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Prune supplementation for 12 months alters the gut microbiome in postmenopausal women†‡

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Prunes have health benefits, particularly in postmenopausal women. It is likely that the gut microbiome mediates some of these effects, but its exact role remains to be elucidated. This study aims to characterize the effect of prune supplementation on the gut microbiome of postmenopausal women. The fecal microbiome of 143 postmenopausal women ages 55–75 who met the compliance criteria in a randomized controlled trial of a 12-month dietary intervention in one of three treatment groups – no prunes (n = 52), 50 g prunes per day (n = 54), or 100 g prunes per day (n = 37) – was characterized at baseline and at the 12-month endpoint using 16S rRNA gene sequencing and QIIME2. Additional outcomes included assessment of select urinary phenolic metabolites and inflammatory markers. After 12 months, microbiomes of women consuming 50 g prunes had decreased evenness in bacteria taxa (Pielou's Evenness, Kruskal–Wallis p = 0.026). Beta diversity comparisons indicated significant differences in microbiomes among prune treatments (Bray–Curtis PERMANOVA, p = 0.005), and the effect was different at each prune dose (p = 0.057). Prunes enriched some bacterial taxa such as the family Lachnospiraceae (LEfSe LDA = 4.5). Some taxa correlated with urinary phenolic metabolites and inflammatory markers. *Blautia* negatively correlated with total urinary phenolics (r = −0.25, p = 0.035) and Lachnospiraceae UCG-001 negatively correlated with plasma concentrations of IL-1β (r = −0.29, p = 0.002). Differing gut microbiomes and correlation of some taxa with select phenolic metabolites and inflammatory markers, particularly Lachnospiraceae, after prune consumption suggest a potential mechanism mediating health effects. The microbiome differences at each dose may have implications for the use of prunes as a non-pharmacological whole food intervention for gut health.

1. Introduction

Prunes confer health benefits including increased gastric motility,[1,2] improved cardiovascular health,[3] and bone loss prevention in postmenopausal women.[4,5] The gut microbiome may mediate health effects of prunes by influencing nutrient uptake and prune metabolites in the lower gut.[6] Further, the gut microbiome can influence the host immune system through direct immune stimulation, modulation of gut wall permeability, and production of immunomodulatory metabolites.[7] Gut microbiome-mediated immune responses are implicated in the development of diabetes, cardiovascular disease, inflammatory bowel disease, cancer,[7] and postmenopausal osteoporosis.[8,9] Immune disfunction is a hallmark of aging,[10] and increases disease risk in postmenopausal women. Thus, the interaction between prunes, the gut microbiome, immunologically active metabolites, and host inflammatory markers in postmenopausal women merits investigation.

Prune phenolics are of particular interest because of their potential to modulate antioxidant and anti-inflammatory responses.[5,11] Prune phenolics are primarily composed of caffeoylquinic acids, otherwise known as chlorogenic acids.[1] While abundant in the fruit, chlorogenic acids are poorly absorbed in the upper intestine and become available to the gut microbiota in the lower intestine. The gut microbiota metabolize these compounds[12] to a diverse array of low molecular weight phenolic metabolites.[11,12] These microbial phenolic metabolites are more bioavailable to the host[13] and are

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believed to reduce the host inflammatory response[14] and inhibit development of inflammatory diseases.

Prunes may exert unique effects on the gut microbiome due to their nutrient composition and effects on gastric motility. In addition to phenolics, prunes are rich in fiber, simple sugars, and micronutrients.[1] Fiber has been shown to impact the human microbiome (reviewed in ref. 15). Animal studies suggest that simple sugars[16,17] and various micronutrients including vitamins and free elements (reviewed in ref. 18) can influence the microbiome. Other fruits have previously been shown to influence gut microbiome composition, but these effects are dependent on the fruit's nutrient composition.[20] Thus, these compositional differences in prunes may exert a different effect than other fruits on the composition of the microbiome.

Taken together, the unique nutrient composition, phenolic content, effects on the gastrointestinal tract, and empirical health benefits of prunes warrant further investigation into prunes' effect on the gut microbiome. To date, there have been no long-term and only two short-term studies that have investigated the effect of acute prune consumption on the microbiome in humans.[2,19] In men and women age 18–53 years, consumption of prune juice for four weeks increased the colony count of beneficial bacterial populations, particularly *Bifidobacterium* and *Lactobacillus* spp., while lowering the colony count of potentially harmful bacteria, including *Escherichia coli* and *Clostridium perfringens*, suggesting that prunes may positively modulate the gut microbiome.[19] In another study in men and women age 18–65 years, consuming 80 g and 120 g of whole prunes per day for four weeks increased bifidobacteria.[2] These studies have a number of caveats that may limit their applicability to postmenopausal women, including: (1) younger, mixed-gender cohorts, who may respond differently to prunes than postmenopausal women; (2) the use of culture-dependent methods, which are only capable of measuring a fraction of microbial diversity;[21] and (3) the short-term intervention length. The health effects of prunes in postmenopausal women are observed on the scale of months to years,[4] and the effects of dietary interventions on the gut microbiome may differ in the long-term. It is therefore necessary to investigate the effect of long-term prune consumption on the gut microbiome.

The purpose of this study was to characterize the effect of 12-months of prune consumption on the gut microbiome, prune phenolic metabolism, and inflammatory markers in postmenopausal women. We hypothesized that 12 months of prune consumption would change the gut microbiome of postmenopausal women, altering excreted phenolic metabolites and host inflammatory markers. Specifically, we aimed to (1) determine the changes in gut microbiome composition of postmenopausal women after a 12-month prune intervention, (2) quantify if microbiome differences vary with prune dose, and (3) identify correlations between the gut microbiome, select phenolic metabolites, and host inflammatory markers. To address these aims, postmenopausal women were assigned to 50 g prune per day, 100 g prune per day, or a no prune control in a 12-month, parallel arm, randomized controlled trial (RCT). The fecal microbiome was analyzed pre- and post-prune intervention using 16S rRNA gene amplicon sequencing. Additional measures collected pre- and post-intervention included select urinary phenolic metabolites and host inflammatory markers.

2. Materials and methods

2.1. Study design

2.2.1. Study overview. The Prune Study (Clinical Trials NCT02822378) is a parallel arm randomized controlled trial to study the effects of a 12-month prune intervention on bone health (bone outcomes published elsewhere[4]) and the gut microbiome of postmenopausal women. A detailed description of the design of this study has been published.[22] Briefly, postmenopausal women aged 55–75 years were enrolled at Pennsylvania State University (PSU) and randomized into one of three treatments: 50 g prunes (*i.e.*, 4–6 prunes), 100 g prunes (10–12 prunes), or a no prune control group. Prunes were consumed daily for 12 months in the two treatment groups. Two hundred thirty-five women enrolled (78 control, 79 50 g, 78 100 g) and 183 women completed the 12-month intervention (70 control, 67 50 g, 46 100 g). Fecal microbiome analysis was conducted on 155 of these women (58 control, 58 50 g, 39 100 g), due to missing or insufficient fecal sample amounts collected from some subjects. Of these, 143 women (52 control, 54 50 g, 37 100 g) were compliant with the treatment (see section Compliance), and this was the subset of subjects analyzed to determine the effect of prune treatment on the microbiome. A CONSORT diagram is available in Fig. S1.‡ Measured outcomes used in this study are the fecal microbiome, select phenolic metabolites, and inflammatory markers.

