Effects of high dose aspartame-based sweetener on the gut microbiota and bone strength in young and aged mice

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Abstract

In a recent study examining the effects of manipulating the gut microbiome on bone, a control group of mice in which the microbiome was altered using a non-caloric, aspartame-based sweetener resulted in whole bone strength being 40% greater than expected from geometry alone, implicating enhanced bone tissue strength. However, the study was not designed to detect changes in bone in this control group and was limited to young male mice. Here we report a replication study examining how changes in the gut microbiome caused by aspartame-based sweetener influence bone. Male and female C57Bl/6 J mice were untreated or treated with a high dose of sweetener (10 g/L) in their drinking water from either 1 to 4 mo of age (young cohort; $n = 80$) or 1 to 22 mo of age (aged cohort; $n = 52$). Sweetener did not replicate the modifications to the gut microbiome observed in the initial study and did not result in an increase in bone tissue strength in either sex at either age. Aged male mice dosed with sweetener had larger bones (+17% femur section modulus, $p<.001$) and greater whole bone strength (+22%, $p=.006$) but the increased whole bone strength was explained by the associated increase in body mass (+9%, $p<.001$). No differences in body mass, whole bone strength, or femoral geometry were associated with sweetener dosing in males from the young cohort or females at either age. As we were unable to replicate the gut microbiota observed in the initial experiment, it remains unclear if changes in the gut microbiome can enhance bone tissue strength. Although prior work studying gut microbiome–induced changes in bone with oral antibiotics has been highly repeatable, the current study highlights the variability of nutritional manipulations of the gut microbiota in mice.

Keywords: bone, aspartame, microbiome, aging, biomechanics

Lay Summary

To replicate prior findings and to better understand how changes in the gut microbiome caused by aspartame-based sweetener influence bone, mice (both male and female) were treated with high doses of a calorie-free sweetener in their drinking water until the age of 4 mo (young) or 22 mo (aged). Body weight, bone strength, geometry, and microbiome composition were measured in mice receiving sweetener and compared to mice that were untreated. Sweetener did not change in the gut microbiome as much as observed in our prior study and no changes in bone tissue strength were seen at either age in either sex. Aged male mice receiving sweetener had increased body weight and increased whole bone strength as compared to untreated aged male mice. Sweetener treatment did not affect body weight or bone strength and geometry of males in the young cohort or females at either age. Our work highlights the challenges of replicating microbiota composition across studies via dietary manipulations to study bone and the need to preserve live communities of microbiota to improve replication.

Introduction

The composition of the gut microbiota has been shown to influence both bone quantity and quality.[1-4] Specifically, the gut microbiota has been associated with bone formation and resorption through modulation of inflammatory responses[5,6] and can influence mineralization and collagen structure of bone.[7] A standard approach to studying the effects of the gut microbiome on the physiology of mice is to apply a cocktail of antibiotics (ampicillin + neomycin + vancomycin + metronidazole) in drinking water resulting in removal of 99.9% of microbes as indicated by a 400-fold decrease in bacterial DNA in feces.[8,9]

In a recent study from our group, we applied the cocktail of 4 antibiotics to explore the effect on bone. However, in our hands, use of this cocktail of antibiotics resulted in

insufficient water intake by the mice. To ensure adequate intake of water, a non-caloric aspartame-based sweetener was included with the cocktail of 4 antibiotics. A group of mice receiving only the sweetener was included as an additional control. Surprisingly, the group receiving only the sweetener showed a 40% greater whole bone strength after accounting for geometry when compared to untreated mice. The effect was attributed to alterations in the mechanical properties of the bone matrix (not just bone size/density).[1] This finding was exciting in that it suggested that alterations to the gut microbiota could increase the mechanical performance of bone matrix. Bone tissue strength is a major contributor to bone fragility but has not previously been explored as a therapeutic target.[10] However, this prior study was limited in that it was not designed to examine the effects of the aspartame-based sweetener, it only examined male mice, and it included only one time point (collected at 4 mo of age after 3 mo of dosing). As a result, it remained unclear how changes in the gut microbiome caused by chronic sweetener influenced whole bone strength and if the effect we previously observed was limited to one sex or age.

Aspartame, a key constituent of the sweetener blend that we administered, is a non-caloric sweetener that is hydrolyzed in the small intestine and does not reach the colon in its intact form.[11] Although oral absorption of aspartame is negligible, in humans, aspartame consumption has been linked to changes in liver function,[12] neurological function,[13,14] glucose tolerance, and obesity,[15,16] suggesting that either aspartame-induced changes in the gut microbiota or byproducts of the breakdown of aspartame within the gut can influence host health. The World Health Organization has classified aspartame as a possible carcinogen based on limited evidence associated with liver cancer in clinical and murine studies.[17]

The goal of this work was to determine the effect of aspartame-based sweetener on the gut microbiota and bone strength. Specifically, we sought to determine: (1) the effect of changes in the gut microbiota caused by aspartame-based sweetener on bone strength and geometry across age and sex, (2) the effect of sweetener on the composition of the gut microbiota, and (3) identify the microbial taxa associated with changes in bone strength following dosing with sweetener.

Materials and methods
Mouse strains
Animal procedures were approved by the local Institutional Animal Care and Use Committee. Male and female C57Bl/6 J mice were purchased from Jackson Laboratory and bred using trio breeding. Pups were weaned at 3 wk of age and randomly housed by sex ($n = 3$–4/cage). Sterile cages contained ¼-inch corn cob bedding (The Andersons' Lab Bedding) and cardboard hut (Ketchum Manufacturing). Breeding was carried out in a barrier (specific pathogen free) facility and upon weaning (3 wk of age), mice were transferred to a conventional facility in the same building. Animals were bred and raised at the same institution, in the same barrier (specific pathogen free) and conventional facility as in Luna et al.,[1] but in different rooms.

Diet and aspartame-based sweetener
Mice received standard laboratory chow (Teklad LM-485 Mouse/Rat Sterilizable Diet Irradiated, Envigo Diets; ingredient list outlined in Table S1) and reverse osmosis sterilized water ad libitum with or without 10 g/L zero-calorie sweetener (Equal: aspartame, dextrose, maltodextrin, acesulfame potassium; Merisant Company).[1] Sweetener laced water was freshly prepared and replenished every 3 d.

