## Title

Microbiome—induced increases and decreases in bone matrix strength can be initiated after skeletal maturity



## Journal

Journal of Bone and Mineral Research, 39(11)

## ISSN

0884-0431

## Authors

Liu, Chongshan
Cyphert, Erika L
Stephen, Samuel J
et al.

## Publication Date

2024-10-29

## DOI

10.1093/jbmr/zjae157

*Journal of Bone and Mineral Research*, 2024, **39**, 1621–1632
https://doi.org/10.1093/jbmr/zjae157
Advance access publication: September 30, 2024
**Research Article**

# Microbiome–induced increases and decreases in bone matrix strength can be initiated after skeletal maturity

**Chongshan Liu[1,2], Erika L. Cyphert[1,2], Samuel J. Stephen[3], Bowen Wang[3], Angie L. Morales[2], Jacob C. Nixon[2], Nicholas R. Natsoulas[2], Matthew Garcia[2], Pablo Blazquez Carmona[4], Albert C. Vill[5], Eve Donnelly[6,7], Ilana L. Brito[5], Deepak Vashishth[3,8], Christopher J. Hernandez[1,9,*]**

[1]Departments of Orthopaedic Surgery and Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA, United States
[2]Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY, United States
[3]Shirley Ann Jackson, PhD Center for Biotechnology and Interdisciplinary Studies, Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States
[4]Escuela Superior de Ingeniería, Universidad de Cádiz, Spain
[5]Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY, United States
[6]Department of Material Science and Engineering, Cornell University, Ithaca, NY, United States
[7]Reseach Division, Hospital for Special Surgery, New York, NY, United States
[8]Rensselaer - Icahn School of Medicine at Mount Sinai Center for Engineering and Precision Medicine, New York, NY, United States
[9]Chan Zuckerberg Biohub, San Francisco, CA, United States

*Corresponding author: Christopher J. Hernandez, Department of Orthopaedic Surgery, 513 Parnassus Ave S-1161, San Francisco, CA 94143, USA (christopher.hernandez@ucsf.edu).

## Abstract

Recent studies in mice have indicated that the gut microbiome can regulate bone tissue strength. However, prior work involved modifications to the gut microbiome in growing animals and it is unclear if the same changes in the microbiome, applied later in life, would change matrix strength. Here we changed the composition of the gut microbiome before and/or after skeletal maturity (16 weeks of age) using oral antibiotics (ampicillin + neomycin). Male and female mice ($n = 143$ total, $n = 12$-17/group/sex) were allocated into five study groups: (1) Unaltered, (2) Continuous (dosing 4-24 weeks of age), (3) Delayed (dosing only 16-24 weeks of age), (4) Initial (dosing 4-16 weeks of age, suspended at 16 weeks), and (5) Reconstituted (dosing from 4-16 weeks following by fecal microbiota transplant from Unaltered donors). Animals were euthanized at 24 weeks of age. In males, bone matrix strength in the femur was 25%-35% less than expected by geometry in mice from the Continuous ($p = 0.001$), Delayed ($p = 0.005$), and Initial ($p = 0.040$) groups as compared to Unaltered. Reconstitution of the gut microbiota led to a bone matrix strength similar to Unaltered animals ($p = 0.929$). In females, microbiome-induced changes in bone matrix strength followed the same trend as males but were not significantly different, demonstrating a sex-dependent response of bone matrix to the gut microbiota. Minor differences in chemical composition of bone matrix were observed with Raman spectroscopy. Our findings indicate that microbiome-induced impairment of bone matrix in males can be initiated and/or reversed after skeletal maturity. The portion of the femoral cortical bone formed after skeletal maturity (16 weeks) was small; suggesting that microbiome–induced changes in bone matrix occurred without osteoblast/osteoclast turnover through a yet unidentified mechanism. These findings provide evidence that the mechanical properties of bone matrix can be altered in the adult skeleton.

**Keywords:** biomechanics, bone matrix, bone modeling, microbiome, systems biology - bone interactors

## Lay Summary

This study looked at how changes in the gut microbiome affect bone strength in adult mice. The gut microbiome of male and female mice was altered either before or after skeletal maturity. In male mice, those with altered microbiomes had weaker bones (a 25%-35% reduction). Alterations to the gut microbiome after skeletal maturity had the same effect as lifelong changes, and restoration of an altered gut microbiome after skeletal maturity reversed the effect. Female mice showed a similar trend, but the changes were not statistically significant. The study concluded that changes in the gut microbiome can weaken bone strength in adult male mice in as short as two months, but this effect can be reversed by restoring the microbiome. These changes seem to occur without removal and replacement of bone tissue using the common bone remodeling processes, suggesting an unknown mechanism. This research provides new evidence that gut bacteria can affect bone strength suggesting the possibility that the microbiome can influence bone fragility.

**Received:** January 5, 2024. **Revised:** July 25, 2024. **Accepted:** September 26, 2024

## Introduction

Osteoporosis is a disease characterized by low bone mineral density and increased risk of fragility fracture. Although several effective pharmaceutical interventions are available to increase bone mineral density and reduce fracture risk, prolonged use of existing therapeutics results in either diminishing returns or increased risk of adverse side effects.[1] Hence, new methods to address bone fragility are essential to advancing fracture prevention beyond current capabilities. The mechanical properties of bone tissue (the mineralized matrix) contribute to bone strength but are not directly addressed by existing therapeutics, although recent discoveries suggest the possibility of agents that enhance bone matrix.[2] Here we examine the effect of the gut microbiome on the strength of bone matrix.

The mammalian gut microbiome consists of bacteria, archaea, viruses, fungi, and protozoa.[3] Changes in the constituents of the gut microbiome are associated with clinical conditions[4] and can influence bone quantity and quality.[5–7] We have previously shown that alterations to the composition of the gut microbiome can lead to impaired strength of cortical bone tissue.[8–10] Specifically, when the composition of the gut microbiota is modified in mice through chronic dosing with specific antibiotics, the strength of cortical bone is reduced independent of geometry, indicating impairment of bone matrix.

Antibiotics are among the most powerful manipulations of the composition of the gut microbiome. Oral antibiotics rapidly change the composition of the gut microbiota by suppressing the growth of susceptible organisms.[11] In the days to months after an oral antibiotic regimen ends, the composition of the gut microbiota can return to that seen before treatment, although the time required for full recovery can be lengthy.[12] The composition of the gut microbiome can be rapidly reconstituted by applying a fecal microbiota transplant from a donor.[13]

There are two key limitations to prior studies examining the effects of the gut microbiome on bone matrix strength. First, our prior work manipulated the gut microbiota from weaning until euthanasia at skeletal maturity (16 weeks of life in mice),[9,10] a period when skeletal acquisition is most rapid and the majority of the bone volume within the cortical diaphysis is formed.[14] It is therefore not clear if a change in the gut microbiota alters all the bone matrix or only regions of bone matrix formed after the microbiota is altered. If the gut microbiome only regulates bone tissue strength at the time of matrix synthesis, changes in the gut microbiota later in life (when bone formation rates are lower) are unlikely to lead to functionally important changes in tissue strength, limiting the ability of the gut microbiota to address bone fragility in adults. Second, our prior work only examined male mice and it is unclear if changes in the gut microbiome have similar effects in females.

The goal of this line of investigation is to understand the effects of the gut microbiome on bone strength and fragility. Specifically, we address the following research questions: (1) How do changes in the composition of the gut microbiota applied after skeletal maturity influence bone matrix strength and (2) How does the effect of the gut microbiome on bone matrix strength differ between males and females? We hypothesize that the state of the gut microbiota alters bone matrix strength at the time of matrix formation, and therefore expect manipulation of the gut microbiota after skeletal maturity, when less bone volume is formed, to have only minor effects on bone tissue strength assessed using whole bone testing.

## Materials and methods

### Study design

The study was designed to achieve a minimum of $n = 12$ animals/group (power of 0.80 to detect an effect size of 0.88 with $\alpha = 0.05$ using variance in bone matrix strength from prior work[9]). However, breeding was unexpectedly successful, and all available animals were used in the study. At weaning, pups from different breeding cages were placed in cages at random by sex. Later those cages were randomly assigned into five treatment groups per sex: Unaltered ($n = 13$ M, $n = 14$ F), Continuous ($n = 17$ M, $n = 14$ F), Initial ($n = 18$ M, $n = 18$ F), Reconstituted ($n = 12$ M, $n = 12$ F), and Delayed ($n = 14$ M, $n = 16$ F). Experimental mice were bred from the same cohort of breeders in three separate breeding rounds (2 months and then 6 months after the first).