2.2.2. Recruitment, screening & eligibility. All study procedures were performed at the Pennsylvania State University Clinical Research Center from June 2016 to February 2021. Informed consent was obtained during an in-person visit. Eligible participants were postmenopausal women aged 55–75 years, not severely obese (BMI < 40 kg m^{-2}), healthy (as determined by screening questionnaire and metabolic panel), non-smoking, ambulatory, and had a bone mineral density T-score at the lumbar spine, total hip, and/or femoral neck between 0.0 and −3.0. Exclusion criteria included consumption of any natural dietary supplement containing phenolics, consumption of >1 cup per day of blueberries or apples for at least 2 months prior to study entry, a history of bone fractures after age 50, significant chronic disease, and consumption of certain medicines affecting bone metabolism, as described previously.[22]

2.2.3. Prune intervention. Participants were randomly assigned to one of three treatment groups: a no prune control group, 50 g prunes per day (*e.g.*, 4–6 prunes), and 100 g prunes per day (10–12 prunes) for 12 months. Prunes were supplied by the California Prune Board. Prune phenolic composition,

determined as previously described,[23] varied little between years. The main phenolic constituents were chlorogenic acid derivatives (Table S1‡). Prune groups underwent a "run-in" period to slowly increase prune consumption in their diet, as previously described.[22] All participants were supplemented as necessary to meet the required intake of 1200 mg calcium and 800 IU vitamin D_3 daily (Nature Made Pharmavite LLC, West Hills, CA). This supplementation was related to the primary outcome of the trial, bone health. Due to the nature of the intervention, it was not possible to blind participants and study staff to the treatment, but data analysts and outcome assessors were blinded.

2.2.4. Compliance. Compliance was determined from self-report logs of prune and/or calcium and vitamin D_3 consumption logs, completed each day. Compliance was calculated as the reported consumed prunes or supplements divided by the prescribed amounts of prunes or supplements (%). "Compliant" participants were those who consumed >80% of prescribed prunes (for the 50 g and 100 g prune groups) or >80% of prescribed supplements (for the control group) during the entirety of the 12-month intervention.

2.3. Anthropometrics

Height was measured in centimeters using a stadiometer. Total body weight was measured on a physician's scale (Seca, Model 770, Hamburg, Germany) during screening and each monthly visit. BMI was calculated as body mass divided by height squared (kg m^{-2}).

2.4. Diet assessment

To assess kilocalorie intake and dietary composition, participants measured and recorded all food and beverages consumed in a 3-day window encompassing two weekdays and one weekend day. Diet diary data were coded and analyzed using Nutritionist Pro software (Axxya Systems, Redmond, WA). Daily nutrient intake was averaged over the 3-day recording window.

2.5. Phenolics

Phenolic metabolites were measured from a 48-hour pooled urine sample every three months. To minimize background from non-prune phenolics, participants were asked not to consume phenolic-rich foods such as coffee, fruits, and vegetables for 12 hours before urine collection. All phenolic analyses were conducted at the North Caroline State University Plants for Human Health Institute (Kannapolis, NC). Upon arrival 1 mL of the pooled 48-hour urine sample was stored at −80 °C. Urinary samples were analyzed to determine total phenolics and targeted phenolic metabolites as previously described in detail.[22] Briefly, total urinary phenolics were determined from a 48-hour pooled urine sample after solid phase extraction (SPE) extraction by the Folin–Ciocalteu microplate method[24] and corrected for creatinine content (colorimetric assay kit 500701, Cayman chemical, Ann Arbor, MI).[25] A targeted set of 21 prune-derived phenolic metabolites were measured by ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) using a Waters UPLC Acquity H Class system equipped with a Xevo TQD Mass Spectrometric detector as described previously.[22]

2.6. Inflammation assessment

Inflammatory markers were measured at baseline and post-intervention. Detailed methods have been published.[22] C-reactive protein (CRP) in serum was quantified in duplicate using an Immulite (Siemens Healthcare, Munich, Germany) high-sensitivity CRP kit as per manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described[22] and counted for use in functional and phenotypic analyses as previously described.[26]

2.6.1. *In vitro* inflammatory cytokine secretion assay. PBMCs (2×10^5 mL^{-1}) were stimulated with 0.625 μg mL^{-1} lipopolysaccharide (LPS) (Sigma-Aldrich, St Louis, MO)[27] in round-bottomed 96-well plates, and supernatants (70 μL per replicate) were harvested after 4 h incubation at 37 °C and frozen at −80 °C until analysis. Cytokines and chemokines (IL-1β, IL-6, IL-8, TNF-α, and MCP-1) in plasma and supernatants harvested from LPS-stimulated PBMCs were measured using the V-PLEX Proinflammatory Panel 1 Human Kit and V-PLEX Human MCP-1 kit (Meso Scale Diagnostics, LLC, Rockville, MD) as per the manufacturer's instructions. Supernatants were diluted 1 : 5 for MCP-1 measurement and 1 : 20 for IL-1β, IL-6, IL-8, TNF-α measurements in dilution buffer. Each assay was performed in duplicate.

2.6.2. Flow cytometric analysis. PBMCs were washed twice in PBS at 4 °C. Fc receptors on PBMCs were blocked by incubation with Fc block (Human TruStain FcX, Biolegend, San Diego, CA) at 5 μL per 1×10^6 cells at 25 °C for 5 minutes. PBMCs were stained with fluorescence-labeled mouse anti-human antibodies (1 μg per 1×10^6 cells) to the following cell surface markers: CD3, CD14, CD282 (TLR-2) and Human Leukocyte Antigen-DR isotype (HLA-DR). Antibody isotype controls included: mouse IgG2a and mouse IgM. CD282 and CD14 were purchased from Biolegend, and all remaining antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Following incubation with the conjugated antibodies for 30 minutes at 4 °C, cells were washed twice in PBS and then fixed in cytofix (BD Biosciences) for flow cytometric analyses as previously described.[28] A total of 50 000 events were acquired with BD LSR-Fortessa (BD Biosciences). Data were analyzed and plotted using FlowJo software v10 (FlowJo, LLC). Live lymphoid and myeloid cells were gated on forward *versus* side scatter. The percent of live cells that were CD14+ and HLA-DR+ was quantified to measure the percentage of CD14+/HLA-DR+ monocytes within the live cell population. The median fluorescent intensities (MFI) of HLA-DR and CD282 expression on triple-positive CD14+/HLA-DR+/CD282+ cells were calculated to assess monocyte activation.

2.7. Fecal microbiome analysis

2.7.1. DNA extraction & quantification. Homogenized fecal samples were stored at −80 °C until DNA extraction. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals) and bead-beating according to the manufac-

turer's instructions. Extracted DNA quality was assessed using 0.8% agarose gel electrophoresis and a NanoDrop One spectrometer (Thermo Scientific). DNA was quantified using a NanoDrop 3300 spectrofluorometer (Thermo Scientific).

2.7.2. Amplification of 16S rRNA gene. The V3–V4 region of the 16S rRNA gene was amplified by PCR with primers 343F: TAC GGR AGG CAG CAG and 804R: CTA CCR GGG TAT CTA ATC C. PCR was conducted using the Q5 High-Fidelity Master Mix (New England Biolabs), with 10 ng of DNA template per 50 μl reaction, and the following cycling conditions: 5 minutes at 95 °C; 15 cycles of 30 seconds at 94 °C, 20 seconds at 58 °C, and 20 seconds at 72 °C; 10 minutes at 72 °C. PCR products were purified using the Axy-Prep Mag PCR clean-up kit (Axygen®, Corning) and subjected to a second PCR phase, in which amplicons were barcoded using forward and reverse 8-base pair primer tags. The second PCR phase used the following cycling conditions: 5 minutes at 95 °C; 5 cycles of 30 seconds at 94 °C, 20 seconds at 64 °C, and 20 seconds at 72 °C; 10 minutes at 72 °C. PCR products were again purified using the Axy-Prep Mag PCR clean-up kit and quantified using the QuantiFluor dsDNA System (Promega) and a NanoDrop 3300 spectrofluorometer. Purified PCR products were then pooled in equal molar concentrations and 2 × 250 paired end sequencing was performed using an Illumina MiSeq instrument at the Purdue Genomics Facility.

