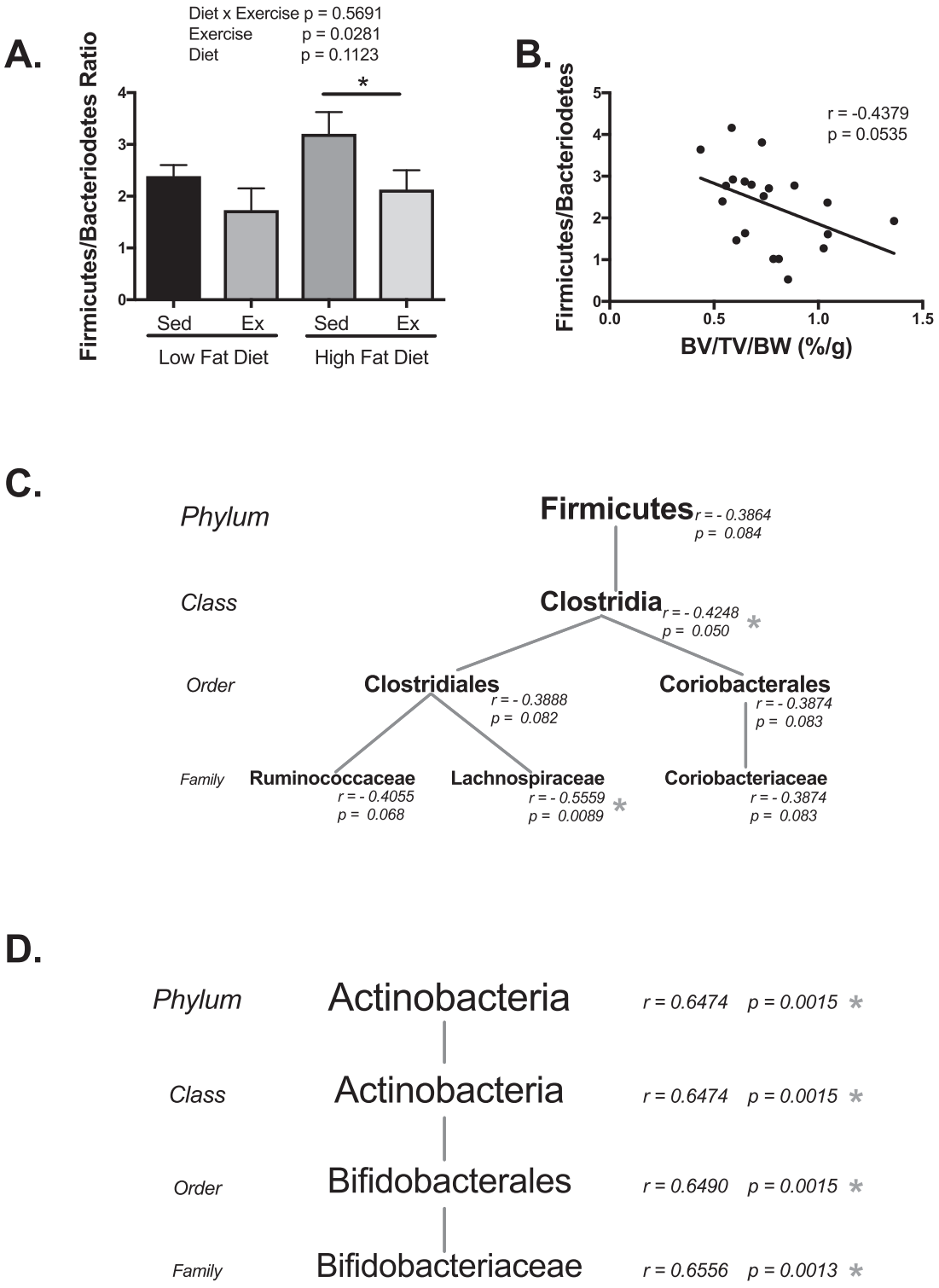


**Fig. 6.** Correlation between microbiome and bone volume fraction. Male mice were fed with either low fat or high fat diet for 14-weeks and allowed to voluntarily exercise using a running wheel in cages (for exercise mice only). Fecal material was collected at time of harvest and analyzed for microbiota composition. A) *Firmicutes/Bacteriodetes* ratio for each group. Statistical analysis was performed by 2-way ANOVA with Fisher post-test. *$p \leq 0.05$. B) Pearson's correlation analyses for *Firmicutes/Bacteriodetes* ratio compared to BV/TV/BW. Each dot represents one mouse. C) Pearson's correlation analyses were used to examine further relationships between the Firmicutes family of bacteria (sub-phylum) populations and BV/TV corrected for body weight. D) Pearson's correlation analyses were used to examine relationships between subpopulations of the Actinobacteria bacterial phylum and BV/TV corrected for body weight. Gray stars denote significant correlations. n = 6, 6, 5, 5.

light on the role of the gut-bone signaling axis in contributing to load induced bone health.

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