Animals in the Unaltered group received standard drinking water. Dosing groups received antibiotics via drinking water (1 g/L ampicillin and 0.5 g/L neomycin),[9] an intervention we have previously shown results in reductions in bone matrix strength.[9] Ampicillin and neomycin exhibit low oral bioavailability, hence the antibiotics are poorly distributed systemically and the effect of dosing is primarily due to changes in the constituents of the gut microbiota.[15] The Continuous group was dosed from 4-24 weeks of age. The Delayed group received standard water until 16 weeks of age, and antibiotic laced water from 16-24 weeks. The Initial group was dosed from 4-16 weeks of age, animals then received only standard drinking water for the remainder of the experiment with no intervention to ensure recovery of the microbiota. The Reconstituted group was dosed from 4-16 weeks of age, at which point a fecal microbiota transplant from sex- and age-matched untreated mice was applied to rapidly repopulate the gut microbiota to that of an Unaltered animal (see Supplemental Materials for preparation of the fecal microbiota transplant) (Figure 1A). These experimental groups include two groups in which the gut microbiota was unaltered during rapid bone growth from 4-16 weeks of age (Unaltered and Delayed), three in which the gut microbiota was altered from 4-16 weeks of age (Continuous, Initial and Reconstituted), and three in which the microbiota was changed after 16 weeks of age (Delayed, Initial, and Reconstituted). Fluorescent bone formation markers were injected at 12-, 16-, and 24- weeks of age to identify regions of bone formed at different stages of the experiment (Figure 1B and C). Fecal pellets were collected at 16 weeks of age (immediately before any change in antibiotic dosing), and one day prior to euthanasia (time of day for collection was not controlled) (see Supplemental Materials for detailed methods of gut microbiome analysis). Animals were euthanized at 24 weeks of age. Serum, cecal content, femurs, and tibiae were collected immediately after euthanasia and stored at −80°C. Perigonadal fat pads were collected and weighed. Serum samples were sent to Biomarkers Core at Duke Molecular Physiology Institute for biomarkers quantification.

### Mechanical characterization of bone

The femurs were harvested, wrapped in PBS-soaked gauze and plastic wrap, and stored at −80°C prior to analysis. Femora were submitted to imaging using microcomputed tomography

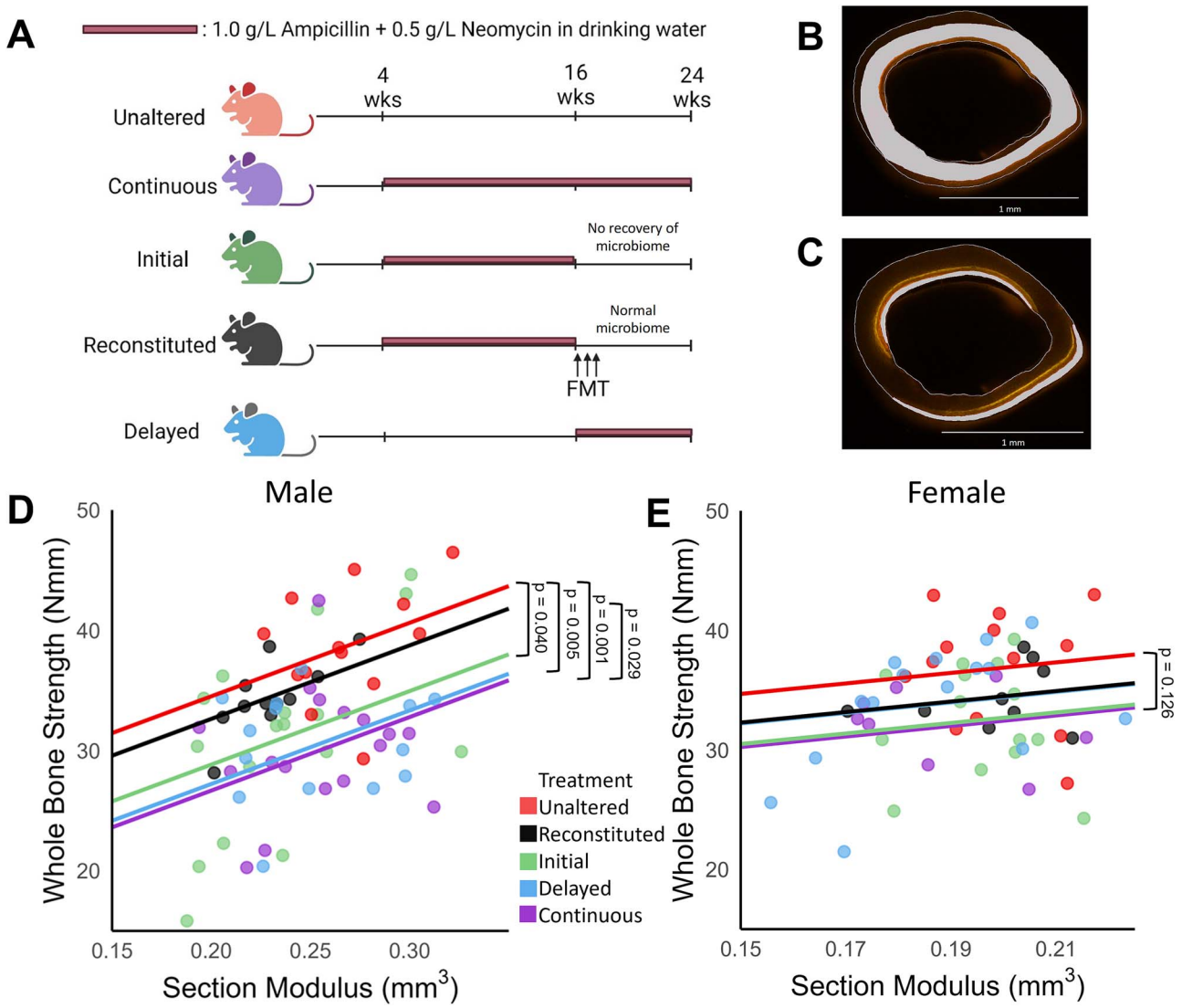


**Figure 1.** (A) Male and female mice were randomly divided into 5 experiment groups in which antibiotics were added to drinking water during different periods of life (highlighted). The mouse femur mid-diaphysis is shown with shaded regions indicating cortical bone formed during (B) 4-16 weeks of age or (C) 16-24 weeks of age as indicated by bone formation labels. (D, E) Graphical illustrations of the ANCOVA analysis comparing whole bone strength among groups after accounting for differences in section modulus are shown. The *p*-values indicate pairwise comparisons within the ANCOVA. In males, dosing before or after skeletal maturity impaired bone matrix strength, but reconstitution of the gut microbiota restored bone matrix strength. Females showed similar trends but no significant differences among groups.

to determine cross-sectional geometry at the mid-diaphysis (including moment of inertia and section modulus) and to measure tissue mineral density using standard methods[16,17] (see Supplemental Materials for detailed methods). The right femora were thawed to room temperature and submitted to mechanical testing (detailed methods in Supplemental Materials). Femora were loaded to failure in three-point bending with the posterior side under tension. Whole bone strength was expressed as the maximum moment (half of the peak load multiplied by half of the span length).[18] Differences in bone matrix strength between groups were detected as differences in whole bone strength after accounting for differences in section modulus using analysis of covariance (ANCOVA). This approach circumvents the assumptions made when calculating ultimate stress using beam theory assumptions.[19] Additionally, tissue strength was calculated using the maximum moment divided by the section modulus. Work to failure was calculated as the total area enclosed within the force-versus-deflection curve, spanning from zero deflection to the maximum deflection.

## Femur dynamic Histomorphometry

After mechanical testing, the proximal end of the fractured femur was dehydrated in ethanol then embedded in polymethyl methacrylate, sectioned and polished to 500 $\mu$m thickness. A fluorescent image of the femur cross section was taken at 5× magnification for each sample to visualize the fluorescent markers (IX83, Olympus, Tokyo, Japan). The mineral apposition rate was measured as the average distance between formation markers divided by the days in between the injection days (33 days for the 12-16 weeks region and 59 days for the 16-24 weeks region).

## Material characterization using Raman spectroscopy

Point spectra were collected from the cross-sections of mid diaphysis of the femur. Raman spectra were collected using a Raman spectrometer equipped with a 785 nm red laser (Ramascope 2000, Renishaw, Mountain View, CA). Raman spectra were acquired at 50× magnification with 0.75 NA and

25% laser power (~75 mW power), 15-second integration time and 5 accumulations. Spectral range was 380 $cm^{-1}$ – 1800 $cm^{-1}$, and grating was 1200 g/cm. Three spectra were taken at regions formed between 12 and 16 weeks of age (as indicated by the fluorescent markers, 60 $\mu$m from the anterolateral side) and three measurements were made at regions formed between 16 and 24 weeks of age (as indicated by fluorescent markers, 30 $\mu$m from the antero-lateral side). Spectra were analyzed to determine crystallinity, mineral-to-matrix ratio (measured as $\nu_1PO4^{3-}$/Proline, $\nu_2PO4^{3-}$/Amide III, and $\nu_1PO4^{3-}$/Amide III), Type-B carbonate substitution, carbonate-to-phosphate ratio, carboxymethyl-lysine, glycosaminoglycans to CH2 proteoglycan content, Amide I collagen maturity as demonstrated previously[20–24] and in greater detail in the Supplemental Materials.

