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Effects of Adding Lean Red Meat to a U.S.-Style Healthy Vegetarian Dietary Pattern on Gut Microbiota and Cardiovascular Risk Factors in Young Adults: a Crossover Randomized Controlled Trial

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A B S T R A C T

Background: Limited research evidence exists on the effects of red meat on gut microbiota in human adults.
Objective: We aim to assess the effects of consuming a Healthy U.S.-Style Dietary Pattern (HDP), without or with unprocessed or processed lean red meats, on gut microbiota and fecal short-chain fatty acid (SCFA) levels in healthy young adults. Secondary outcomes are cardiovascular disease risk factors.
Methods: We conducted a randomized, controlled, crossover trial with 3 3-wk dietary interventions, each separated by a 5-wk washout period with habitual dietary intake. Nineteen participants (8 females, age 26 ± 4 y old, BMI 23 ± 3 kg/m^2) consumed 3 study diets in random order: 1) healthy lacto-ovo vegetarian diet (LOV); 2) LOV plus 3 ounces/d of cooked unprocessed lean red meat (URM); and 3) LOV plus 3 ounces/d of cooked processed lean red meat (PRM). Fecal and fasting blood samples were collected before and during the last 2 wk of each intervention. We measured fecal bacterial community structure using 16S rRNA amplicon sequencing (V4 region, primers 515F-806R). Community diversity, structure, and taxonomic composition were computed using Mothur v.1.44.3.
Results: The addition of unprocessed or processed lean red meats to a LOV HDP did not influence short-term changes in bacterial taxonomic composition. Independent of red meat intake, the HDP led to changes in 23 bacteria; reductions in serum total cholesterol (TC) and LDL-C concentrations; but no changes in fecal SCFA, serum triglycerides, HDL-C concentrations, TC/HDL-C ratio, or blood pressures. With data from all 3 diet interventions combined, changes in some bacteria were associated with improvements in TC, LDL-C, triglycerides, and HDL-C concentrations, and TC/HDL-C ratio.
Conclusions: Healthy young adults who adopt an HDP that may be vegetarian or omnivorous, including lean red meat, experience short-term changes in gut microbial composition, which associate with improvements in multiple lipid-related cardiovascular risk factors.
NCT03885544, https://clinicaltrials.gov/ct2/show/NCT03885544?cond=NCT03885544&draw=2&rank=1

Keywords: beef, pork, gut microbiome, healthy dietary pattern, healthy eating pattern, cardiometabolic risk, RCT, omnivorous diet, fecal short-chain fatty acid

Introduction

Red meat intake is inconsistently associated with an increased risk for cardiovascular diseases (CVD) [1]. Potential confounding factors contributing to such inconsistency include, but are not limited to, mixed red meat categorization (e.g., red meat vs. red and processed meat) [2], study designs (e.g., observational vs. experimental studies, mixed comparator food groups) [3], nutrient contents (e.g., fat and sodium), processing for food preservation, and method of cooking [1]. Accumulating evidence from randomized controlled feeding trials (RCTs) indicates beneficial effects of consuming a healthy dietary pattern,

Abbreviations: AMOVA, analysis of molecular variance; BCFA, branched-chain fatty acid; HEI, Healthy Eating Index; HOMOVA, homogeneity of molecular variance; LEfSe, linear discriminant analysis; LOV, lacto-ovo vegetarian; NDSR, Nutrition Data System for Research; OTUs, operational taxonomic units; PRM, lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat; RCTs, randomized controlled feeding trials; SCFA, short-chain fatty acid; URM, lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat.

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independent of the amount of unprocessed lean red meat, on CVD risk factors among older adults with elevated CVD risk [4,5]. However, it remains unclear how unprocessed versus processed lean red meats consumed in a healthy dietary pattern affects CVD risk factors among young adults without diagnosed disease.

Gut microbiota has emerged as a potential mediator between dietary intake and CVD risk [6–11]. Before starting the current study, an RCT in adults showed that consuming a primarily animal-based diet for 3 wk increased the abundance of gut bacteria associated with inflammatory bowel disease compared with a primarily plant-based diet [9]. Another RCT in adults comparing red meat and whole grain within a habitual diet showed that red meat intake reduced *Clostridium sp.* abundance; still, the health effects of such changes were unclear without being species-specific as the genus *Clostridium* contains a large group of species with inconsistent health effects [12]. Results of an RCT in infants indicated that consuming a high-protein meat-based diet beneficially shifted gut microbiota by increasing the abundance of *Clostridium* Group XIVa, a short-chain fatty acid (SCFA) producer [13]. Subsequent to starting this study, a systematic review of beef protein effects on the gut microbiota underscored the paucity of and need for research on red meat and gut microbiota in humans [14]. Thus, this study was conducted to assess the effects of adding unprocessed or processed lean red meats to a Healthy U.S.-Style Vegetarian Dietary Pattern on gut microbiota (primary), fecal short- and branched-chain fatty acids (primary), and CVD risk factors (secondary) in young adults without disease diagnosis. Using a crossover RCT with controlled feeding, we assessed the gut microbiota composition at community, operational taxonomic units (OTUs), and genus levels.

Methods

Ethics

Participants received monetary compensation for the completion of study procedures. The study was registered at ClinicalTrials.gov (NCT03885544) before participant recruitment for the cohort with 3 intervention arms. The study protocol and materials were approved by the Purdue University Biomedical Institutional Review Board (IRB protocol # 1709019738A003).