2.7.3. Sequence analysis. Sequences were trimmed to remove adapters and barcodes, and then analyzed using QIIME2.[29] After demultiplexing, DADA2[30] was used to trim bases for quality, merge paired-end reads and group sequences into amplicon sequence variants (ASVs). A taxonomy classifier, trained using the SILVA database (version 138),[31] was used to assign taxonomies to representative 16S rRNA gene sequences. Sequences were then filtered to remove chloroplast and mitochondrial sequences, sequences unidentified at the phylum level, and features present in less than 10 samples. For all diversity analyses, sequences were rarified to the same depth, chosen to maximize retained features while minimizing excluded samples. Differences in alpha and beta diversity were tested among the treatment groups pre- and post-intervention (control *vs.* 50 g *vs.* 100 g). Alpha diversity (metrics used: observed features, Pielou's evenness, Faith's phylogenetic diversity[32]) and beta diversity (metrics used: Jaccard, Bray–Curtis, unweighted Unifrac,[33] and weighted Unifrac) were analyzed using QIIME2. Alpha diversity measures the richness and/or evenness of taxa within a sample, while beta diversity measures the difference in taxa distribution among samples. Beta diversity was visualized using Principal Coordinates Analysis (PCoA) plots in *R* using the package ape (v5.4-1). For all four beta diversity metrics, beta diversity dissimilarity within each treatment group was calculated using pairwise comparisons of microbiomes within each treatment group using QIIME2. Analysis of Composition of Microbes (ANCOM)[34] was used to find differences in the log-transformed counts of taxa between treatment groups, and linear discriminant analysis (LDA) effect size (LEfSe)[35] was used to find differences in the relative abundance of taxa among treatment groups.

2.8. Statistical testing

Significant differences in alpha-diversity were tested using Kruskal–Wallis and *post hoc* pairwise testing was conducted using FDR-corrected Kruskal–Wallis. Significant differences in beta diversity were determined using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. *Post hoc* pairwise testing for beta diversity was also tested using FDR-corrected PERMANOVA with 999 permutations. Permutational analysis of multivariate dispersions (PERMDISP) was used to calculate whether significant differences in beta diversity could be due to differences in the variation of treatment groups instead of true differences in group means. For quantitative human health variables, normality was tested using Shapiro-Wilk's method. Inflammatory markers were cleaned for outliers and normalized using log- or square root-transformations, as necessary. Unless otherwise noted, all significance testing among groups was done using ANOVA and subsequent pairwise testing by Tukey's HSD. Correspondence between bacterial genera, prune treatment and phenolic metabolites at week 52 was determined using Canonical Correspondence Analysis (CCA). Linear correlations between the log-count of individual taxa at species-level and host variables were determined using Spearman's rank correlation coefficient in *R* with the package corrplot. Multiple linear regression to associate excreted phenolics with log counts of microbial genera was conducted in *R*. The genera selected for this analysis were the eight most explanatory taxa for each phenolic metabolite as determined using the eleaps function in the *R* package subselect. For all analyses, *P*- or *q*-values less than 0.05 were considered significant, and those less than 0.10 were considered a trend.

3. Results

3.1. Demographics

The gut microbiome can be influenced by many demographic variables.[36,37] Demographics for all 235 enrolled participants are published elsewhere;[4] trends presented here for the 143 compliant subjects (consumed >80% of assigned prune treatment or vitamin D_3/Ca supplements) are similar. The average age was 62.3 years (range: 55–75) at baseline and 99% (n = 141) were Caucasian. Participants were mostly healthy or overweight (72 healthy, 49 overweight, 22 obese) with an average BMI of 25.6 kg m^{-2} (range: 18.6–39.2; Table 1). Notably, participants in the 50 g prune group had a significantly higher BMI than participants in the other groups. Data for all 155 participants whose microbiomes were sequenced, including non-compliant participants, are available in the ESI.‡ In short, the microbiome results for 155 subjects were similar to those presented for compliant participant samples only.

3.2. 16S rRNA gene sequencing results

In total, 310 fecal samples representing baseline and post-intervention samples from 155 subjects (58 control, 58 50 g, 39 100 g) were sequenced of which the results for the 143 compli-

Table 1 Demographics of compliant participants whose fecal microbiomes were sequenced

	Control	50 g prune	100 g prune	*p*
N	52	54	37	
Age (years)	62.3 ± 4.7	63.4 ± 4.7	62.3 ± 5.3	0.960
Years since menopause	11.9 ± 6.9	11.8 ± 6.6	12.2 ± 7.6	0.961
BMI (kg m^{-2})	24.4 ± 3.4	26.7 ± 4.9	25.7 ± 3.0	0.025[a]
Normal	32 (62%)	24 (44%)	16 (43%)	
Overweight	15 (29%)	17 (31%)	17 (46%)	
Obese	5 (10%)	12 (24%)	4 (11%)	
Race				
Caucasian	52 (100%)	52 (96%)	37 (100%)	
Asian	0 (0%)	2 (4%)	0 (0%)	
Daily dietary fiber at baseline (g)	20.6 ± 7.2	21.7 ± 11.2	24.3 ± 15.3	0.828
Daily dietary fiber at week 52 (g)	23.2 ± 8.3	21.8 ± 8.6	26.7 ± 14.1	0.375
Estimated daily fiber from prunes (g)	0	3.94	7.89	

Data are mean ± standard deviation. Significant differences among groups at baseline determined using Kruskal–Wallis. [a] Control and 50 g Prune group differed using pairwise Wilcoxon ranked sum test ($p = 0.039$).

ant subjects are presented here. After processing of MiSeq Illumina sequencing results by DADA2 and filtering, a total of 15 892 968 reads were retained, with a median of 41 914 reads (range: 11 940–308 076) per sample. Most diversity measures require the same number of reads to compare samples. To ensure sufficient sequence coverage (Fig. S2‡) while maximizing retained samples, a sampling depth of 11 940 reads was chosen for all diversity analyses. This depth retained all samples and 3 701 400 (23.29%) reads.

3.3. Alpha diversity

Alpha diversity, a measure of gut microbial species richness (the number of taxa) and evenness (the even distribution of taxa) within each sample, was analyzed among the treatment groups (143 total: 52 control, 54 50 g, 37 100 g).

3.3.1. Baseline alpha diversity. To determine whether the treatment groups had similar microbiomes prior to prune intervention, baseline differences in the among the treatment groups were analyzed. Significant differences in alpha diversity metrics among the randomized treatment groups at baseline were found using the metrics Observed features (considers richness only; Fig. 1A), Shannon diversity (considers richness and evenness; data not shown), and FaithPD (phylogenetic diversity; data not shown) metrics ($p < 0.03$), but not Pielou's evenness (considers evenness only; $p = 0.177$; Fig. 1B). This indicates that differences in diversity within treatments at baseline were likely driven by richness rather than evenness. Specifically, at baseline, the group randomized to the 50 g prune treatment had lower Observed features ($q = 0.013$), Shannon diversity ($q = 0.03$) and FaithPD ($q = 0.009$) than the group randomized to the 100 g prune treatment. These results indicate that the participants randomized to the 50 g prune group had lower richness prior to intervention. Importantly, there were no significant differences between the participants randomized to the no prune control and the two prune treatment groups.

3.3.2. Post-prune treatment & alpha diversity. At the 12-month endpoint, alpha diversity analyses indicated no significant differences in observed features ($p = 0.40$; Fig. 1C) or FaithPD ($p = 0.29$; data not shown). However, there were significant differences in Pielou's evenness ($p = 0.026$; Fig. 1D) and Shannon ($p = 0.044$; data not shown) among treatments. The 50 g prune group had a slightly lower alpha diversity than the control and 100 g prune groups (Pielou's evenness, significant, $q < 0.045$; Shannon, trend, $q < 0.052$). These differences indicate that the 50 g prune group had a more uneven taxa distribution after 12 months that was not seen at baseline. This effect was not observed in the 100 g prune group.

3.4. Beta diversity

Beta diversity, a comparison of diversity among samples, was analyzed to identify microbiome differences among the treatment groups.

3.4.1. Baseline beta diversity. At baseline there was a significant difference among treatment groups using the unweighted Unifrac metric (compares phylogeny; $p = 0.012$), but not using Bray–Curtis (compares taxa relative abundances; Fig. 2A), Jaccard (compares presence/absence of taxa; data not shown), or weighted Unifrac (compares taxa phylogeny and relative abundance; data not shown). The difference at baseline was probably due to the differing phylogenetic structure of the group randomized to the 50 g prune treatment (unweighted Unifrac, pairwise $q < 0.05$) prior to the prune intervention.