Study design
The study included both males and females and examined bones from young (4 mo of age) and aged (22 mo of age) animals. Pups were divided into 4 groups based on age and treatment (number of animals in each treatment group is outlined in Table S2): (1) chronic sweetener dosing from 1 to 4 mo of age, (2) untreated control 4 mo of age, (3) chronic sweetener dosing from 1 to 22 mo of age, and (4) untreated control 22 mo of age (Figure 1A). A sample size of 12 animals per group had a statistical power of 0.80 to detect an effect size of 0.88 in bone tissue strength with $\alpha = 0.05$. Larger sample sizes were bred to maintain the sample size even after age-related attrition; all available animals were used for the study resulting in differences in sample size among groups. Dosing consisted of reverse osmosis sterilized water containing zero-calorie aspartame sweetener (10 g/L; freshly prepared every 3 d).[1] Weekly mixing of bedding between cages of the same sex and treatment group was applied from 1 to 3 mo of age to reduce cage-to-cage variation in the microbiota.[18] Mice were euthanized at either 4 or 22 mo of age, and femora were dissected and preserved by wrapping in gauze soaked with PBS and plastic wrap stored at $-80°C$.

Geometric measurements of femoral cortical bone
Femora were thawed to room temperature and scanned using micro-computed X-ray tomography at a 10-μm voxel size resolution with a mineral calibration phantom, 100 kV with a 1 mm aluminum filter, and an exposure time of 700–900 ms (Bruker SkyScan 1276 mouse CT, Bruker Corporation). Images were processed using a standard methodology, and geometric measurements were made from the diaphyseal cross-section in the micro-computed tomography images.[1,19] Images were analyzed using a custom Java code and run in Fiji (v.2.3.051) with a BoneJ plug-in. Briefly, a Gaussian blur was applied ($\sigma = 1$), the mid-slice of the mid-diaphysis was determined, and a global threshold was applied using the Otsu method.[20,21] Cortical cross-sectional area, cortical thickness, moment of inertia (I) about the medial-lateral axis (direction of loading), distance from the neutral axis to the edge of the bone surface (c), and section modulus (I/c) were measured.

Biomechanics
The mechanical properties of the mouse femur were determined by applying 3-point bending until failure. Mechanical testing in 3-point bending was performed as follows: the right femur was thawed, hydrated with PBS, and tested using 3-point bending in anterior-to-posterior direction to failure (loading rate = 0.1 mm/s, span length = 7.5 mm between outer loading pins) using a materials testing device (858 Mini Bionix; MTS). Force vs displacement curves were collected using an 11.34 kg (25 lbs) load cell (MLP-25, Transducer Techniques; weekly manual calibrations) and linear variable differential transducer at 100-Hz sampling. Data were processed using a custom script in Matlab (R2021b, MathWorks) using a smoothing function to minimize noise. The maximum breaking force and displacement were recorded. A 10% reduction in slope method was used to identify the yield force and yield displacement.[22] The maximum breaking force was used to calculate whole bone strength (max force × span
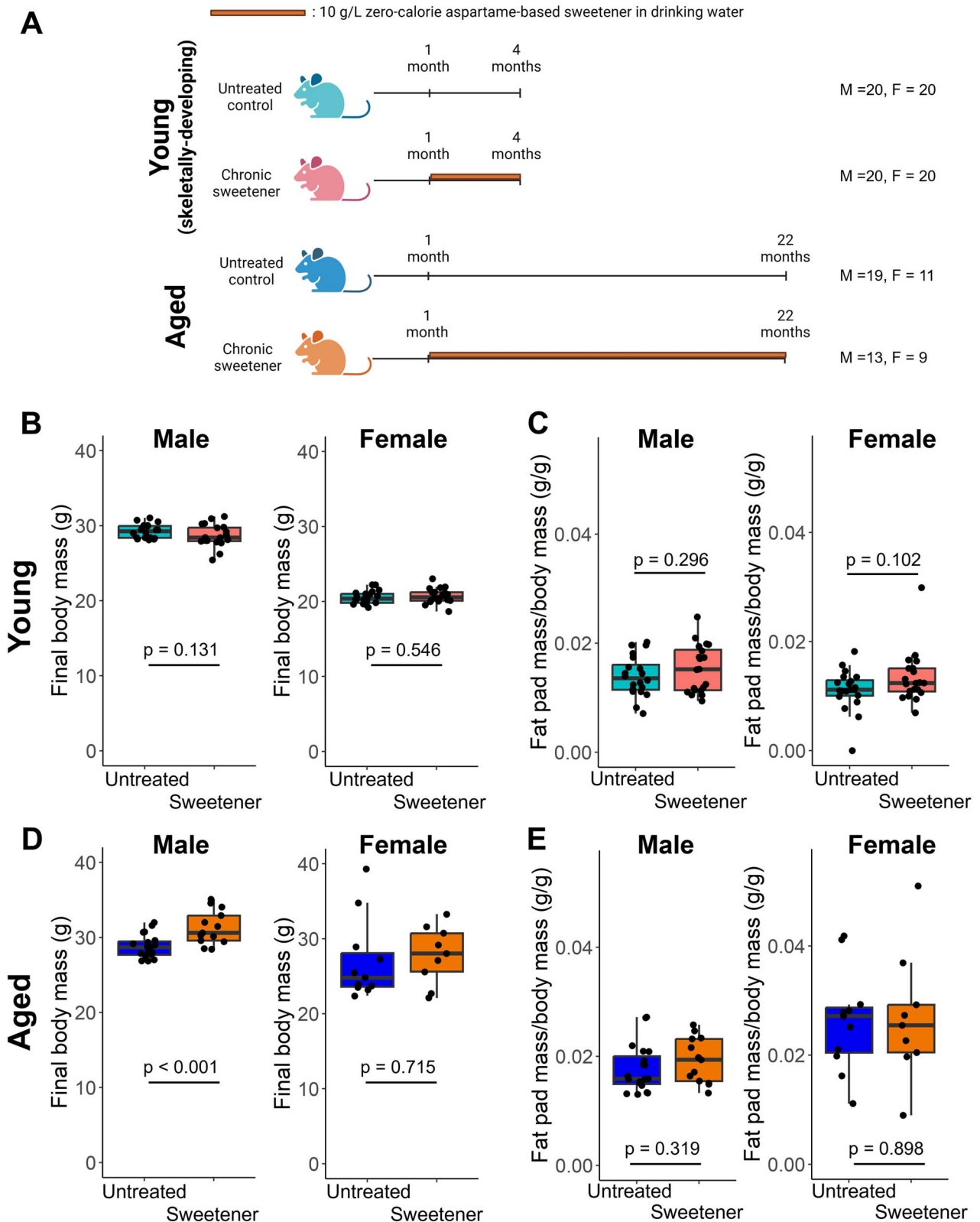
Figure 1. Aged male mice receiving sweetener had greater body mass. (A) Study design. (B and C) Sweetener treatment to mice in the young cohort had no effect on body mass or fat pad mass. (D) Body mass at euthanasia was greater in aged males chronically treated with sweetener but not aged females. (E) Sweetener treatment did not influence perigonadal fat pad mass in aged mice. A one-way ANOVA was used to calculate differences in body mass and normalized fat pad mass between treatment groups in each age and sex.

length/4). Bone tissue strength was calculated using whole bone strength and geometric measurements from micro-computed X-ray tomography (whole bone strength/section modulus).