### Gut microbiome analysis

Microbial composition of fecal samples was analyzed using 16S rRNA amplicon sequencing. DNA extraction, purification, library preparation, and sequencing were performed by the UC San Diego Microbiome Core utilizing previously published protocols[24] and analyzed using QIIME using established methods (see Supplemental Materials for detailed methods).

### Statistical analysis

Differences in the Alpha diversity (Shannon index) of the gut microbiome, fat pad weight, bone geometry, bone mechanical properties, and serum concentration measurements of different treatment groups were evaluated using a one-way analysis of variance (ANOVA). A post hoc Dunnett test was used to determine significance between different treatment groups relative to the Unaltered group. Differences in whole bone strength not explained by variation in section modulus were detected by ANCOVA implemented with a generalized linear model, (Supplemental Table S1). Similarly, characteristics of bone geometry were analyzed raw as well as after adjusting for animal body weight using a regression based approach.[25] Permutational multivariate analysis of variance (PERMANOVA) was used to determine differences between the Bray–Curtis beta diversity (microbiome composition) among treatment groups. A linear mixed effect model was applied to examine the effect of the treatment and tissue age. A Dunnett's test was used to adjust for the $p$ values from the Raman data. Unless explicitly specified, statistical tests were performed with a significant level of alpha = 0.05. Pearson's product–moment correlation analysis was used to establish relationships between the averaged Raman measurements of the tissue formed between the 12-16 weeks and 16-24 weeks age and bone measurements. Analyses were conducted using R Statistical Software (v 4.0.3; R Core Team 2020).

## Results

### Bone geometry and mechanical properties

Males in the Continuous group had an impaired bone matrix strength that could not be explained by geometry ($p = .001$ ANCOVA) corresponding to a 35.2% reduction in whole bone strength compared to bones from Unaltered mice (percent reductions described here are calculated from the coefficients of the generalized linear model ANCOVA, see Supplemental Table S1, and observed graphically as the intercept of the lines in Figure 1D, E). Similarly, whole bone strength was reduced in the Delayed (32.7% reduction, $p = .005$) and Initial (25.5% reduction, $p = .040$) groups compared to Unaltered mice with a similar section modulus. The Reconstituted group exhibited a matrix strength similar to that of the Unaltered group ($p = .929$) and greater than that of Continuous group ($p = .029$). No significant differences in cortical bone cross-sectional area, moment of inertia, or section modulus of the mid-diaphyseal cortical bone were observed with or without adjustment for body weight (Supplemental Table S2).

In females, differences in bone matrix strength among groups followed a pattern similar to that seen in males, but no significant differences were detected ($p = .126$ or greater, power = 51.7%, Figure 1E). No significant differences in cortical bone geometry (cross-sectional area, moment of inertia, section modulus) were observed among female groups either as measured or after adjustment for body weight (Supplemental Table S2).

No noticeable differences in the mineral apposition rate were observed among treatment groups (Supplemental Table S3). The tissue mineral density in the cortical region of the femur was increased in the Delayed group compared with the Unaltered group in males ($p < .001$). There were no significant differences among other groups in males or among any groups in females (Supplemental Table S3).

### Changes in the composition of the gut microbiota

In both males and females, the composition of the gut microbiome (Beta diversity) in Continuous, Delayed, and Initial groups was clustered together and differed from that in mice in the Unaltered or Reconstituted mice (Figure 2A and B). A PERMANOVA detected differences among treatment groups with males and females ($p < .001$). While there were differences in the composition of the microbiome between Unaltered and Reconstituted groups, the gut microbiota of the Reconstituted group clustered more closely to that of Unaltered mice (shifted to the right on the most influential principal coordinate). Both males and females demonstrated similar trends in Shannon diversity metrics among groups with Continuous, Initial, or Delayed groups having a significantly lower Shannon Diversity than the Unaltered or Reconstituted groups (Figure 2C and D; $p < .001$).

Fecal microbiota transplant changed the gut microbiota in the Reconstituted group; at 16 weeks (before removal of antibiotics) the composition was similar to that of the Initial group and Continuous group but at 24 weeks clustered close to that of the Unaltered group (see Figure 3A and B; Supplemental Figures S2 and S3), demonstrating recovery of the microbiota after transplant. In the Initial group, the composition of the gut microbiota resembled that of the Continuous group at both 16 and 24 weeks of age, indicating little recovery of the microbiota after 16 weeks, despite termination of antibiotic dosing. At 16 weeks of age, the Delayed group resembled that of the Unaltered group, but after two months of dosing the microbiota resembled that of the Continuous group. Only small changes in the composition of the gut microbiota with time were observed in the Unaltered and Continuous groups.

A combinatorial approach of microbial biomarker analyses (LEfSe and MaAsLin2) was used to identify microbial features associated with a normal (Unaltered and Reconstituted groups) versus impaired bone matrix strength (Continuous, Delayed and Initial groups) in male mice at 24 weeks of

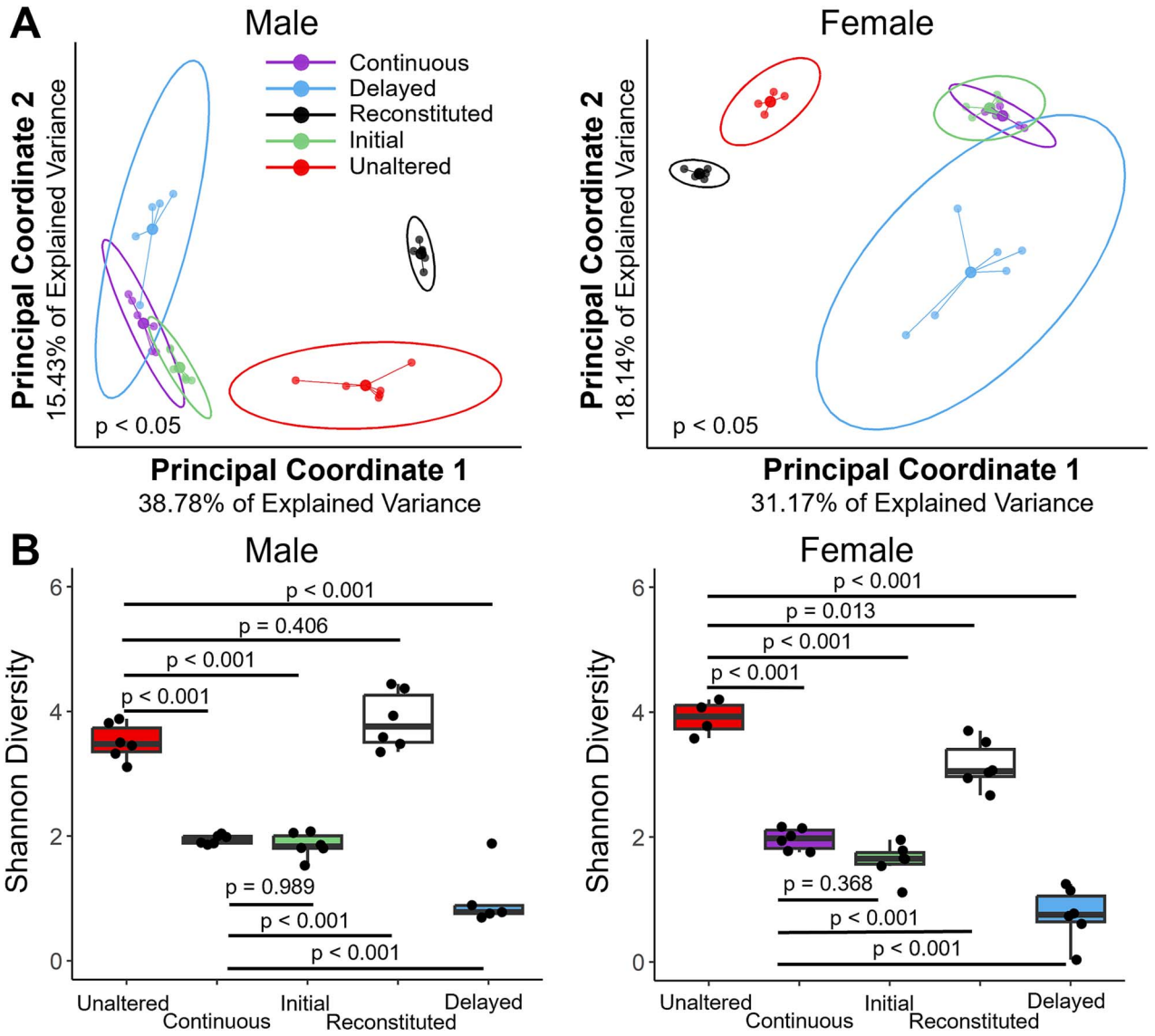


**Figure 2.** The composition of the gut microbiome for the five groups is shown for both sexes. The gut microbiome in animals receiving antibiotics at the time of euthanasia (Continuous, Delayed) was similar to that in the Initial group. The gut microbiome in the reconstituted group was similar to that of the Unaltered group. The results are consistent with both beta diversity (male in A, female in B) and alpha diversity (male in C, female in D).