3.4.2. Post-prune treatment & beta diversity. After a 12 month prune intervention statistical differences in the microbiomes of control, 50 g prune, and 100 g prune groups were observed using all four beta diversity metrics tested (Bray–Curtis, Jaccard, weighted Unifrac, significant, $p < 0.04$; unweighted Unifrac, trend, $p = 0.06$). The PCoA plot of the Bray–Curtis metric (representative of all the metrics) indicates there is overlap of microbiomes among treatments but the centroids (diamonds in Fig. 2B) illustrate the differences. PERMDISP was not significant for any metric ($p > 0.189$), indicating that these differences are due to true differences in the means of groups and not due to dispersion differences. Only Bray–Curtis, which considers taxa abundance, and weighted Unifrac, which considers taxa phylogeny as well as abundance, detected pairwise differences between interventions. Bray–Curtis detected significant differences between control and the 50 g prune groups ($q = 0.037$), as well as between control and the 100 g prune groups ($q = 0.037$). The difference between 50 g and 100 g prunes was a trend ($q = 0.057$). For weighted Unifrac, all three comparisons trended significant ($q = 0.076$). These results indicate that differences in the community due to prunes are caused by differing abundances of taxa, rather than a substantially different set of taxa among the groups. The difference observed between 50 g and 100 g prune groups indicate that the two prune doses exert different effects on the

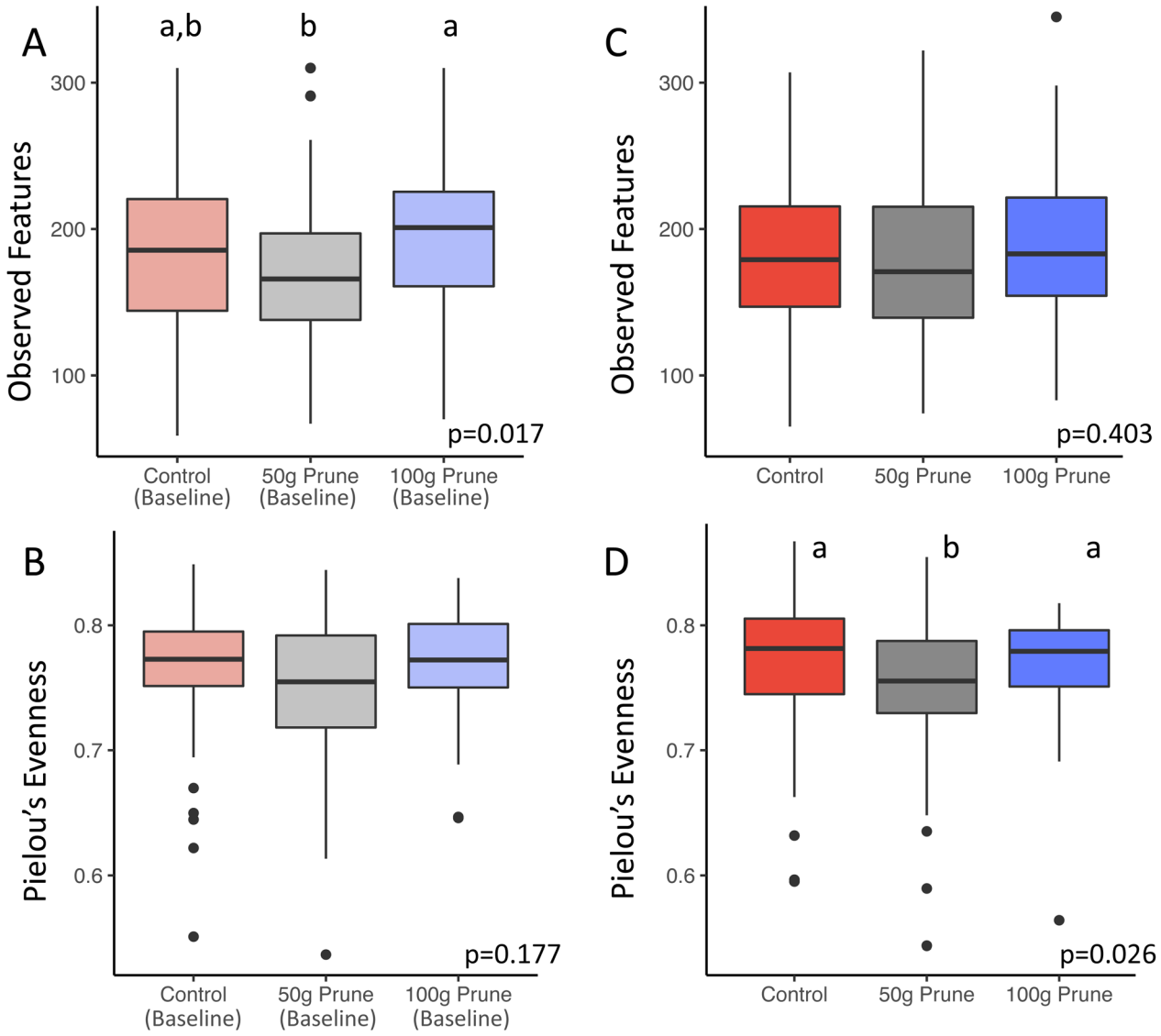

Fig. 1 Boxplots represent the medians and upper and lower quartile values of within sample (alpha) diversity metrics before (A and B) and after (C and D) prune consumption. (A and C) Observed Features, (B and D) Pielou's Evenness. Dots represent outliers. Overall Kruskal–Wallis *p*-values are presented and if pairwise FDR-corrected Kruskal–Wallis significance ($p < 0.05$) was found it is denoted with different letters above boxes (n = 52 control, 54 50 g prune, 37 100 g prune).

gut microbiome. To better illustrate the decrease in beta diversity dissimilarity after prune consumption, the averages of pairwise comparisons within groups were calculated and violin plots were used to show that microbiomes became more similar after consuming prunes within the 50 and 100 g prune groups (all four beta diversity metrics, ANOVA $p < 9 \times 10^{-9}$, Tukey HSD $p < 0.002$; Fig. 2C example using Bray Curtis). Pairwise comparisons indicated significant differences at 12-months for control *vs*. 50 g and control *vs*. 100 g prune ($p < 1 \times 10^{-7}$), but not for 50 *vs*. 100 g prunes ($p = 0.23$).

3.4.3. Post prune treatment & differential taxa. LEfSe identified taxa that differed between control, 50 g prune, and 100 g prune groups at 12-months. LEfSe identified 75 taxa as differentially abundant at LDA > 2 (Table S2‡). Those with LDA > 3 are depicted in Fig. 3A. The most differentially abundant taxon as determined by LEfSe (Fig. 3B) was the family Lachnospiraceae (LDA = 4.5), which was most abundant in the 50 g prune group. Other highly differentially abundant taxa include the genus *Blautia* (LDA = 4.3, most abundant in 50 g prune group; Fig. 3C), the order Oscillospirales (LDA = 4.3, least abundant in the 50 g prune group; Fig. 3D), and the genus *Anaerostipes* (LDA = 4.2, increases with prune dose; Fig. 3E). There were no significantly different taxa identified by ANCOM (data not shown).

3.5. Phenolics

Post-prune intervention, comparisons of excreted urinary phenolic metabolite concentrations indicated no differences in