16S rRNA gene sequencing analysis

16S rRNA gene sequencing was used to determine the composition of the microbiota from fecal samples collected at 4 mo (young cohort) or 22 mo of age (aged cohort). The University of California San Diego Microbiome Core carried out DNA extraction, purification, library preparation, and sequencing. DNA was isolated and purified using a liquid handler robot (MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, Thermo Fisher Scientific). For quality control, blanks and mock communities were used throughout the processing pipeline (Zymo Research Corporation). 16S rRNA gene amplification was completed using a protocol from the Earth Microbiome Project.[23] Unique forward primer barcodes (Illumina) were used to amplify the V4 region of the 16S rRNA gene (515fB-806r; forward – GTGYCAGCMGCCGCGGTAA, reverse – GGACTACN-VGGGTWTCTAAT[24,25]). Amplification was individually carried out on each sample as a single reaction (94°C 3 min, 94°C 45 s ×35, 50°C 60 s ×35, 72°C 90 s ×35, 72°C 10 min, 4°C hold), equal volumes of each amplicon were pooled, and libraries were sequenced on Illumina MiSeq using paired-end 150 bp cycles.[26] Quality control trimming and taxonomic classification were carried out using QIIME2 (v. 2020.6) with a SILVA database (SSU r138-1).[27] Reads were assigned at the genus level and normalized with a single rarefaction step (feature count cut-off 46 067; range of feature sizes in samples: 46 068–233 105; average and standard deviation of feature size in samples: $110\,390 \pm 59\,424$).[28] Feature count cut-off was determined using standard rarefaction methods in an effort to maximize the number of features relative to the percent of samples retained.[4,28,29] Alpha (Shannon index) and beta diversity (Bray–Curtis dissimilarity) were calculated using the amplicon sequence variant (ASV) table from QIIME2 output using the vegan package (v. 2.5–7) in RStudio.[4,30] A principal coordinate analysis was carried out on the Bray–Curtis dissimilarity matrix by sex and treatment group. Microbiome Multivariate Associations with Linear Models (MaAsLin2) was carried out to identify univariate associations between treatment groups, sex, and microbial abundance.[31] Specifically, genera-level taxa were used in the MaAsLin2 analysis that had >10% prevalence and post hoc q-values associating treatment groups and microbes were calculated with the Benjamini–Hochberg method.[31] A secondary differential abundance analysis (ALDEx2 R package) was performed using a rarefied ASV table (separated by sex) to create Monte Carlo simulations of Dirichlet distributions of each sample.[32,33] The distributions were transformed using a centered log ratio transform, and a generalized linear model was generated based on treatment (sweetener vs untreated).[33] Volcano plots were generated by sex, and significant ASVs ($p<.05$; magnitude of fold change > |1|) are shown in upper left and right corners.

Statistical analysis

Values are shown as the mean ± standard deviation. Statistical analyses were performed using RStudio (v. 1.4.1106, 2021).[34] One-way ANOVA was used to calculate significance between treatment groups by age and sex in body mass, fat pad mass, bone biomechanics, femoral geometry, alpha diversity, and microbial abundance. For parameters that were correlated (section modulus and whole bone strength), an analysis of covariance was performed to detect differences in whole bone strength between groups when accounting for differences in section modulus.[1] Permutational multivariate analysis of variance (adonis2 function) was used to calculate differences in beta diversity (microbiota composition) by treatment group.[35] A Pearson's correlation matrix between femoral geometry, body mass, fat pad mass, and biomechanics was performed (95% confidence intervals are shown; p-values <.05 considered significant). Femoral geometry and bone biomechanics are shown without (main figure) and with normalization (Supplementary Table) by body mass. Normalization by body mass was performed using a regression-based approach.[36]

Results

Thirty-one percent of the aged females (22 mo age) dosed with sweetener died prior to the end of the study (deaths occurred between 9 and 22 mo of age; consistent with a previous study[37]), survivorship among untreated females and males from both groups was 100%. Staff veterinarians did not determine the cause of the premature death of the aged females dosed with sweetener.

Aged male mice receiving sweetener had greater body mass

Aged male mice (22 mo age) receiving chronic high dose sweetener (10 g/L) had greater body mass (Figure 1D; $p<.001$) and comparable perigonadal fat pad mass (normalized by body mass; Figure 1E; $p=.319$) relative to untreated mice. Young (4 mo) males and females (both young and aged) receiving sweetener did not have differences in body mass or fat pad mass relative to untreated mice (Figure 1B and C).

Aged male mice receiving sweetener had greater whole bone strength and femoral geometry

Aged male mice treated with sweetener had greater whole bone strength (Figure 2E; $p=.006$), maximum load (Table 1; $p=.006$), and work to failure (Table 1; $p=.028$) relative to mice not receiving aspartame. Additionally, sweetener-treated aged male mice had greater femoral geometry including section modulus (Figure 2F; $p<.001$), cross-sectional area (Figure 2G; $p=.021$), and moment of inertia (Figure 2H; $p<.001$) than untreated mice. Differences in whole bone strength and femoral geometry were not detected in young males (4 mo age) or in females at either age. There were no differences in femur length, yield load, or yield displacement at either timepoint in either sex (Table 1).

When normalized by body mass, differences in cross-sectional area and whole bone strength between aged males with and without sweetener were not significantly different (Table S3).[36] Femoral geometry (cross-sectional area, moment of inertia, and section modulus) was positively correlated with body mass and fat pad mass in both aged males (Table S4) and females (Table S5). Additionally, cross-sectional area, moment of inertia, and section modulus were positively correlated with whole bone strength in both sexes in aged mice.