age. The LEfSe analysis identified an increased abundance of *Bacteroides, Akkermansia*, and *Parabacteroides* and decreased abundance of *Oscillospira*, *Prevotella*, *Ruminococcus*, and *Coprococcus* in groups with impaired bone matrix strength (Continuous, Delayed, Initial) as compared to Unaltered and Reconstituted (Figure 3C). Animals in the Reconstituted group had increased abundance of *Oscillospira*, and *Ruminococcus* and decreased abundance of *Akkermansia*, *Bacteroides*, and *Prevotella* as compared to the Unaltered group. The MaAsLin analysis identified a strong positive association of *Akkermansia* (Continuous, Delayed, Initial groups) and *Enterococcus* (Delayed group) relative to the Unaltered group. Negative associations were identified between the *Lactobacillus* (Continuous, Delayed, Initial groups), *Lactococcus* (Continuous, Delayed, Initial, Reconstituted groups), *Coprococcus* (Continuous, Delayed, Initial groups), *Oscillospira* (Continuous, Delayed, Initial groups), *Turicibacter* (Continuous, Delayed, Initial groups), *Prevotella* (Continuous, Delayed, Initial, Reconstituted groups), and *Allobaculum* (Continuous, Delayed groups) relative to the Unaltered group (Figure 3D). Animals in the Reconstituted group had a strong positive association with *Desulfovibrio*, *Bilophila*, and genera from the *Bacillota* phylum including *Roseburia*, *Dorea*, *Coprococcus*, *Anaerostipes*, *Ruminococcus*, *Oscillospira*, *Butyricicoccus*, and *Lactobacillus* relative to the Unaltered group.

## Sexual Dysmorphism in the composition of the gut microbiota

In males and females, the composition of the gut microbiome (Beta diversity) in the Continuous and Unaltered groups was comparable across sexes at 16 weeks of age (Figure 4A; $p = .097$ by sex, $p < .001$ by group). While there were significant differences in the composition of the microbiome between Unaltered and Continuous groups, the gut microbiota within the same treatment group was comparable between sexes. However, there were noticeable

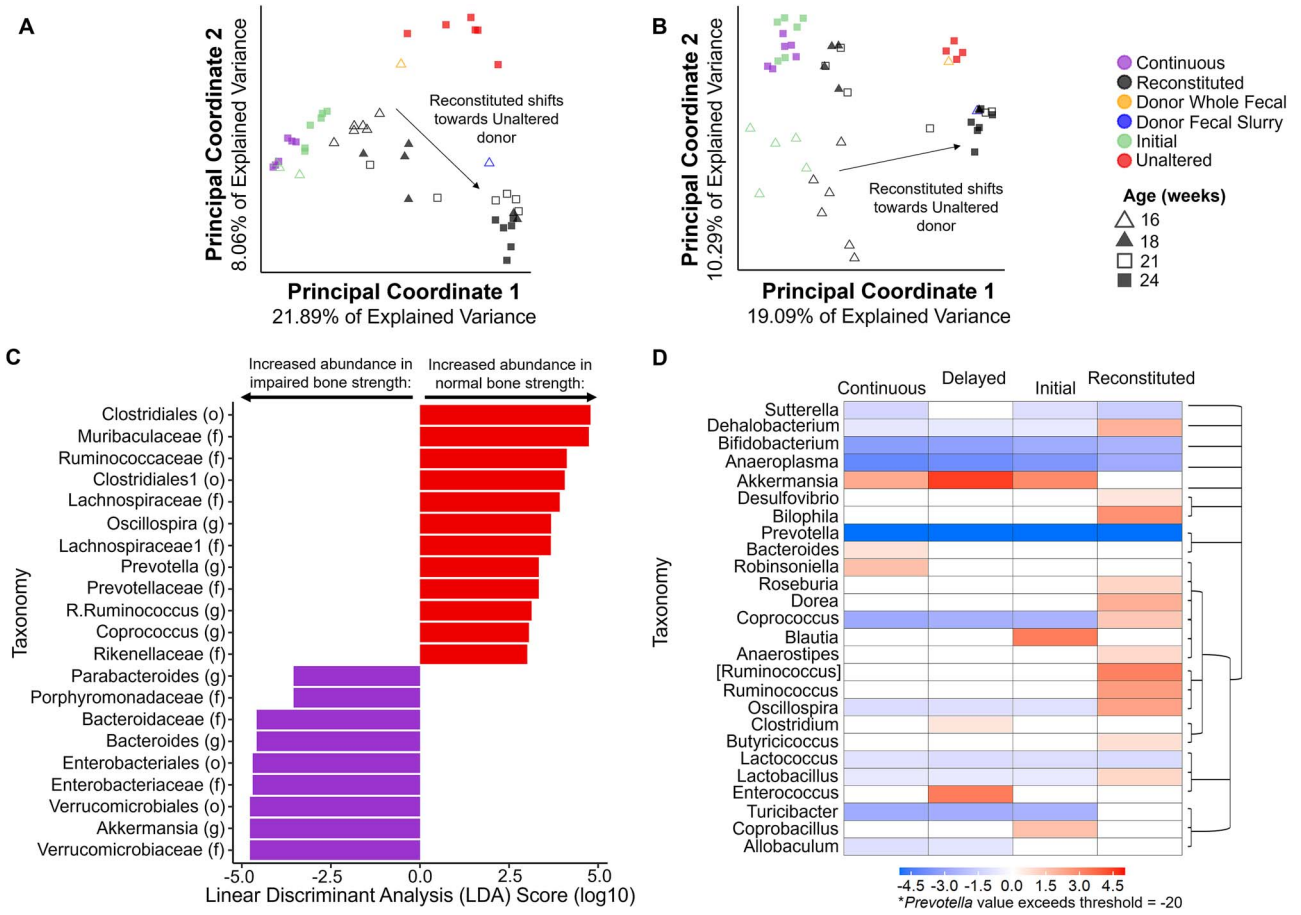


**Figure 3.** (A, B) principal coordinate analysis of the microbiota of fecal samples collected at different time points between 16-24 weeks is shown. In the Reconstituted group, the composition of the microbiota shifted from that similar to the Continuous group at 16 weeks of age to one more similar to the Unaltered group at 24 weeks of age (shift to the right). Fecal pellets collected from donors (whole fecal) and processed for transplant (slurry) are similar to those of the Unaltered group. (C) The linear discriminant analysis of effect size comparing groups with impaired bone matrix strength (Continuous, Initial, Delayed) to groups with normal bone matrix strength (Unaltered and Reconstituted) is shown. Taxa are listed along with rank (f = family; g = genus, etc.). (D) The results of the Microbiome Multivariate Associations with Linear Models (MaAsLin) analysis are shown with taxonomies (relative to the Unaltered group).

differences in the microbiome between Continuous males and Continuous females at 24 weeks of age (Figure 4B, $p = .022$ by sex, $p < .001$ by group). A LEfSe analysis was used to determine differentially abundant taxa between sexes in the Continuous group at 24 weeks of age (Figure 4C). The LEfSe analysis identified increased abundance of *Akkermansia* and *Robinsoniella* and a decreased abundance of *Enterococcus* and *Bacteroides* genera in the Continuous females relative to the Continuous males.

## Bone tissue composition

No significant differences among different treatment groups were observed in the measured $\nu_1PO4^{3-}$/Amide III mineral to matrix ratio (Figure 5A and B), crystallinity (Figure 5C and D) and type B carbonate substitution (Figure 5E and F) when compared to the Unaltered group. The collagen maturity was slightly reduced in the tissue formed between 16 and 24 weeks of age in the Continuous group when compared with the Unaltered group ($p = .031$) in the males. No other significant differences were observed among the other treatment groups in the tissue formed between 12 and 16 weeks of age (Figure 5G) or in the female groups (Figure 5H). There were no significant differences among treatment groups in the mineral to matrix ratio measured by $\nu_2PO4^{3-}$/Amide III, carboxymethyl-lysine, and proteoglycan content (Supplemental Figure S4).

A correlation analysis was performed between the averaged Raman spectroscopy and measurements of bone geometry and mechanical performance (Supplemental Table S4). In the males, carboxymethyl-lysine was positively correlated with both the maximum bending moment (correlation coefficient = 0.530, [0.161 0.769], $p = .008$) and the section modulus (correlation coefficient = 0.591, [0.246 0.803], $p = .003$). In the females, none of the measurements achieved with Raman spectroscopy were correlated to the bone measurements.