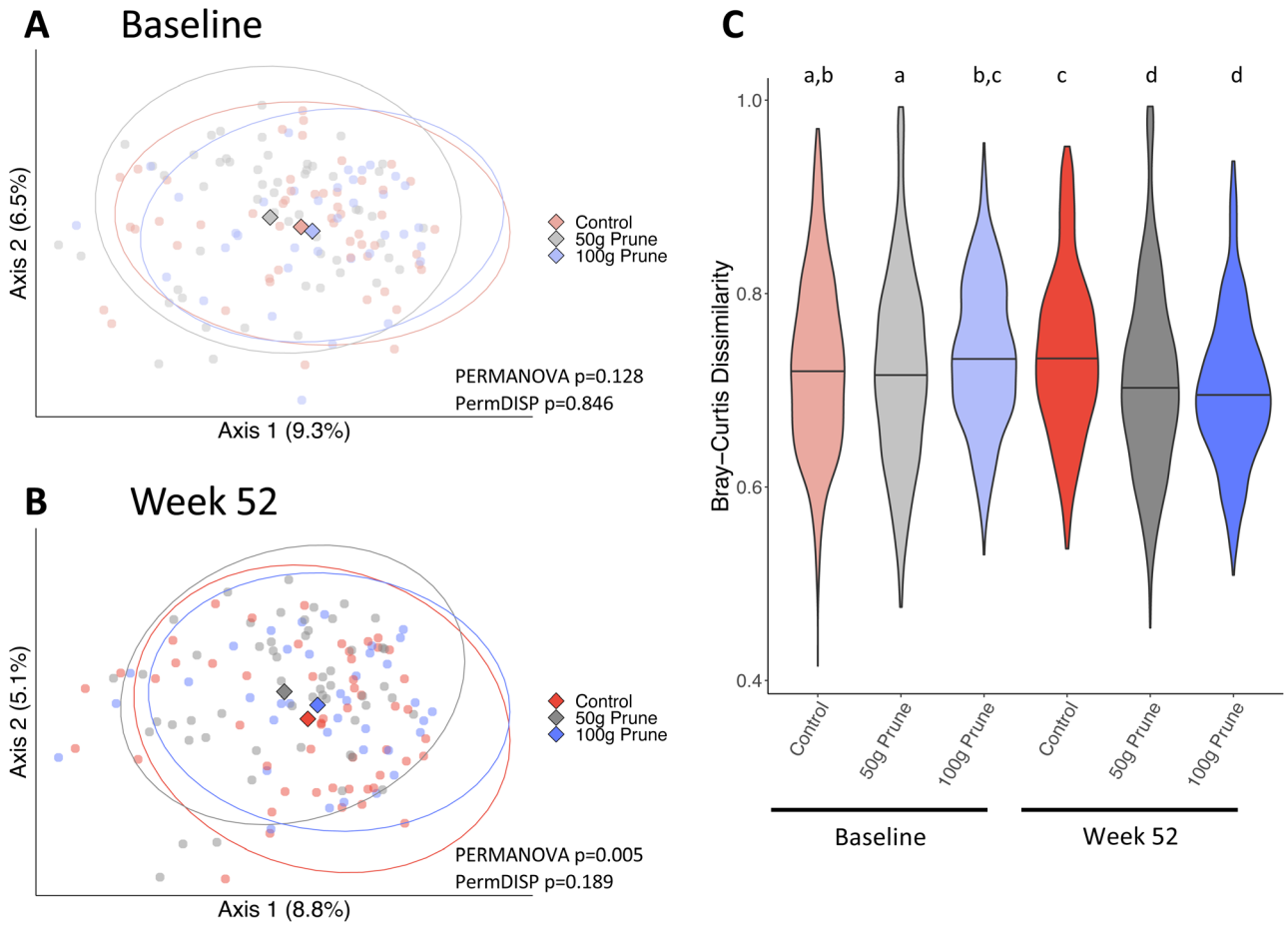

Fig. 2 Among sample (beta) diversity at baseline (A) and week 52 (B) (n = 52 control, 54 50 g prune, 37 100 g prune). Principal coordinate plots of the beta diversity dissimilarity metric Bray–Curtis. Each dot represents one microbiome, color coded by treatment. The centroids of each treatment group are represented by diamonds. Ellipses represent spread of the data. Numbers in parentheses indicate the percent of variation explained by each axis. PERMANOVA and PERMDISP *p*-values presented. (C) Bray–Curtis dissimilarity values from pairwise comparisons of samples within each treatment group at baseline and week 52 (sample *n* = 52 control, 54 50 g prune, 37 100 g prune; number of comparisons = 1326 control, 1431 50 g prune, 666 100 g prune). Violin plot shows the distribution of dissimilarity values and lines represent the median. Overall significance was detected (ANOVA $p < 2 \times 10^{-16}$) and letters represent pairwise significant differences (Tukey HSD $p < 0.1$).

total urinary phenolics (measured by Folin–Ciocalteu and corrected for creatinine) between groups, but some differences were observed in individual metabolites. The targeted phenolic metabolite method used in this study measured 21 specific phenolic metabolites including individual flavonoids, hydroxycinnamic acids, hydroxybenzoic acids and hippuric acids. Metabolites of interest 4-hydroxybenzoic acid, 3-hydroxyhippuric acid, and hippuric acid were identified because they differed in the first 12 weeks of the intervention (data not shown). It should be noted that sample size is different for each metabolite due to some samples being below the detection limit (4-hydroxybenzoic acid n = 52, 3-hydroxyhippuric acid n = 78, hippuric acid n = 110, total phenolics n = 142). Post-intervention, hippuric acid was differentially excreted in urine (Kruskal–Wallis, p = 0.0002). Hippuric acid was significantly increased in the 50 g and 100 g prune groups compared to control (Fig. 4; pairwise Wilcoxon $p < 0.01$). In addition, 4-hydroxbenzoic acid trended lower with increasing prune dose (p = 0.09), and 3-hydroxyhippuric acid trended higher with increasing prune dose (p = 0.05), although upon *post-hoc* testing, only the 50 g prune group had increased 3-hydroxyhippuric acid excretion compared to control (p = 0.043).

3.6. Associations between microbiota and phenolics

We conducted canonical correspondence analysis (CCA) to determine relationships between the whole microbial community (log counts of bacterial genera) and urinary phenolics post-prune intervention. Axis 1 explains 6% of the variation in the data (Fig. 5). The vectors running parallel to this axis indicate the microbiome differences among the prune treatments were primarily driven by hippuric acid and 4-hydroxybenzoic acid. Vectors along Axis 2, which explains 4% of the variation, indicate differences among the microbiomes of the prune groups were driven primarily by total phenolics and 3-hydroxyhippuric acid. The overall model was not significant (p = 0.375).