The biomechanical analysis from the young (4 mo age) male and female mice suggested that chronic sweetener did not affect bone strength or geometry in either sex during
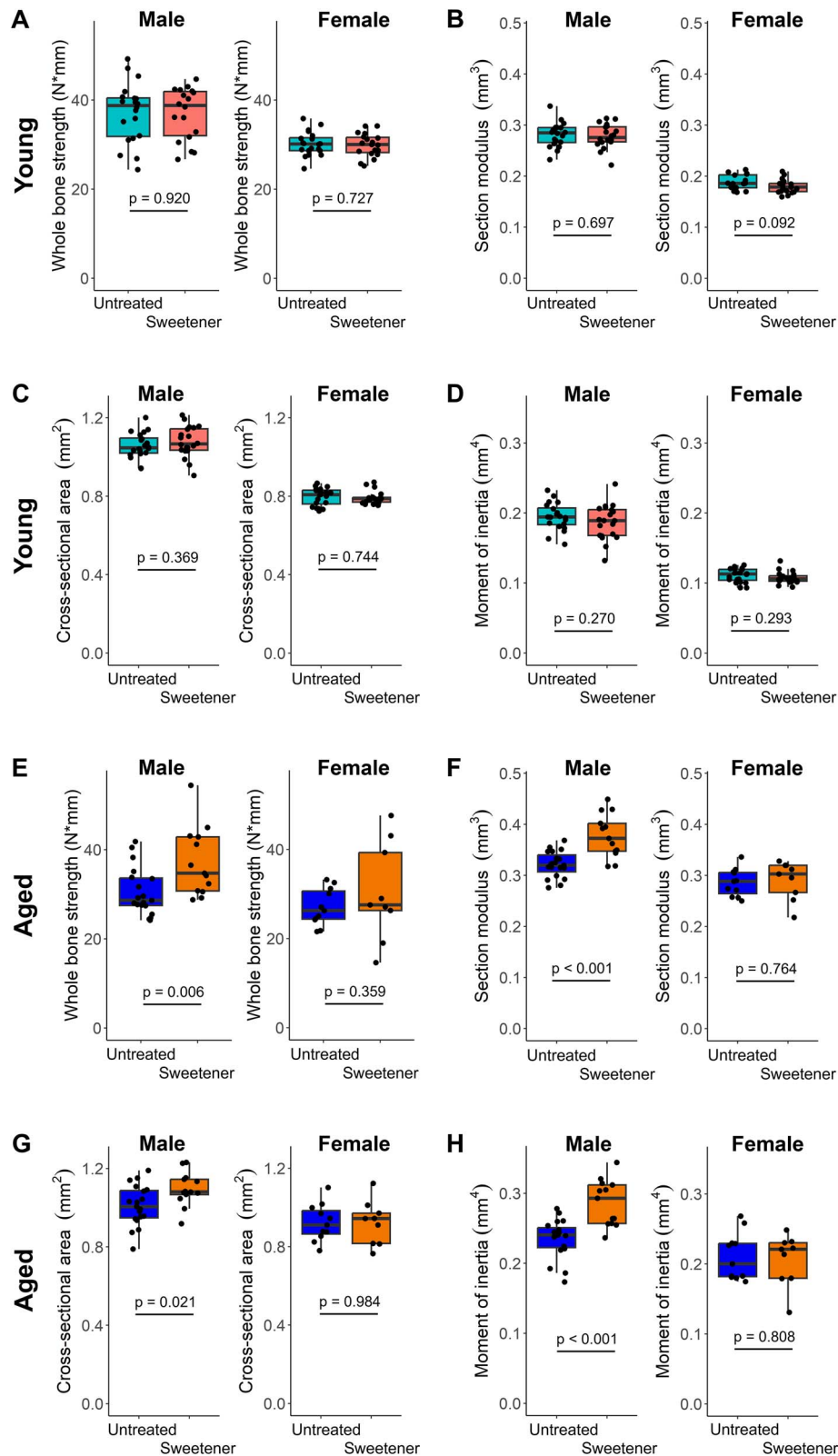
Figure 2. Aged male mice treated with sweetener had greater whole bone strength and femoral geometry. (A–D) Sweetener treatment to mice in the young cohort had no effect on whole bone strength, section modulus, cross-sectional area, or moment of inertia. (E–H) Whole bone strength, section modulus, cross-sectional area, and moment of inertia were greater in aged males treated with sweetener but not females. A one-way ANOVA was used to calculate differences in biomechanics and femoral geometry between treatment groups in each age and sex.

Table 1. Measurements of bone geometry and biomechanics are shown.

Group	Femur length (mm)	Tissue strength (N/mm²)	Distance to neutral axis (mm)	Maximum load (N)	Yield displacement (mm)	Stiffness (N/mm)	Yield load (N)	Work to failure (N*mm)
Males – Aged (22 mo)								
Chronic sweetener	16.394 ± 0.285	99.216 ± 20.251	0.759 ± 0.024	19.847 ± 4.072	0.127 ± 0.034	100.290 ± 18.198	11.334 ± 2.556	7.925 ± 2.714
	p=.794	p=.551	p=.071	p=.006	p=.679	p=.516	p=.594	p=.028
Unaltered	16.361 ± 0.381	95.315 ± 16.254	0.733 ± 0.046	16.275 ± 2.834	0.134 ± 0.047	95.237 ± 23.208	10.827 ± 2.602	5.485 ± 3.080
Males – Young (4 mo)								
Chronic sweetener	16.003 ± 0.332	132.370 ± 25.873	0.669 ± 0.052	19.509 ± 3.116	0.146 ± 0.028	131.123 ± 19.389	15.613 ± 2.646	5.162 ± 3.559
	p=.127	p=.979	p=.093	p=.961	p=.382	p=.386	p=.795	p=.868
Unaltered	16.119 ± 0.190	132.586 ± 23.326	0.692 ± 0.026	19.561 ± 3.475	0.135 ± 0.047	136.482 ± 18.733	15.313 ± 4.030	4.955 ± 4.146
Females – Aged (22 mo)								
Chronic sweetener	16.111 ± 0.256	103.754 ± 31.899	0.706 ± 0.051	16.212 ± 5.821	0.138 ± 0.055	105.396 ± 35.614	12.019 ± 6.046	5.935 ± 3.203
	p=.604	p=.422	p=.325	p=.359	p=.935	p=.845	p=.770	p=.034
Unaltered	16.187 ± 0.365	94.917 ± 14.656	0.732 ± 0.060	14.429 ± 2.213	0.137 ± 0.025	102.846 ± 21.369	12.618 ± 2.007	2.997 ± 2.306
Females – Young (4 mo)								
Chronic sweetener	15.467 ± 0.272	166.541 ± 14.613	0.598 ± 0.029	15.833 ± 1.354	0.131 ± 0.040	97.389 ± 12.831	10.595 ± 1.777	4.241 ± 2.579
	p=.560	p=.185	p=.441	p=.647	p=.094	p=.001	p=.748	p=.968
Unaltered	15.509 ± 0.248	159.252 ± 17.276	0.591 ± 0.030	15.798 ± 1.770	0.111 ± 0.032	111.562 ± 12.687	10.406 ± 1.876	4.275 ± 2.726

Statistical comparisons are only made within sex and age groups. Results are shown as the mean ± standard deviation. Measurements normalized for differences in body mass are shown in Table S3.

early growth. No differences in biomechanical parameters were associated with sweetener treatment after normalizing by body mass.