## Serum markers

The levels of serum procollagen I N-terminal propeptide (P1NP), a marker indicative of bone formation, exhibited distinct patterns across treatment groups. In males, the Reconstituted ($p = .019$) and Delayed ($p = .021$) groups showed lower levels compared to the Unaltered group. In females, the Continuous ($p = .041$) and Initial ($p = .002$) groups had higher levels of P1NP in comparison to the Unaltered group (Figure 6A, E). No significant differences in the serum tartrate-resistant acid phosphatase 5b (TRAP-5b), a marker indicative of bone

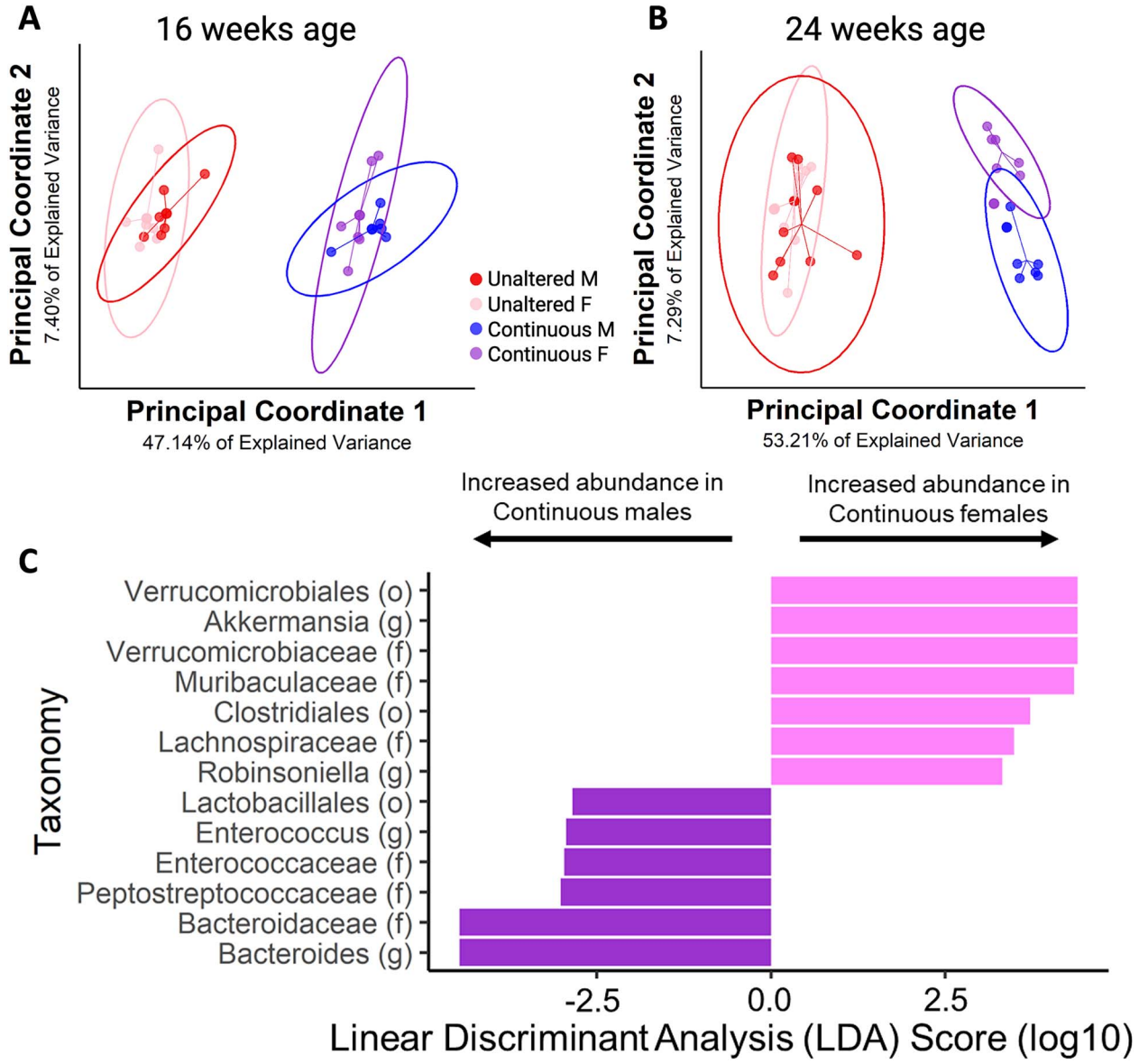


**Figure 4.** Differences in the gut microbiota between males and females are shown. Continuous antibiotic treatment exerted a differential sex-dependent effect on the composition of the microbiome during aging. (A) At 16 weeks of age no sex-dependent differences were apparent, but (B) at 24 weeks of age the antibiotics differentially influenced the microbiome of males and females. (C) Continuously treated females had an increased abundance of *Akkermansia* and *Robinsoniella* and decreased abundance of *Enterococcus* and *Bacteroides* relative to the males.

resorption were observed among groups in both males and females (Supplemental Figure S6B, E). To confirm that there was not a change in osteoclast activity, we also measured the serum carboxy-terminal collagen crosslinks (CTX) in the Unaltered, Continuous, Initial, and Delayed groups. There were no significant differences among groups in the male groups, but the serum CTX level was increased in the female Delayed group as compared to the Unaltered group ($p = .010$) (Supplemental Figure S5). Alterations to the gut microbiome did not lead to changes in serum Insulin-like Growth Factor 1 (Supplemental Figure S6A, D). However, the proinflammatory cytokine Tumor Necrosis Factor Alpha (TNF-$\alpha$) was significantly decreased in the Reconstituted groups when compared to the Unaltered groups in both males ($p < .001$) and females ($p = .001$) (Figure 6B, F). There were no significant differences in the adiponectin level among treatment groups in males, but in females the Reconstituted group showed reduced values compared to the Unaltered groups ($p = .011$) (Figure 6C, G). In males, insulin levels were greater than the Continuous group as compared to Unaltered ($p < .001$) but no differences were detected among groups in females (Supplemental Figure S6C, F). No significant differences in leptin levels were observed among groups in males. In females, the Unaltered group had higher leptin levels compared to Continuous ($p < .001$), Initial ($p = .009$), Reconstituted ($p = .017$), and Delayed ($p < .001$) (Figure 6D, H).

In males, the Interleukin 6 (IL-6), insulin, TNF-$\alpha$, and adiponectin were correlated to several measurements of bone and body composition. IL-6 was positively correlated with the femur length ($r = 0.386$, [0.037 0.651], $p = .032$). Insulin was positively correlated with body weight ($r = 0.357$, [0.027 0.617], $p = .035$) and adiponectin was negatively correlated with body weight ($r = -0.407$, [−0.652 −0.086], $p = .015$). Serum TNF-$\alpha$ was negatively correlated with maximum