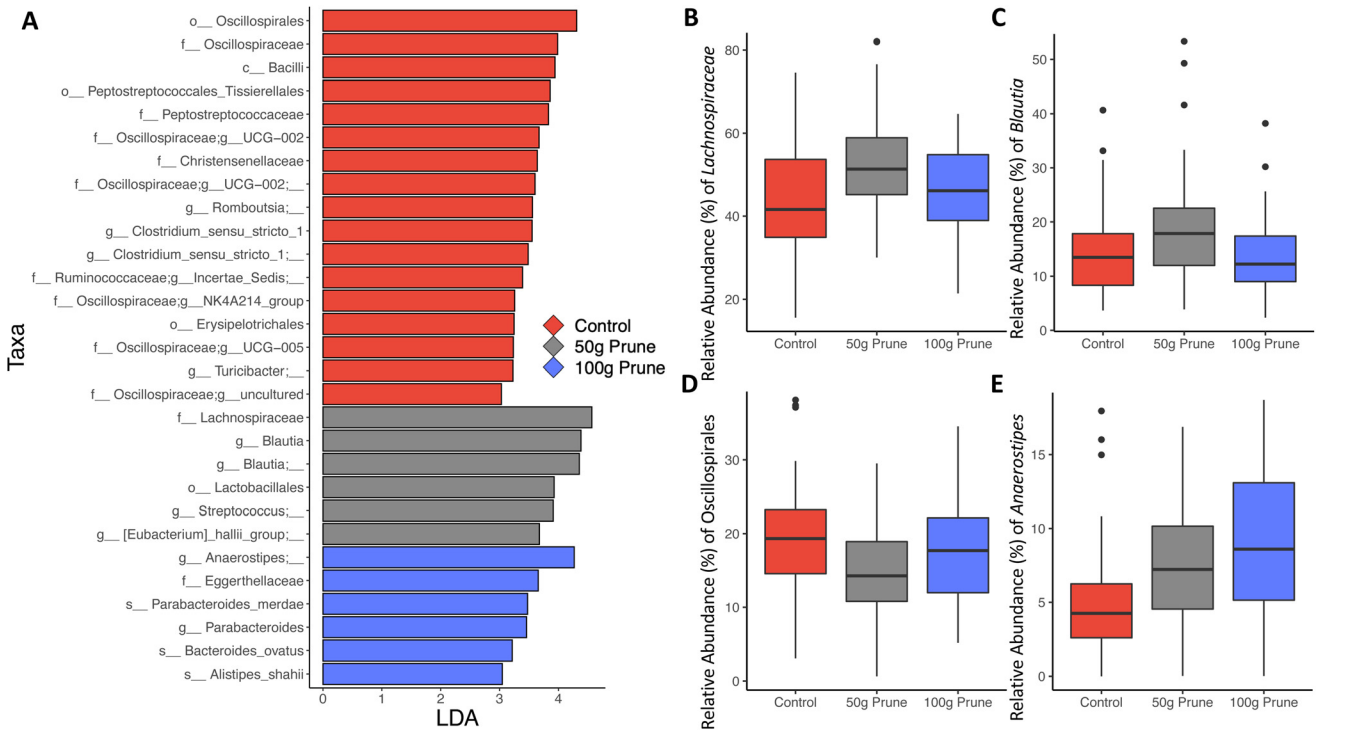

Fig. 3 The effect of prunes on the differential abundance of gut microbiome taxa. (A) Differentially abundant taxa identified by Linear Discriminate Analysis Effect Size (LEfSe) analysis of bacterial communities from prune treatments. Only lowest taxonomic assignments of taxa with an LDA > 3 are presented. Bars are colored by treatment group in which that taxon is most abundant. (B–E) Boxplots representing the medians and upper and lower quartile values of relative abundances of most differentially abundant taxa identified by LEfSe: (B) *Lachnospiraceae*, (C) *Blautia*, (D) Oscillospirales, (E) *Anaerostipes*. Dots represent outliers. Data presented are from compliant subjects at week 52 (n = 52 control, 54 50 g prune, 37 100 g prune).

Because associations between phenolics and the total gut microbiome were not statistically significant, we then conducted linear correlations between phenolics and individual taxa. For this analysis, all samples (compliant and non-compliant, baseline and week 52, n = 310) were included to increase power. Four taxa were significantly correlated or trended towards correlations with phenolic metabolites after Bonferroni correction (Fig. S3‡). All correlations explained a limited proportion of the variation in the data (Spearman's r^2 < 0.12). A bacterium in the family Lachnospiraceae correlated positively with total phenolics (p = 0.05, r^2 = 0.06). A bacterium in the genus *Blautia* correlated negatively with total phenolics (p = 0.03, r^2 = 0.06). Two taxa correlated negatively with 3-hydroxyhippuric acid: a bacterium in the family Oscillospiraceae (p = 0.02, r^2 = 0.11), and a bacterium in the genus *Hydrogenanaerobacterium* (p = 0.08, r^2 = 0.10).

Because phenolic metabolism is likely conducted by multiple genera, we used multiple linear regression to determine whether multiple taxa may explain observed outcomes in urinary phenolic metabolites, and which genera were most explanatory (Table 2). All samples (compliant and non-compliant, baseline and week 52, n = 310) were included to increase power. All eight taxa identified for the multiple linear regression models were significant for total urinary phenolics and 4-hydroxybenzoic acid. Seven and five taxa were significant for 3-hydroxyhippuric acid and hippuric acid, respectively. Eight taxa significantly ($p = 1 \times 10^{-4}$) explained 10% of the variation in total urinary phenolics, including *Lactobacillus* (coefficient: 18.06, p = 0.009) and two Lachnospiraceae genera (p < 0.03). Eleven percent of the variation in hippuric acid was significantly (p = 0.001) explained by eight taxa, including the *[Eubacterium] ruminantium* group (coefficient: −130.51, p = 0.002). Thirty-two percent of the variation in 3-hydroxyhippuric acid was significantly ($p = 2 \times 10^{-10}$) explained by eight taxa, with the most significantly associated genera being *Megamonas* (coefficient: 28.20, $p = 6 \times 10^{-6}$). Twenty-eight percent of the variation in 4-hydroxybenzoic acid was explained by a model incorporating eight taxa ($p = 2 \times 10^{-6}$), including some genera of the families Eggerthellaceae, Lachnospiraceae, and Ruminococcaceae.

3.7. Associations between microbiota and inflammatory markers

Linear correlations between host inflammatory markers and microbial taxa were conducted. As with the phenolic analysis, this analysis included both compliant and noncompliant samples, pre- and post-intervention, in order to increase power (n = 310). One taxon was significantly correlated after Bonferroni correction. Lachnospiraceae UCG-001 was negatively correlated with plasma concentrations of IL-1β (p = 0.002, r = −0.29; Fig. S4A‡) and IL-6 (p = 0.09, r = −0.25; Fig. S4B‡). We did not use CCA to determine associations with inflammatory markers due to the large number of missing samples.

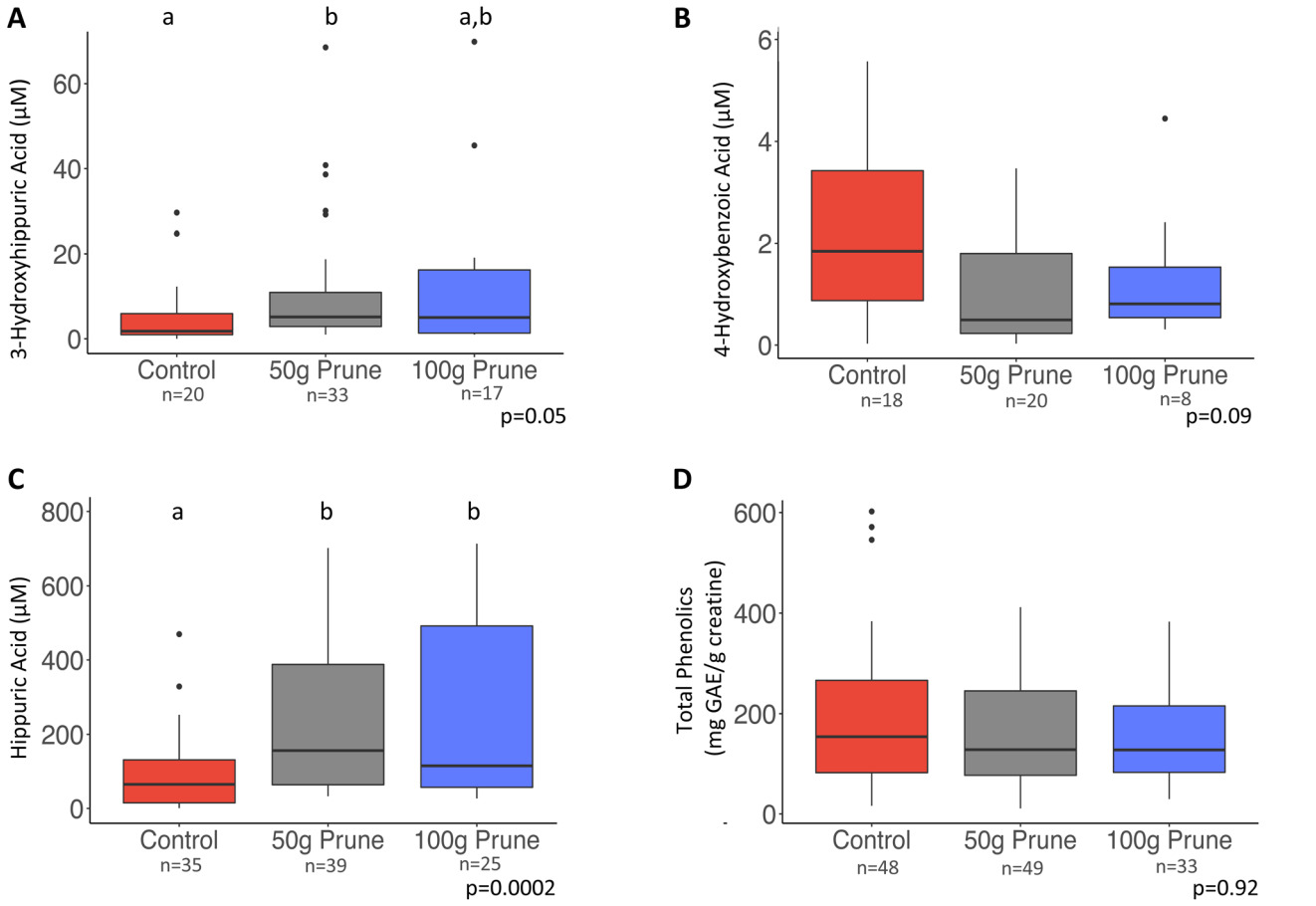

Fig. 4 Boxplots represent the medians and upper and lower quartile values of excreted urinary phenolics at week 52: (A) 3-hydroxyhippuric acid, (B) 4-hydroxybenzoic acid, (C) hippuric acid, and (D) total phenolics, Dots represent outliers. Overall comparison Kruskal–Wallis p-values are presented and if significant pairwise comparisons (pairwise Wilcoxon $p < 0.05$) were found, they are marked by different letters above boxes.