Sweetener dosing led to a distinct gut microbiota from untreated, but not as different as seen in prior work

Since we did not observe the difference in whole bone strength in the young mouse cohort as we had observed in our prior work, we looked in greater detail at changes in the gut microbiota associated with sweetener in the current study. Both aged males and females receiving chronic sweetener had a significantly different microbiota composition (Bray–Curtis beta diversity) relative to untreated (M: $p=.007$; F: $p=.026$) (Figure 3A, Figure S1). The differences in beta diversity in aged males receiving aspartame appeared to be caused by a few individual mice that had a drastically different taxonomic abundance of microbes compared to untreated mice (Figure 3B). Sweetener did not appear to affect alpha diversity (Figure S2: observed richness, Shannon diversity) in either aged males or females. Notably, differences in the gut microbiota associated with sweetener were substantially smaller than were seen in the study by Luna et al.[1] (Figure 4). MaAsLin analysis of the samples from the current study found that *Odoribacter* was significantly decreased in aged males and females receiving sweetener relative to untreated mice (Figure 3C; M: $p=.002$; F: $p=.052$). Differential abundance analysis by ALDEx2 determined that 1.8% (15/852) and 2.4% (21/875) of the ASVs identified in aged males and females were significantly different in abundance ($p<.05$; magnitude of fold change $>$ |1|) in mice that received sweetener relative to untreated mice (Figure 3D). ASVs with significantly different abundance in aged males and females belonged to either *Bacteroides* or *Firmicutes* phyla (Tables S6 and S7).

Discussion

In this study, we evaluated the effects of long-term high dose sweetener on body mass, bone, and microbiota composition in young (4 mo age; equivalent ~12.5 yr age in humans) and aged (22 mo age; equivalent ~67 yr age in humans) cohorts of male and female mice. Our goal was to evaluate how consumption of high dose sweetener influenced bone strength and the microbiome across different stages of skeletal growth and development. Compared to our prior study,[1] dosing with sweetener generated significant, but much smaller changes in the composition of the gut microbiota (Figure 4). Since dosing with sweetener did not result in the same microbiota across studies, our findings are unable to support or refute our prior observation that changes in the gut microbiota can increase bone tissue strength above what is seen in mice with an unaltered gut microbiota.

Our prior study dosing mice with the same sweetener resulted in a substantially different composition of the gut microbiota and an increase in bone tissue strength (Figure 4)[1] that were not observed in this more comprehensive follow-up study. We implemented several methods to increase repeatability of the gut microbiota, including breeding mice in the same facility, using the same food, water, and sweetener. Further, to reduce the risk of contamination, we prepare sweetener-laced solutions in bottles that are regularly sterilized. It is possible
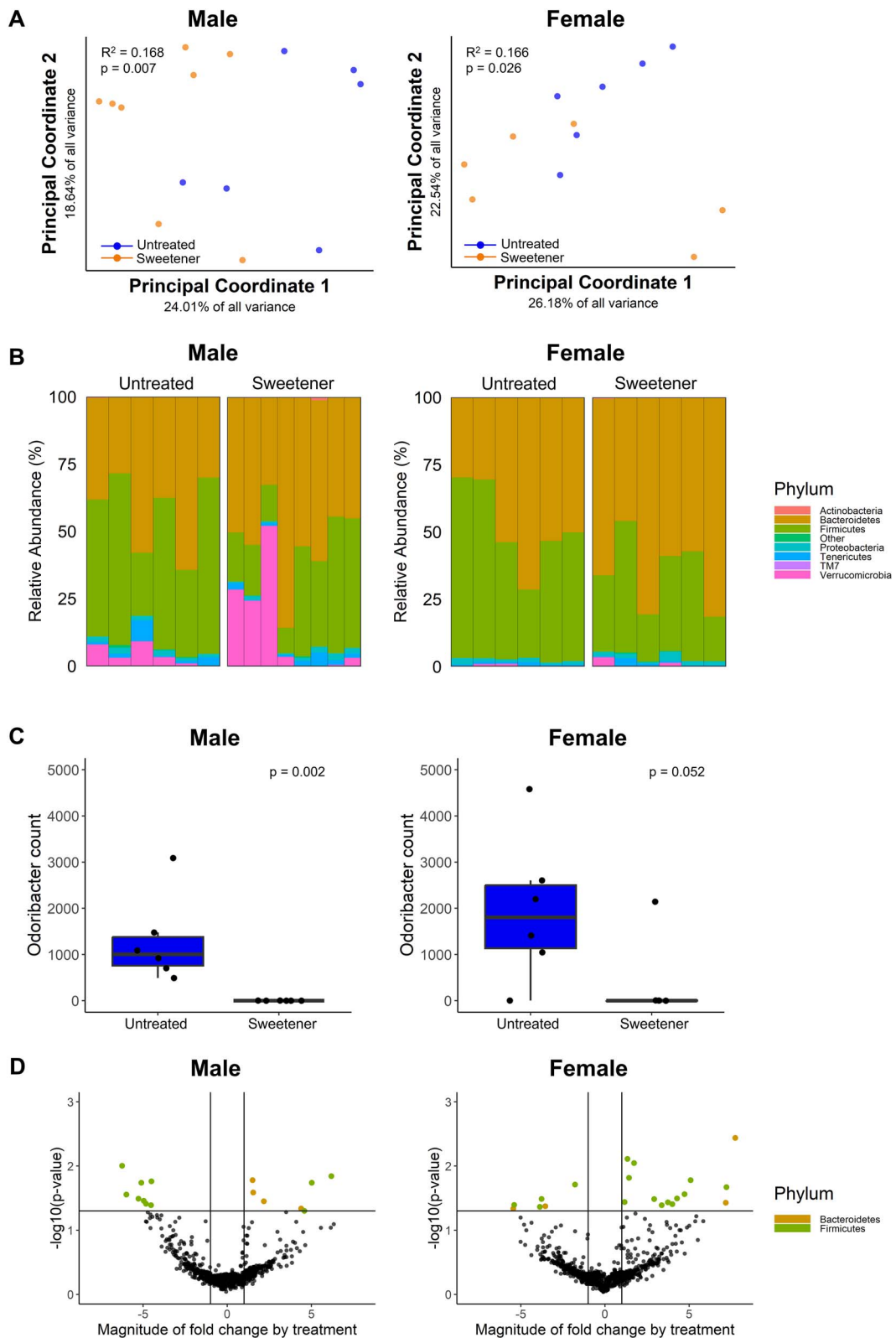
Figure 3. Aged male and female mice receiving chronic sweetener had a different microbiota than untreated mice. (A) Both aged males and females receiving sweetener had a different composition of the microbiota (Bray–Curtis beta diversity) than untreated mice. (B) A subset of aged male mice receiving sweetener had a drastically different relative abundance of taxa compared to untreated mice. (C) Sweetener treatment decreased the abundance of *Odoribacter* in both aged males and females relative to untreated. (D) Differential abundance analysis by ALDEx2 identified significant ASVs from the *Bacteroides* and *Firmicutes* phyla in both males and females.
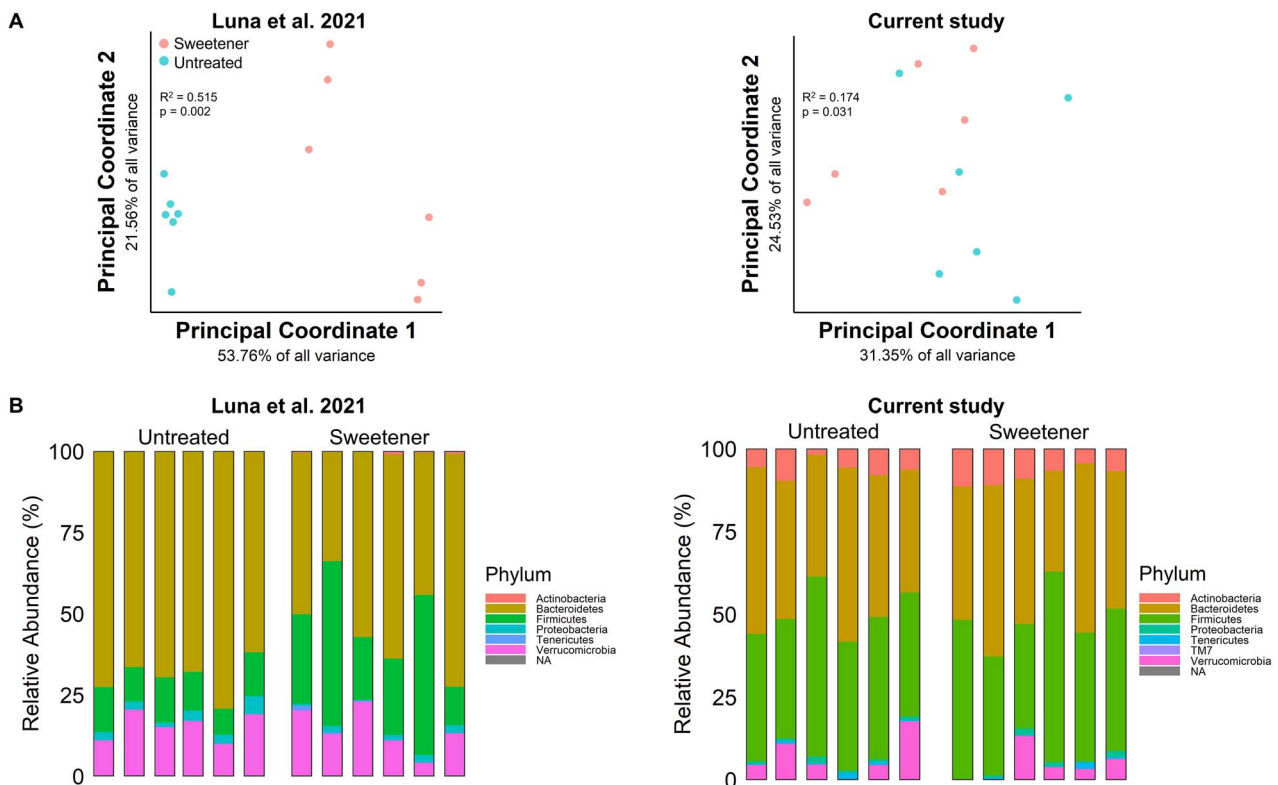
Figure 4. Across 2 independent studies in male mice treated with sweetener from 1 to 4 mo of age, sweetener had a different effect on the microbiota (Bray–Curtis beta diversity, relative abundance). (A) In our 2021 study (Luna et al.[1]), male mice receiving sweetener had a large difference in the composition of the microbiota relative to untreated mice (*p*=.02). In our current follow-up study (Current study), male mice receiving sweetener had a microbiota composition more similar to that of the unaltered mice relative to Luna et al. (*p*=.031). (B) In our 2021 study (Luna et al.[1]), male mice had decreased *Actinobacteria*, decreased *Firmicutes*, and increased *Bacteroidetes* relative to male mice in our current follow-up study (Current Study).