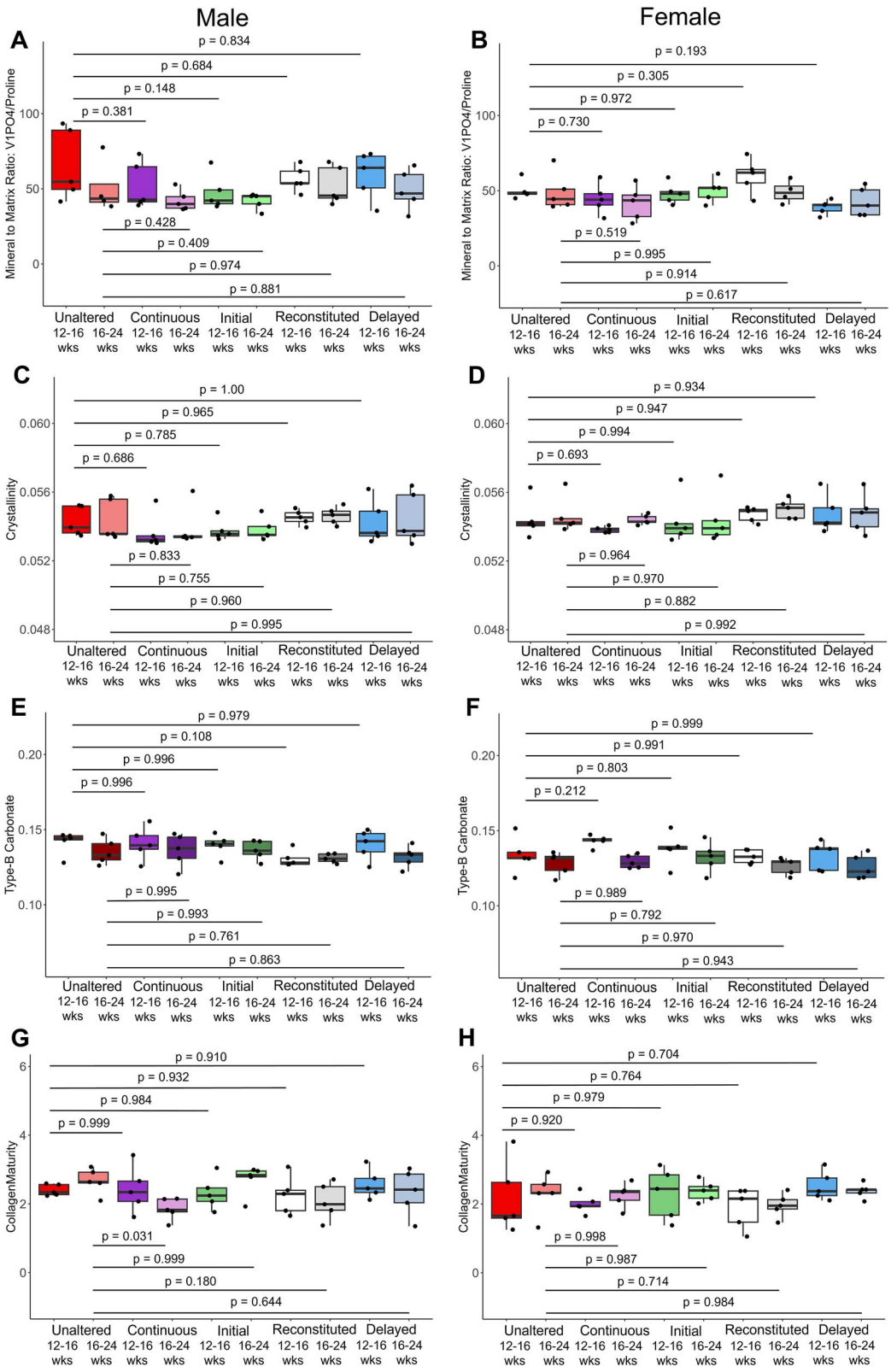


**Figure 5.** The mineral to matrix ratio, crystallinity and type B carbonate measured by Raman spectroscopy in bone matrix is shown. The mineral to matrix ratio, quantified by the area ratio of the v1 phosphate peak and Proline, remained unchanged in both male (A) and females (B). Crystallinity did not show statistical differences across treatment groups in both males (C) and females (D). Type-B carbonate substitution did not show significant changes among treatment groups in both males (E) and females (F). The collagen maturity was decreased in the Continuous males in the tissue formed between 16-24 weeks of age when compared to the Unaltered males (G). There were no observed significant changes in the female collagen maturity (H).

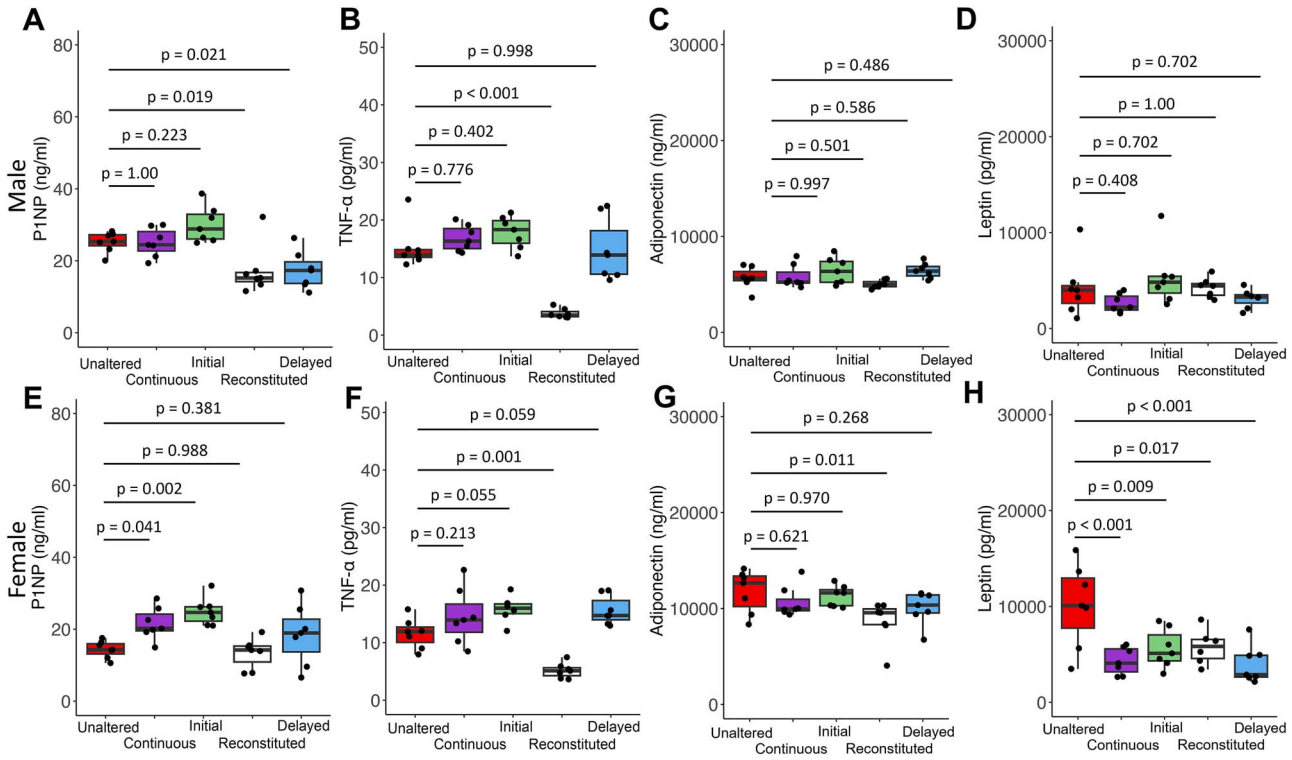


**Figure 6.** Serum markers are shown for (A, E) bone formation (P1NP); (B, F) TNF-$\alpha$; (C, G) adiponectin; and (D, H) leptin for both sexes.

bending moment (r = −0.373, [−0.628 −0.046], $p = .027$), body weight (r = −0.330, [−0.598 −0.003], $p = .053$), and tissue strength (r = −0.401, [−0.648 0.078], $p = .017$) (Supplemental Table S5).

In females, several serum markers were correlated with body weight and the work to failure (Supplemental Table S6). Body weight was positively correlated to the level of adiponectin and leptin and negatively correlated to P1NP and TRAP5b. There were no detectable differences in the level of estrogen between treatment groups (Supplemental Figure S7).

## Discussion

Our findings indicate that changes in the composition of the gut microbiome after skeletal maturity can decrease or improve bone matrix strength to the same degree as changes in the gut microbiota applied throughout growth and maturation. Since the amount of bone matrix formed after skeletal maturity is much smaller than that formed during growth[14] (Figure 1B and C), our hypothesis that the gut microbiome influenced the composition of bone matrix only at the time of matrix synthesis is unlikely to be true. We observed a similar trend in females but did not observe significant differences among groups suggesting that if modification to the gut microbiota alters bone matrix strength in female mice, the effect is smaller than that seen in males and could not be detected with this sample size.

Several lines of evidence support our finding that microbiome-induced changes in bone matrix strength in males were not limited to matrix formed after a change in the microbiota. First, bone matrix strength was altered when we applied changes in the gut microbiome after 16 weeks of age, when the bone formation rate within the cortex is greatly reduced.[14] If the gut microbiome exclusively influenced the properties of the matrix during bone formation, we would expect that the bone matrix strength in the Delayed group would resemble that of the Unaltered group, since only a small region of the femur cross-section was formed following the alteration of the gut microbiota (Figure 1C), however, instead bone matrix strength in the Delayed group was more similar to that of the Continuous group. Similarly, bone matrix strength in the Reconstituted group was expected to be more similar to that in the Continuous group since the majority of bone matrix in the femur was formed while the gut microbiota was altered (Figure 1B), yet bone matrix strength in the Reconstituted group was more similar to that of the Unaltered group. In contrast, the Initial group displayed a gut microbiome and reduced bone strength more similar to the Continuous group despite removal of the oral antibiotics for the same period as the Reconstituted group.

One possible explanation for our findings was that mechanical failure of a whole bone under bending initiated at the regions of greatest tensile stress (in this case on the outmost posterior side), hence improvements in bone matrix strength only at the periosteal surface could have effects on measures of whole bone strength. However, bone tissue formed on the periosteum of the mouse femur after 16 weeks of age occurred primarily on the anterior side of the bone, not the posterior side of the bone[14] (Figure 1B and C). Hence, changes in bone tissue in small regions of bone formed after 16 weeks of age could not explain the changes in bone matrix strength assessed here. Furthermore, serum markers of bone remodeling did not suggest that turnover of bone matrix after 16 weeks of age could explain the changes in bone matrix strength: the bone formation marker P1NP, was reduced in the groups in which the microbiome was changed after 16 weeks of age (Reconstituted and Delayed) and there were no differences in the bone resorption marker TRAP 5b among the treatment groups. Hence, histomorphometry and serum markers suggest that it is unlikely that there was substantial bone turnover

after 16 weeks of age. Together, these findings demonstrated that an alteration in the gut microbiome changed bone matrix strength in as short as two months without substantial osteoclast/osteoblast turnover.

Our findings suggest that microbiome-induced changes in bone matrix strength cannot be explained by Raman spectroscopy-based measures of matrix composition in regions of bone formed after 12 weeks. Groups that received antibiotics to alter their gut microbiome had reduced bone matrix strength but differences in Raman spectroscopy metrics were small and not consistent with observed changes in bone matrix strength. We consider the differences in Raman spectroscopy metrics to be too small to be influential: posthoc power analysis suggests that a sample size of $n = 44$ per group would be to detect differences with $p = .05$, and a power of 0.80. Previous work on the gut microbiome and bone strength revealed that the alteration to the gut microbiome leads to decreased crystallinity.[26] We believe the differences among studies were caused by sample-to-sample variation. The failure of Raman spectroscopy metrics to detect differences in matrix composition suggests that other modalities (ie proteomics) may be required to determine the changes in matrix composition that explain alterations in bone matrix strength.