4. Discussion

Postmenopausal women consuming prunes for 12 months had statistically different gut microbiomes compared to controls. In particular, subjects consuming 50 g and 100 g prune per day over 12 months had different beta diversity compared to controls post-intervention, and these differences were driven by changing abundances of microbial taxa rather than selection for a substantially different set of taxa. This is not unexpected, because the macro- and micronutrients in prunes are not totally unique to prunes. At baseline, subjects were consuming sugars, fibers, and phenolics in other foods, and already possessed taxa that could utilize these compounds. However, prune consumption altered the relative amounts and composition of fibers and phenolics delivered to the gut, likewise altering the microbial community. This changing relative abundance (as opposed to selection for a new set of taxa) indicates that many postmenopausal women already harbor gut bacteria that can provide beneficial effects upon consuming prunes and can be enriched with prune consumption. Further, we noted that within-group dissimilarity decreased for the prune groups. This may indicate that prunes exert a selection pressure on the microbiome,[38,39] enriching specific taxa that metabolize prunes. These differences may be due in part to differential dropout rates among the treatment groups due to prune intolerance (Fig. S1‡), which may be influenced by microbiome makeup.[40] Prune intolerant microbiomes would not have dropped out of the control group. However, we observed differences in the 50 g prune group, in which only four out of 79 allocated participants dropped out due to prune intolerance. In addition, differences at the endpoint could be due to the prune supplement's effect on diet, rather than prunes themselves. All treatment groups consumed similar amounts of total fiber (Fig. 1) at week 52, indicating that prunes may have displaced other sources of fiber. While there were differences in the microbiomes among treatment groups at baseline, these differences were due to differences in presence of some taxa rather than relative abundances (as noted post-intervention). The differences at baseline are attributable to random chance, caused by differences in baseline diets, environments, and/or host factors.[37,41]

This study demonstrates that the addition of prunes to diets for 12 months has similar outcomes to other fruit intervention and microbiome studies. Other studies in fruits have

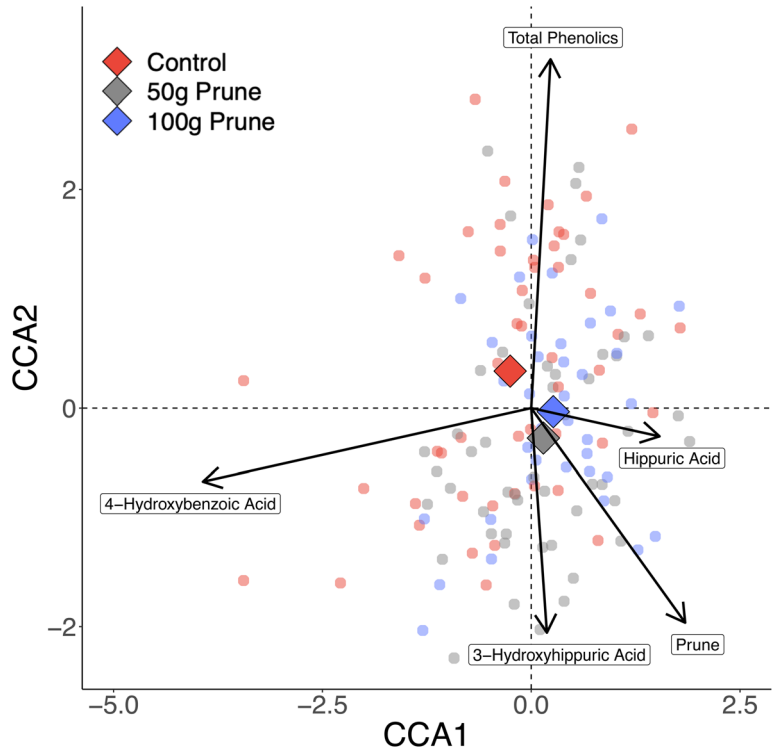

Fig. 5 Canonical Correspondence Analysis (CCA) of log transformed bacterial genera counts, prune treatment group and phenolic metabolite concentrations from compliant participants at week 52 (n = 52 control, 54 50 g prune, 37 100 g prune). Each dot represents a microbiome and the centroids for each treatment group are represented by diamonds. Each arrow shows direction and strength of associations with host variables. Samples clustered with arrows are associated with that host variable. The model was not significant (p = 0.375).

reported that phenolics and fibers alter the gut microbiome species composition, and select for commensal taxa (reviewed in ref. 42). In particular, phenolics enrich for *Bacteroides* and Lactobacillales, as noted here, and fiber increases Lactobacillales and decreases clostridia, also noted here.[43] However, these studies are usually short-term, acute interventions. In long-term studies, the gut microbiome may return to a baseline-like composition after three months, such as in a study of a low-carbohydrate and low-fat dietary interventions in humans.[44] In contrast, we observed sustained differences in the microbiome after 12 consecutive months of prune consumption. In addition, we identified significant correlations between select phenolic metabolites and a few taxa, but these associations were weak. This may be due to confounding variables, which are especially plentiful in human microbiome studies. However, upon testing multiple taxa using multiple linear regression, we were able to greatly improve the percent of variation in phenolic metabolites explained, indicating that phenolic metabolism is cooperative and influenced by multiple bacterial organisms. Associations may be due to phenolic metabolism by these organisms, as is likely the case for known phenol metabolizers such as *Eggerthella*,[45] or inhibition of their growth by phenolics. In addition, we were able to determine *via* CCA that prune treatment, total phenolics, and hippuric acid were associated with the gut fecal microbiome makeup. This finding is supported by the beta diversity differences between the prune groups, and the correlations between microbial taxa and phenolic metabolites. These results indicate that prune consumption and associated phenolics altered the microbial community, supporting other studies.[20]

The two prune doses of 50 g and 100 g exerted differential effects on the gut microbiome. Beta diversity analysis using Bray Curtis indicated there were differences in the overall community abundances between the 50 g and 100 g prune doses. There were different patterns of taxa enrichment and suppression at each dose, *i.e.*, some taxa were most abundant at 50 g, and lower in both control and 100 g, indicating that prune dose is a factor contributing to microbiome structure and composition. This was further supported by the lower evenness measure of alpha diversity in subjects consuming 50 g prune per day compared to the other groups. An important caveat to this finding is that the 50 g prune group had a slightly higher BMI, and that alpha diversity differed among the groups at baseline and remained different at 12 months. This has important implications for the dosing of dietary supplements and may partly explain why beneficial effects seen at one prune dose are not always seen at higher prune doses. Similarly, in an ovariectomized rat model of postmenopause, different doses of blueberry phenolics enriched different sets of taxa, and caused altered metabolism of dietary compounds.[46] The observed differences in taxa at each dose may be driven by inhibition of metabolism or cell division at high phenolic

Table 2 Multiple linear regression of excreted urinary phenolics as a function of the log counts of the eight most explanatory genera for each metabolite