that differences in gut microbiota before treatment and/or subtle differences in composition of the chow or sweetener received from the manufacturer prevented the same changes in the composition of the gut microbiota reported by Luna et al.[1] There was more than 1 yr of time between the start of the current study and the initial study reported by Luna et al.[1] and as a result we were not able to use the same lot or batch of sweetener. High variability in the individual response of the microbiome to dietary-induced manipulations has been observed by others,[16,38,39] contributing to the challenge of replicating dietary-induced manipulations to the microbiota composition across studies. In contrast, our prior work using oral antibiotics to manipulate the gut has shown high repeatability in both changes in the gut microbiota and bone phenotype, with similar results observed in 3 independent studies in our facility[1,2,40] and one additional study at a separate facility.[41] Our findings highlight a technical limitation in studying the effects of a complex microbiota on host physiology: the microbial community is often difficult to replicate, sometimes requiring a fecal microbiota transplant to directly demonstrate the effects of the gut microbiome on a phenotype. Stored microbiota are a limited resource that, when expended, cannot be further replicated. However, a recent advancement with in vitro culture techniques addresses this limitation by enabling storage and subsequent growth of complex donor communities[42] (such an approach also enables sharing with other labs). We are now implementing the development of these "stock" microbial communities in our laboratory and encourage the community to also maintain viable, frozen stool-derived microbial communities where possible.

Although the changes in the gut microbiota in the current study are much smaller than those seen by Luna et al., in the aged cohort, the composition of the microbiota differed in both males and females receiving the sweetener relative to untreated mice with decreased abundance of *Odoribacter* in mice that received sweetener. *Odoribacter* is a member of *Bacteroidetes* phylum and *Porphyromonadaceae* family that has previously been shown to be decreased with aspartame treatment.[16] Additionally, human-derived isolates of *Odoribacter* sp. have been used as probiotics to consume excess succinate to modulate glucose tolerance and inflammation in mice[43] and decreased abundance of *Odoribacter* with sweetener treatment may partially explain a greater body mass in aged male mice.[44] The secondary differential abundance analysis via ALDEx2 was confirmatory of the results from MaAsLin2 and identified significant ASVs in aged males and females from the *Bacteroides* phylum and *Porphyromonadaceae* family.