Our findings highlight sex-related differences in response to antibiotic-induced manipulation of the gut microbiota. Microbiome-induced alterations in bone matrix strength were clear in males (25%-35% change in bone tissue strength), but were too small to be detected with this sample size in females ($p = .126$, power = 51.7%). A potential contributor is differences in the response of the microbiome to dosing. The composition of the gut microbiota in the Unaltered groups did not differ between males and females at either of the time points examined. However, the Continuous groups (with the most drastic changes in the gut microbiota and bone) showed differences in the composition of the gut microbiota between males and females at 24 weeks of age. Hence, the microbiota in females appeared to respond differently to dosing, potentially explaining why the differences in bone matrix strength among female groups were more subtle.

Several facts support the idea that changes in bone matrix strength in males are caused by alterations of the gut microbiota, not the direct effects of the antibiotics on host tissues. First, the antibiotics used (ampicillin and neomycin) have low/zero oral bioavailability and therefore are not widely distributed to bone through the systemic circulation. Second, in the Initial group antibiotic dosing was suspended at 16 weeks of age, yet the microbiota and bone matrix strength at 24 weeks was similar to the Continuous group. If the antibiotics had a direct effect on bone, the matrix strength in the Initial group would have been more similar to that of the Unaltered and Reconstituted groups. This finding is consistent with our prior work which indicated that microbiome-induced changes in bone matrix strength could be caused by alterations to the gut microbiota caused by neomycin alone,[10] yet when neomycin was dosed along with three other antibiotics causing removal of 99% of the microbiota, bone matrix strength was not reduced as compared to Unaltered groups. These results suggest that microbiome-induced impairment of bone matrix strength depends on the microbial population not the antibiotic.

Our analysis of the constituents of the fecal microbiota identifies several microbial taxa associated with changes in bone matrix strength. The five study groups showed two different phenotypes of bone matrix strength, normal bone matrix strength (Unaltered and Reconstituted) and impaired bone matrix strength (Initial, Delayed, and Continuous). Mice in the Reconstituted and Unaltered groups had increased abundance of genera from the *Bacillota (Firmicutes)* phylum relative to mice in the Initial, Delayed, and Continuous groups. This finding was corroborated across several metrics including relative abundance, LEfSe, and MaAsLin analyses. Both analyses consistently reveal significantly higher abundances and strong positive associations between the *Bacillota*: *Coprococcus*, *Lactobacillus*, *Oscillospira*, and *Ruminococcus*, in the Unaltered and Reconstituted groups as compared to the Initial, Delayed, and Continuous groups. Additionally, both analyses indicate increased abundance and strong positive associations between *Akkermansia* and *Bacteroides* in the Initial, Delayed, and Continuous groups compared to Unaltered and Reconstituted groups. Finally, the MaAsLin analysis identified a strong positive association of *Bilophila* and *Desulfovibrio* in the Reconstituted group relative to other treatment groups.

Although we did not measure microbial genes or metabolites, many of the genera with increased abundance in mice with normal bone strength are associated with two factors often associated with healthy gut microbiota: production of short chain fatty acids and modification of bile acids. *Oscillospira*, *Coprococcus*, and *Ruminococcus* produce the short chain fatty acid butyrate.[27,28] *Lactobacillus* produces lactic acid and L-Ornithine to maintain the gut mucosal barrier[29] and elicits an immunomodulatory effect on bone.[30] *Desulfovibrionaceae* and *Bilophila* produce hydrogen sulfide[31] which can mitigate bone loss by suppressing RANKL/OPG osteoclastogenesis.[32] Furthermore, *Prevotella* (a producer of the short chain fatty acid propionate) showed increased abundance and were positively associated with the Unaltered and Reconstituted groups.[33] Mice in the Initial, Delayed, and Continuous groups had increased abundance of opportunistic microbes when the microbial diversity was depleted by dosing, including *Akkermansia* (all three groups), *Bacteroides* (Continuous group), and *Enterobacteriaceae* (Delayed group).[34] Greater *Akkermansia* abundance has been linked to lower bone density in other studies. Our prior work (Luna et al. 2021)[10] associated a decrease in *Akkermansia* abundance with microbiome-induced impairment of bone strength. Although *Akkermansia* is widely considered to reduce inflammation at the gut lining, a mechanistic link to bone matrix strength has yet to be proposed. Hence it is unclear if the observed correlation between *Akkermansia* abundance and bone matrix strength is causative. While these observations were notable, it remains unclear how these differences in microbial abundance may have led to impaired matrix strength.

There were several limitations to our study. First, a study published during the review of this manuscript showed that the composition of the gut microbiome changes throughout the day.[35] The current study did not control the time of fecal pellet collection and may therefore have variability in the microbiota related to time of day of collection. However, the magnitude of change in the composition of the gut microbiome caused by antibiotics in the current study was much larger than that seen from variation in time of day of fecal collection[35] suggesting a limited effect of collection time of day on our results. Second, three-point bending is not an

optimal testing methodology for assessing bone tissue mechanical properties because bone cross-sectional geometry is irregular and the matrix is inhomogeneous[18] yet traditional calculation of matrix strength (maximum moment divided by section modulus) assumes the cross-sectional geometry is uniform and material composition is homogeneous. To avoid these limitations, we used ANCOVA to detect differences in whole bone strength that could not be explained by geometry. Hence, a more precise and micro-level mechanical testing approach is needed to accurately assess bone matrix strength. Lastly, we did not measure the microarchitecture of trabecular bone. The current study focuses on mechanical properties of bone matrix, which was measured in the femur diaphysis and not in regions with trabecular bone. Since our prior work did not observe changes in trabecular microarchitecture with this manipulation of the gut microbiome,[9] we did not include that analysis in the current study.

The bone matrix strength in the female and male responded differently to oral antibiotics. Complex interactions between the gut microbiome and circulating sex hormones have noted,[36] however, no trends in the estrogen levels were observed among the female treatment groups were associated with altered bone tissue strength. Other studies have observed an effect of the gut microbiome on bone loss following estrogen depletion in mice (ovariectomized models).[37] It would be an interesting study to examine if the effect of estrogen depletion in mice is mediated by changes in the gut microbiome.

Lastly, despite our comprehensive analysis encompassing bone composition, circulating bone turnover markers, hormones, and inflammation markers, the current study provides only limited insight into the factors that link the microbial taxa in the gut to bone or the specific changes in bone matrix caused by the microbiome. The challenge of identifying mechanistic links between the gut microbiome and organ phenotype remains the greatest challenge in the field of microbiome. Understanding how the gut microbiota might alter the strength of bone matrix is further complicated by the fact that mechanisms regulating bone matrix strength are not as well studied compared to mechanisms that regulate bone volume/density.[38] We have an ongoing follow up study using shotgun metagenomics approaches in similar groups to identify more accurate functional profiles of the microbiota and thus may provide deeper understanding of mechanism.

In summary, our findings demonstrate that the composition of the gut microbiome can influence the mechanical properties of bone matrix after skeletal maturity, suggesting that changes in the gut microbiota later in life (in adults) can alter bone matrix strength either reducing bone matrix strength to enhance bone fragility, or even improving bone matrix strength.

## Acknowledgments

Dr. Teresa Porri for acquisition of micro-CT imaging data of the right femora. Janet Hueber for acquisition of serum data. We thank Mr. Raymond Dove for Nanoscale Characterization Core usage at Rensselaer Polytechnic Institute.

## Author contributions

Christopher J. Hernandez had primary responsibility for final content. All authors read and approved the final manuscript.

Chongshan Liu (Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing—review & editing), Erika L Cyphert (Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Sam J Stephen (Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Bowen Wang (Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Angie L Morales (Formal analysis, Investigation), Jacob C. Nixon (Formal analysis, Investigation), Nicholas R Natsoulas (Investigation), Matthew Garcia (Investigation), Pablo Blazquez Carmona (Investigation), Albert Vill (Formal analysis, Writing—original draft), Eve Donnelly (Conceptualization, Writing—original draft, Writing—review & editing), Ilana L. Brito (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Deepak Vashishth (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), and Christopher J. Hernandez (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing – review & editing).

Chongshan Liu and Erika L. Cyphert contributed equally to this work.

## Supplementary material

Supplementary material is available at JBMR Plus online.

## Funding

This work was supported by NIH R01AG067997 (C.J.H.), NIH F32AG076244 (E.L.C.), and the Chan Zuckerberg Biohub. Imaging data was acquired through the Cornell Institute of Biotechnology's Imaging Facility, with NIH S10OD025049 for the SkyScan 1276 mouse CT.

## Conflicts of interest

The authors declare no potential conflict of interest.