Total urinary phenolics			
Multiple *R*-squared:	10%		
Adjusted *R*-squared:	8%		
Overall model *P*-value:	$\boldsymbol{1 \times 10^{-4}}$		
	Estimate	**Std. error**	***P*-Value**
Intercept	237.96	46.82	$\boldsymbol{7 \times 10^{-7}}$
Atopobium	−99.85	31.99	***0.002***
[Clostridium] innocuum group	−80.96	33.85	***0.017***
Lactobacillus	−13.15	5.65	***0.021***
Streptococcus	18.06	6.85	***0.009***
Clostridia vadin BB60 group	14.30	6.11	***0.020***
Agathobacter	−11.86	5.28	***0.025***
[Eubacterium xylanophilum] group	−13.39	5.36	***0.013***
Hippuric acid			
Multiple *R*-squared:	11%		
Adjusted *R*-squared:	8%		
Overall model *P*-value:	***0.001***		
	Estimate	**Std. error**	***P*-Value**
Intercept	412.83	368.16	0.263
Eggerthellaceae, unclassified	−181.74	96.44	*0.061*
Eggerthella	−110.19	46.74	***0.019***
[Eubacterium] ruminantium group	−130.51	41.79	***0.002***
Monoglobus	114.24	58.49	*0.052*
Oscillospiraceae, unclassified	−152.84	62.96	***0.016***
Peptococcaceae uncultured	161.50	79.46	***0.043***
Family XIII AD3011 group	98.65	56.41	*0.082*
3-Hydroxyhippuric acid			
Multiple *R*-squared:	32%		
Adjusted *R*-squared:	29%		
Overall model *P*-value:	$\boldsymbol{<2 \times 10^{-10}}$		
	Estimate	**Std. error**	***P*-Value**
Intercept	69.68	22.20	***0.002***
Paraprevotella	5.43	2.06	***0.009***
Erysipelatoclostridium	24.79	7.18	***0.001***
Lachnospiraceae UCG 004	5.06	3.12	0.108
[Eubacterium] eligens group	−6.18	2.13	***0.004***
Oscillospira	8.25	3.19	***0.010***
Ruminococcaceae *incertae sedis*	−9.46	3.84	***0.015***
[Clostridium] methylpentosum group	−6.00	3.43	*0.082*
Megamonas	28.20	6.01	$\boldsymbol{6 \times 10^{-6}}$
4-Hydroxybenzoic acid			
Multiple *R*-squared:	28%		
Adjusted *R*-squared:	23%		
Overall model *P*-value:	$\boldsymbol{<2 \times 10^{-6}}$		
	Estimate	**Std. error**	***P*-Value**
Intercept	13.47	3.46	$\boldsymbol{2 \times 10^{-4}}$
Gordonibacter	1.45	0.57	***0.012***
Erysipelatoclostridium	1.79	0.88	***0.044***
Gemella	−1.79	0.85	***0.036***
[Eubacterium] eligens group	−0.73	0.28	***0.010***
Ruminococcaceae *incertae sedis*	−1.31	0.62	***0.038***
Pygmaiobacter	−3.22	1.27	***0.012***
Megamonas	2.71	0.86	***0.002***
Ralstonia	−1.75	0.75	***0.020***

Estimate represents either the partial slope (β) or the value of the intercept. Std. Error is the standard error of the estimate. Data represents four separate models for each phenolic metabolite. These models used all 310 microbiome samples.

dosages,[47] but could also be due to altered food composition, altered nutrient availability, changes in other taxa altering community dynamics and competition pressure, altered interactions with the host GI tract and immune system, or prune-altered gut transit time.[2] Some combination of these effects are likely responsible for the distinct responses seen at the two different prune doses.

We detected many differentially abundant taxa between the prune groups that support other findings. A previous study has shown that prune juice decreased culturable *Clostridium perfingens*,[19] and we also observed that *Clostridium sensu stricto 1*, of which *C. perfingens* is a member, decreased in relative abundance in the prune groups. In fact, clostridia are generally decreased by fiber supplements.[43] However, we did not observe other differences noted in the prune juice study, such as decreased *E. coli* and increased *Lactobacillus* and *Bifidobacterium* among the prune groups, probably due to differences in the methods used to quantify bacteria (cultivation *vs.* 16S rRNA gene sequencing), as well as different subject demographics and their use of prune juice instead of whole prunes, which differ in fiber content.[19] Fiber can modulate the gut microbiome through selection for fiber-fermenting organisms, and fiber can be fermented by the gut microbiome to SCFAs.[15,48] A four-week study of whole prune consumption observed no differences between intervention groups (control, 80 g, 120 g prune) post-intervention in select fecal bacteria quantified by qPCR. We also did not observe significant differences for any of the taxa tested by those authors.[2] By using 16S rRNA gene amplicon sequencing we were able to get a more complete picture of bacterial taxa impacted by a long term prune intervention.

Lachnospiraceae is likely involved in part of the health effects of prunes. Prunes increased the relative abundance of Lachnospiraceae and genera within that family, such as *Blautia* and *Anaerostipes*. *Anaerostipes* has been previously reported to increase with phenolic-rich fruits, including after a 12-week chokeberry extract intervention in healthy adults.[49] Organisms in the family Lachnospiraceae are fiber fermenters and produce SCFAs, which have anti-inflammatory properties that may benefit the host.[50] Lachnospiraceae and *Blautia* correlated with urinary phenolic metabolites, and may be involved in their metabolism. Lachnospiraceae members have previously been shown to metabolize flavonoids, a class of phenolic compounds.[51] In addition, Lachnospiraceae UCG-001 (although not differentially abundant among the prune groups) was negatively correlated with IL-1β and IL-6, indicating a role for this family in immune signaling in the context of prune consumption. Previous studies have also demonstrated that Lachnospiraceae are potent immunomodulators, maintaining the gut epithelial barrier and likely exerting anti-inflammatory effects against IBD.[51] However, the role of Lachnospiraceae in human health is complex and the family has also been positively correlated with metabolic diseases, including diabetes and obesity.[51] In summary, the Lachnospiraceae is enriched by prunes may be involved in producing anti-inflammatory SCFAs and phenolic metabolites from prunes, and likely plays a role in immune regulation, mediating the health effects of prunes.

Limitations of this study include the presence of confounding variables, low power, and lack of funds to characterize

microbiome function. The gut microbiome is influenced by many variables, including diet, exercise, age,[37] BMI,[52] host genetics,[41,53] and more. Associations between prunes, phenolic metabolism, inflammatory markers, and the gut microbiome may be overshadowed by these confounding variables. Also, the phenolic quantification method utilized in this study was limited to 21 metabolites, chosen based on preliminary assessment of plum metabolism in rodents.[54] While these metabolites are representative of human metabolites and hippuric acid is a well-known marker of phenolic metabolism, it is likely that a broader targeted method may be able to detect additional metabolites of interest. In order to minimize the invasiveness of study procedures, we measured circulating inflammatory markers, some of which may be influenced by factors such as infection, as opposed to intestinal-specific inflammatory measures, such as gut permeability. Future research may be benefitted by incorporating intestinal measures of inflammation in order to develop a more complete understanding of the microbiome-mediated effects of prunes on inflammation. Because of the high dropout rate, confounding variables, and incredible variability of microbiomes, this study may be underpowered to identify more relationships between the gut microbiome, phenolic metabolism, and inflammatory markers. Power analyses have revealed that microbiome-focused intervention studies in human subjects may require several hundred subjects in each treatment group.[36] In fact, *post hoc* power analyses on this dataset (data not shown) indicate we only achieved 50% power and would need to double the number of subjects to achieve 80% power. Strict exclusion criteria for human microbiome studies to reduce uncontrolled variation may be worth considering, although this approach would severely limit generalization of results. In addition, because of the method used to characterize the gut microbiome, we were not able to determine functional differences between microbiomes, as might be possible using approaches such as RNAseq, metabolomics, proteomics, or enzyme assays. Functional and mechanistic studies are required to further define relationships between prunes, the gut microbiome, phenolic metabolites, and inflammatory markers.

5. Conclusions

Taken together, these results indicate that 12-months of daily prune consumption appears to exert beneficial impacts on the gut fecal microbiome of postmenopausal women, such as enriching bacteria in the family Lachnospiraceae. This is a group of known SCFA-producers that we found were associated with immunomodulatory effects and phenolic metabolism. The beta diversity analyses indicate the beneficial effects appear to result from a change in abundances of certain bacteria present in the microbiome. This makes the benefits associated with prune consumption accessible to many individuals, because the changes do not rely on acquiring a rare set of taxa. It is likely that there are many microbes that metabolize phenolics to beneficial metabolites, and prunes may help shift the microbiome towards a community that does so. This study indicates that long term prune consumption results in a sustained change in the gut fecal microbiome and phenolic metabolism associated with health benefits.

Author contributions

MJDS, CR, CHN, NIW, and MGF designed research; MJDS was PI for grant funding research; MGF and SC conducted phenolic metabolite analyses; JD and CR conducted inflammatory marker assays; AMRS and CHN conducted microbiome analyses. AMRS and CHN wrote manuscript. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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