This study has several limitations related to its scope. Specifically, our study was not designed to explain the mechanism of how the increased body mass caused by sweetener increased femoral geometry and whole bone strength in aged male mice. It is conceivable that sweetener increased mouse cage activity leading to increased loading and bone formation. It has also been reported that chronic aspartame impairs the function of intestinal alkaline phosphatase through its byproduct phenylalanine causing translocation of gut microbes and inflammation.[45] Therefore, it is also possible that the aspartame-based sweetener used in our experiment could have inactivated intestinal alkaline phosphatase and interfered with

bone metabolism signaling downstream (TLR4/NF-κB signaling pathways)[45] and/or microbial metabolites or translocated microbes were directly influencing bone metabolism. However, noticeable changes in bone geometry were only seen in the aged males. Further, our study was limited in that it did not measure food intake, which could provide more insight into why sweetener-treated aged male mice had an increased body mass.

Our study involved mice receiving an extraordinarily high dose of sweetener (estimated daily intake 1500 mg/kg, assuming 2–3 mL water consumed/d, human equivalent dose 122 mg/kg).[46] Our dose was within the range of prior carcinogenicity studies in rodents and accounted for differences in the rate of aspartame metabolism in mice vs humans.[47,48] The zero-calorie aspartame-based sweetener used in the study (Equal®) contains approximately 1.7% aspartame by mass, therefore the human equivalent dose of aspartame consumed daily is approximately 2.07 mg/kg (excluding other fillers present in the Equal® sweetener). Assuming a 60 kg adult, our dose of 2.07 mg/kg aspartame would be the equivalent of <1 can diet soda/d (average can diet soda contains 200–300 mg aspartame). The current study was not designed to detect the effects of aspartame-based sweetener on mouse health, so while we cannot make conclusions regarding any detrimental effects of aspartame, we did observe an increase in early mortality in females receiving high dose sweetener, although the cause of death is not clear.

In summary, changes in the gut microbiota induced by high dose sweetener in the current study did not match those observed in our prior study and we subsequently did not observe the same increase in bone tissue strength reported in our prior work. We did observe that aged male mice receiving chronic sweetener had greater body mass, whole bone strength, and femoral geometry relative to untreated mice; an effect that was not observed in aged females and appeared to be explained by increased body mass. Further studies are necessary to elucidate the mechanism and potential role of the microbiome. However, this finding suggests that chronic use of aspartame-based sweetener negatively affects metabolism in males that leads to secondary effects on bone geometry and strength. Our study highlights the need for replication in microbiome studies and the importance of long-term rodent studies to understand the effects of compounds of interest across the lifespan on bone.

Author contributions

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

Erika L. Cyphert (Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing—review & editing), Chongshan Liu (Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Angie L. Morales (Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Jacob C. Nixon (Formal analysis, Investigation), Emily Blackford (Formal analysis, Investigation), Matthew Garcia (Formal analysis, Investigation), Nicolas Cevallos (Formal analysis, Writing—original draft, Writing—review & editing), Peter J. Turnbaugh (Formal analysis, Writing—original draft, Writing—review & editing), Ilana L. Brito (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Sarah L. Booth (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), and Christopher J. Hernandez (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing – review & editing)

Supplementary material

Supplementary material is available at *JBMR Plus* online.

Conflicts of interest

The authors have no conflicts of interest to disclose.

Data availability

Data described in the manuscript, code book, and analytic code will be made publicly and freely available without restriction at https://doi.org/10.5061/dryad.bk3j9kdk6 and raw V4 16S rRNA DNA sequences are available at the NCBI's Sequence Read Archive Database (BioProject ID: PRJNA 1032620; http://www.ncbi.nlm.nih.gov/bioproject/1032620).

References

1. Luna M, Guss J, Vasquez-Bolanos L, et al. Components of the gut microbiome that influence bone tissue-level strength. *J Bone Min Res*. 2021;36(9):1823–1834. https://doi.org/10.1002/jbmr.4341

2. Guss JD, Horsfield MW, Fontenele FF, 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

3. Guss J, Zieman S, Luna M, et al. The effects of metabolic syndrome, obesity, and the gut microbiome on load-induced osteoarthritis. *Osteoarthr Cartil*. 2019;27(1):129–139. https://doi.org/10.1016/j.joca.2018.07.020

4. Cyphert E, Clare S, Dash A, et al. A pilot study of the gut microbiome in spine fusion surgery patients. *HSS J*. 2023;0(0). https://doi.org/10.1177/15563316231201410

5. Yan J, Herzog JW, Tsang K, et al. Gut microbiota induce IGF-1 and promote bone formation and growth. *Proc Natl Acad Sci U S A*. 2016;113(47):E7554–E7563. https://doi.org/10.1073/pnas.1607235113

6. Li J-Y, Chassaing B, Tyagi AM, et al. Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest*. 2016;126(6):2049–2063. https://doi.org/10.1172/JCI86062

7. Vahidi G, Moody M, Welhaven HD, et al. Germ-free C57BL/6 mice have increased bone mass and altered matrix properties but not decreased bone fracture resistance. *J Bone Miner Res*. 2023;38(8):1154–1174. https://doi.org/10.1002/jbmr.4835

8. Desbonnet L, Clarke G, Traplin A, et al. Gut microbiota depletion from early adolescence in mice: implications for brain and

behavior. *Brain Behav Immun*. 2015;48:165–173. https://doi.org/10.1016/j.bbi.2015.04.004

9. Reikvam DH, Erofeev A, Sandvik A, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS One*. 2011;6(3):e17996. https://doi.org/10.1371/journal.pone.0017996

10. Castaneda M, Strong JM, Alabi DA, 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

11. Ruiz-Ojeda FJ, Plaza-Díaz S-LMJ, Gil A. Effects of sweeteners on the gut microbiota: a review of experimental studies and clinical trials. *Adv Nutr*. 2019;10(suppl_1):S31–S48. https://doi.org/10.1093/advances/nmy037

12. Finamor I, Perez S, Bressan C, et al. Chronic aspartame intake causes changes in the trans-sulphuration pathway, glutathione depletion and liver damage in mice. *Redox Biol*. 2017;11:701–707. https://doi.org/10.1016/j.redox.2017.01.019

13. Christian B, McConnaughey K, Bethea E, et al. Chronic aspartame affects T-maze performance, brain cholinergic receptors and Na+, K+-ATPase in rats. *Pharmacol Biochem Behav*. 2004;78(1):121–127. https://doi.org/10.1016/j.pbb.2004.02.017

14. Park S, Belfoul A, Rastelli M, et al. Maternal low-calorie sweetener consumption rewires hypothalamic melanocortin circuits via a gut microbial co-metabolite pathway. *JCI Insight*. 2023;8(10):e156397. https://doi.org/10.1172/jci.insight.156397

15. Suez J, Korem T, Zeevi D, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*. 2014;514(7521):181–186. https://doi.org/10.1038/nature13793