## Data availability

Raw V4 16S rRNA DNA sequences are available at the NCBI's Sequence Read Archive Database (BioProject ID: PRJNA1000601; http://www.ncbi.nlm.nih.gov/bioproject/1000601).

## References

1. Kennel K, Drake M. Adverse effects of bisphosphonates: implications for osteoporosis management. *Mayo Clin Proc*. 2009;84(7):632-638. https://doi.org/10.1016/S0025-6196(11)60752-0
2. Gobron B, Couchot M, Irwin N, Legrand E, Bouvard B, Mabilleau G. Development of a first-in-class unimolecular dual GIP/GLP-2 analogue, GL-0001, for the treatment of bone fragility. *J Bone Miner Res*. 2023;38(5):733-748. https://doi.org/10.1002/jbmr.4792
3. Desselberger U. The mammalian intestinal microbiome: composition, interaction with the immune system, significance for vaccine efficacy, and potential for disease therapy. *J Pathog*. 2018;7(3):57. https://doi.org/10.3390/pathogens7030057
4. Knight R, Callewaert C, Marotz C, et al. The microbiome and human biology. *Annu Rev Genomics Hum Genet*. 2017;18(1):65-86. https://doi.org/10.1146/annurev-genom-083115-022438
5. Yan J, Charles JF. Gut microbiome and bone: to build, destroy or both? *Curr Osteoporos Rep*. 2017;15(4):376-384. https://doi.org/10.1007/s11914-017-0382-z
6. Hernandez C, Guss J, Luna M, Goldring S. Links between the microbiome and bone. *J Bone Miner Res*. 2016;31(9):1638-1646. https://doi.org/10.1002/jbmr.2887
7. Pacifici R. Bone remodeling and the microbiome. *Cold Spring Harb Perspect Med*. 2018;8(4):a031203. https://doi.org/10.1101/cshperspect.a031203

8. Castaneda M, Strong J, Alabi D, Hernandez CJ. The gut microbiome and bone strength. *Curr Osteoporos Rep*. 2020;18(6):677-683. https://doi.org/10.1007/s11914-020-00627-x
9. Guss JD, Horsfield MW, Fontenele F, et al. Alterations to the gut microbiome impair bone strength and tissue material properties. *J Bone Miner Res*. 2017;32(6):1343-1353. https://doi.org/10.1002/jbmr.3114
10. Luna M, Guss J, Vasquez-Bolanos L, et al. Components of the gut microbiome that influence bone tissue-level strength. *J Bone Miner Res*. 2021;36(9):1823-1834. https://doi.org/10.1002/jbmr.4341
11. Jernberg C, Löfmark S, Edlund C, Jansson J. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiol*. 2010;156(11):3216-3223. https://doi.org/10.1099/mic.0.040618-0
12. Ng K, Aranda-Díaz A, Tropini C, et al. Recovery of the gut microbiota after antibiotics depends on host diet, community context, and environmental reservoirs. *Cell Host Microbe*. 2019;26(5):650-665.e4. https://doi.org/10.1016/j.chom.2019.10.011
13. Le Bastard Q, Ward T, Sidiropoulos D, et al. Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice. *Sci Rep*. 2018;8(1):6219. https://doi.org/10.1038/s41598-018-24342-x
14. Ferguson V, Ayers R, Bateman T, Simke S. Bone development and age-related bone loss in male C57BL/6J mice. *Bone*. 2003;33(3):387-398. https://doi.org/10.1016/S8756-3282(03)00199-6
15. Vijay-Kumar M, Aitken J, Carvalho F, et al. Metabolic syndrome and altered gut microbiota in mice lacking toll-like receptor 5. *Science*. 2016;328(5975):228-231. https://doi.org/10.1126/science.1179721
16. Domander R, Felder A, Doube M. BoneJ2 - refactoring established research software [internet]. *Wellcome Open Research*. 2021;6:37. Available from: https://doi.org/10.12688/wellcomeopenres.16619.1
17. Bouxsein M, Boyd S, Christiansen B, Guldberg R, Jepsen K, Müller R. Guidelines for assessment of bone microstructure in rodents using micro–computed tomography. *J Bone Miner Res*. 2010;25(7):1468-1486. https://doi.org/10.1002/jbmr.141
18. Jepsen K, Silva M, Vashishth D, Guo X, van der Meulen M. Establishing biomechanical mechanisms in mouse models: practical guidelines for systematically evaluating phenotypic changes in the diaphyses of long bones. *J Bone Miner Res*. 2015;30(6):951-966. https://doi.org/10.1002/jbmr.2539
19. Dempster D, Compston J, Drezner M, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR histomorphometry nomenclature committee. *J Bone Miner Res*. 2013;28(1):2-17. https://doi.org/10.1002/jbmr.1805
20. Morris M, Mandair G. Raman assessment of bone quality. *Clin Orthop Relat Res*. 2011;469(8):2160-2169. https://doi.org/10.1007/s11999-010-1692-y
21. Mandair G, Morris M. Contributions of Raman spectroscopy to the understanding of bone strength. *Bonekey Rep*. 2015;4:1-8. https://doi.org/10.1038/bonekey.2014.115
22. Unal M, Ahmed R, Mahadevan-Jansen A, Nyman J. Compositional assessment of bone by Raman spectroscopy. *Analyst*. 2021;146(24):7464-7490. https://doi.org/10.1039/D1AN01560E
23. Gamsjaeger S, Srivastava A, Wergedal J, et al. Altered bone material properties in HLA-B27 rats include reduced mineral to matrix ratio and altered collagen cross-links. *J Bone Miner Res*. 2014;29(11):2382-2391. https://doi.org/10.1002/jbmr.2268
24. Soto-Martin EC, Warnke I, Farquharson FM, et al. Vitamin biosynthesis by human gut butyrate-producing bacteria and cross-feeding in synthetic microbial communities. *MBio*. 2020;11(4):e00886-20. https://doi.org/10.1128/mBio.00886-20
25. Lang D, Sharkey N, Lionikas A, et al. Adjusting data to body size: a comparison of methods as applied to quantitative trait loci analysis of musculoskeletal phenotypes. *J Bone Miner Res*. 2005;20(5):748-757. https://doi.org/10.1359/JBMR.041224
26. Guss J, Taylor E, Rouse Z, et al. The microbial metagenome and bone tissue composition in mice with microbiome-induced reductions in bone strength. *Bone*. 2019;127:146-154. https://doi.org/10.1016/j.bone.2019.06.010
27. Yang J, Li Y, Wen Z, Liu W, Meng L, Huang H. Oscillospira - a candidate for the next-generation probiotics. *Gut Microbes*. 2021;13(1):1987783. https://doi.org/10.1080/19490976.2021.1987783
28. Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ. Acetate utilization and butyryl coenzyme a (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl Environ Microbiol*. 2002;68(10):5186-5190. https://doi.org/10.1128/AEM.68.10.5186-5190.2002
29. Qi H, Li Y, Yun H, et al. Lactobacillus maintains healthy gut mucosa by producing L-ornithine. *Commun Biol*. 2019;2(1):171. https://doi.org/10.1038/s42003-019-0424-4
30. Sapra L, Dar HY, Bhardwaj A, et al. Lactobacillus rhamnosus attenuates bone loss and maintains bone health by skewing Treg-Th17 cell balance in Ovx mice. *Sci Rep*. 2021;11(1):1807. https://doi.org/10.1038/s41598-020-80536-2
31. Rey F, Gonzalez M, Cheng J, Wu M, Ahern P, Gordon J. Metabolic niche of a prominent sulfate-reducing human gut bacterium. *Proc Natl Acad Sci USA*. 2013;110(33):13582-13587. https://doi.org/10.1073/pnas.1312524110
32. Liu Y, Yang R, Liu X, et al. Hydrogen sulfide maintains mesenchymal stem cell function and bone homeostasis via regulation of Ca(2+) channel sulfhydration. *Cell Stem Cell*. 2014;15(1):66-78. https://doi.org/10.1016/j.stem.2014.03.005
33. Precup G, Vodnar D. Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review. *Br J Nutr*. 2019;122(2):131-140. https://doi.org/10.1017/S0007114519000680
34. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol*. 2017;10(1):18-26. https://doi.org/10.1038/mi.2016.75
35. Allaband C, Lingaraju A, Flores RS, et al. Time of Sample Collection Is Critical for the Replicability of Microbiome Analyses. *Nature Metabolism*. 2024;6:1282-1293. https://doi.org/10.1038/s42255-024-01064-1
36. Pace F, Watnick P. The interplay of sex steroids, the immune response, and the intestinal microbiota. *Trends Microbiol*. 2021;29(9):849-859. https://doi.org/10.1016/j.tim.2020.11.001
37. Deckard C, Walker A, Hill BJF. Using three-point bending to evaluate tibia bone strength in ovariectomized young mice. *J Biol Phys*. 2017;43(1):139-148. https://doi.org/10.1007/s10867-016-9439-y
38. Alliston T. Biological regulation of bone quality. *Curr Osteoporos Rep*. 2014;12(3):366-375. https://doi.org/10.1007/s11914-014-0213-4