16. Suez J, Cohen Y, Valdes-Mas R, et al. Personalized microbiome-driven effects of non-nutritive sweeteners on human glucose tolerance. *Cell*. 2022;185(18):3307–3328.e19. https://doi.org/10.1016/j.cell.2022.07.016

17. Naddaf M. Aspartame is a possible carcinogen: the science behind the decision. *Nature Brief*. 2023. https://doi.org/10.1038/d41586-023-02306-0

18. Ma BW, Bokulich NA, Castillo PA, et al. Routine habitat change: a source of unrecognized transient alteration of intestinal microbiota in laboratory mice. *PLoS One*. 2012;7(10):e47416. https://doi.org/10.1371/journal.pone.0047416

19. Bouxsein M, Boyd S, Christiansen B, Guldberg R, Jepson K, Muller 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

20. Tassani S, Korfiatis V, Matsopoulos G. Influence of segmentation on micro-CT images of trabecular bone. *J Microsc*. 2014;256(2):75–81. https://doi.org/10.1111/jmi.12159

21. Coutel X, Olejnik C, Marchandise P, et al. A novel microCT method for bone and marrow adipose tissue alignment identifies key differences between mandible and tibia in rats. *Calcif Tissue Int*. 2018;103(2):189–197. https://doi.org/10.1007/s00223-018-0397-1

22. 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 diaphysis of long bones. *J Bone Miner Res*. 2015;30(6):951–966. https://doi.org/10.1002/jbmr.2539

23. Thompson L, Sanders J, McDonald D, et al. A communal catalogue reveals Earth's multiscale microbial diversity. *Nature*. 2017;551(7681):457–463. https://doi.org/10.1038/nature24621

24. Caporaso J, Lauber C, Walters W, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. 2012;6(8):1621–1624. https://doi.org/10.1038/ismej.2012.8

25. Walters W, Hyde E, Berg-Lyons D, et al. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems*. 2015;1(1):e00009–e00015. https://doi.org/10.1128/mSystems.00009-15

26. Marotz C, Sharma A, Humphrey G, et al. Triplicate PCR reactions for 16S rRNA gene amplification sequencing are unnecessary. *BioTechniques*. 2019;67(1):29–32. https://doi.org/10.2144/btn-2018-0192

27. Estaki M, Jiang L, Bokulich N, et al. QIIME2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. *Curr Protoc Bioinforma*. 2020;70(1):e100. https://doi.org/10.1002/cpbi.100

28. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*. 2017;5(1):27. https://doi.org/10.1186/s40168-017-0237-y

29. Mallick H, Ma S, Franzosa E, Vatanen T, Morgan X, Huttenhower C. Experimental design and quantitative analysis of microbial community multiomics. *Genome Biol*. 2017;18(1):228. https://doi.org/10.1186/s13059-017-1359-z

30. Dixon P. VEGAN, a package of R functions for community ecology. *J Veg Sci*. 2003;14(6):927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

31. Mallick H, Rahnavard A, McIver LJ, et al. Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol*. 2021;17(11):e1009442. https://doi.org/10.1371/journal.pcbi.1009442

32. Fernandes A, Reid J, Macklaim J, McMurrough T, Edgell D, Gloor G. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome*. 2014;2(1):15. https://doi.org/10.1186/2049-2618-2-15

33. Nearing JT, Douglas GM, Hayes MG, et al. Microbiome differential abundance methods produce different results across 38 datasets. *Nat Commun*. 2022;13(1):342. https://doi.org/10.1038/s41467-022-28034-z

34. RStudio Team. *RStudio: Integrated Development for R*. RStudio, PBC; 2021: http://www.rstudio.com.

35. Anderson M. Permutational multivariate analysis of variance (PERMANOVA). In: Wiley StatsRef: Statistics Reference Online; 2017. https://doi.org/10.1002/9781118445112.stat07841

36. 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

37. Guru SK, Ying L, Savinova OV, Zhang Y. Long-term consumption of artificial sweeteners does not affect cardiovascular health and survival in rats. *PeerJ*. 2022;10:e13071. https://doi.org/10.7717/peerj.13071

38. Smits SA, Marcobal A, Higginbottom S, Sonnenburg JL, Kashyap PC. Individualized responses of gut microbiota to dietary intervention modeled in humanized mice. *mSystems*. 2016;1(5):e00098–e00016. https://doi.org/10.1128/mSystems.00098-16

39. Salonen A, Lahti L, Salojarvi J, et al. Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J*. 2014;8(11):2218–2230. https://doi.org/10.1038/ismej.2014.63

40. Liu C, Cyphert EL, Stephen SJ, et al. Microbiome-induced increases and decreases in bone tissue strength can be initiated after skeletal maturity. *bioRxiv*. 2024;2024. https://doi.org/10.1101/2024.01.03.574074

41. Castaneda M, Smith KM, Nixon JC, Hernandez CJ, Rowan S. Alterations to the gut microbiome impair bone tissue strength in aged mice. *Bone Reports*. 2021;14:101065. https://doi.org/10.1016/j.bonr.2021.101065

42. Aranda-Díaz A, Ng KM, Thomsen T, et al. Establishment and characterization of stable, diverse, fecal-derived *in vitro* microbial communities that model the intestinal microbiota. *Cell Host Microbe*. 2022;30(2):260–272.e5. https://doi.org/10.1016/j.chom.2021.12.008

43. Huber-Ruano I, Calvo E, Mayneris-Perxachs J, et al. Orally administered Odoribacter laneus improves glucose control and inflammatory profile in obese mice by depleting circulating

succinate. *Microbiome*. 2022;10(1):135. https://doi.org/10.1186/s40168-022-01306-y

44. Lim M, You H, Yoon H, et al. The effect of heritability and host genetics on the gut microbiota and metabolic syndrome. *Gut*. 2017;66(6):1031–1038. https://doi.org/10.1136/gutjnl-2015-311326

45. Gul S, Hamilton A, Munoz A, et al. Inhibition of the gut enzyme intestinal alkaline phosphatase may explain how aspartame promotes glucose intolerance and obesity in mice. *Appl Physiol Nutr Metab*. 2017;42(1):77–83. https://doi.org/10.1139/apnm-2016-0346

46. Nair A, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm*. 2016;7(2):27–31. https://doi.org/10.4103/0976-0105.177703

47. Borghoff S, Cohen S, Jiang X, et al. Updated systematic assessment of human, animal and mechanistic evidence demonstrates lack of human carcinogenicity with consumption of aspartame. *Food Chem Toxicol*. 2023;172:113549. https://doi.org/10.1016/j.fct.2022.113549

48. Okasha E. Effect of long term-administration of aspartame on the ultrastructure of sciatic nerve. *J Microsc Ultrastruct*. 2016;4(4):175–183. https://doi.org/10.1016/j.jmau.2016.02.001