Participant recruitment

Young adults with no diagnosed diseases were recruited from the greater Lafayette area, Indiana, and tested between May 2019 and March 2020. Recruitment was completed after we met the anticipated number of participant enrollment ($N \geq 16$) with a 20% or less expected drop-out rate [5]. Twenty-four participants were recruited after trial registration (March 2019), and 12 completed 3 randomized controlled crossover intervention periods, specifically, the lacto-ovo vegetarian (LOV), the lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red (URM), and lacto-ovo vegetarian plus 3 oz/d of cooked processed red meat (PRM) diets. Among the other 12 participants who did not complete all 3 intervention periods, 5 participants dropped out, and 7 participants were required to discontinue the protocol at the onset of the COVID-19 pandemic in March 2020. These 7 participants completed 1 ($N = 2$) or 2 ($N = 5$) of the 3 intervention periods before March 2020, and their data were included in the final analysis.

The inclusion criteria were male or female, 20 to 35 y old, BMI of 20.0 to 29.9 kg/m^2, fasting serum TC concentration <240 mg/dL, low-density lipoprotein cholesterol concentration <160 mg/dL, triglycerides concentration <400 mg/dL, and glucose concentration <110 mg/dL, systolic/diastolic blood pressure <140/90 mmHg. Participants must have maintained a stable body weight (±3 kg) and physical activity regimen for 3 mo prior, stable medication use for 6 mo prior, as well as not to use medications or supplements (including probiotics, prebiotics, or antibiotics) known to impact gut function. Additional inclusion criteria were nonsmoking; not drinking more than 2 alcoholic drinks per day; nondiabetic; no history of gastrointestinal disorders, surgeries, or cancers; not pregnant and not lactating. Participants had to be willing and able to consume the prescribed diets (representing individuals with a habitual omnivorous diet). All participants attended an in-house health screening, and their profiles were reviewed by a clinical physician for approval to participate in the study based on the inclusion criteria.

Dietary intervention

In order to define the gut microbiota in humans specific to consuming red meat (beef and pork), we specifically used a LOV diet as the control diet for this study to avoid potential confounding from other muscle foods. The LOV diet followed a Healthy U.S.-style Vegetarian Dietary Pattern as recommended by the 2015–2020 Dietary Guidelines for Americans [15]. Both the URM and PRM diets were the same as the LOV diet, except that one 3-ounce portion of red meat (unprocessed or processed) per day, 7 d per wk (21 oz/wk), were also consumed. The URM diet included 1.5 oz/d of beef tenderloin and 1.5 oz/d of pork loin, defined by the American Meat Science Association as meats that "have not been significantly altered compositionally and contain no added ingredients, but may have been reduced in size by fabrication, mincing, grinding, and/or a meat recovery system" [16]; the PRM diet included 3 oz/d of processed beef and pork (i.e., combinations of deli roast beef, ham, corned beef, salami, bacon, or pastrami), which are defined by the 2015–2020 Dietary Guidelines for Americans as meats "preserved by smoking, curing, salting, and/or the addition of chemical preservatives" [15].

Participants were randomly assigned to consume 3 study diets (LOV, URM, and PRM), each for 3 wk, in a crossover order. Following a 5-wk preintervention baseline period, participants were randomly assigned to consume one of the study diets for 3 wk. After a 5-wk dietary 'washout' period, they consumed one of the alternative diets for 3 wk. After the second 5-wk dietary 'washout' period, they consumed the remaining alternative diet for 3 wk. All participants consumed their usual, unrestricted self-chosen diets during baseline and washout periods (Figure 1). Allocation of the order of consuming 3 study diets was conducted by the laboratory manager using an online randomization tool with random permutations of treatments (http://randomization.com). The study investigators were blinded to randomization codes throughout the study, sample processing, and data analysis. Participants were enrolled by the study investigators and assigned to interventions by the laboratory manager.

Participants were provided with all foods and only foods from the prescribed study diets, as well as 4-d-cycle menus with instructions for food preparation that they repeatedly used during each 3-wk controlled feeding period (Supplemental Tables S1A-D). Menus were developed by an experienced research dietitian

using Pronutra software (Viocare, Inc. Princeton, NJ). Each subject's energy requirement was estimated using sex-specific equations published by the Institute of Medicine [17]. The nutrient composition of study diets was analyzed by a research dietitian using the Nutrition Data System for Research (NDSR) Software Food and Nutrient Database [18]. The NDSR food groups were categorized and converted into Dietary Guidelines for Americans food groups based on the Healthy U.S.-Style Dietary Pattern [15]. The 2015 Healthy Eating Index (HEI-2015) scores were also calculated using NDSR outputs [19]. Study foods were picked up weekly by participants with a checklist at one of the designated local grocery stores with an online order placed and pre paid by the research team. Food scales and measuring cups, and spoons were provided to participants for food preparation at home. Adherence with the controlled diet interventions was promoted by using daily menu checkoff lists and frequent online and in-person contact between participants and the research team. Self-reported dietary adherence assessments were only based on the booklets that were available at the end of the study, excluding the booklets misplaced by research staff inadvertently or not completed and returned by participants (but reported consuming all foods via written or verbal communication). Dietary adherence was calculated based on (1) the percentage of prescribed menu items marked as consumed and (2) the percentage of unprescribed food items consumed as recorded on the checkoff lists.

Study visits

Before starting each intervention period, participants completed one 24-h dietary recall per day on 3 nonconsecutive days (2 weekdays and 1 weekend day) by phone with a research dietitian. Collected dietary records were validated on an individual basis using the age- and sex-specific Schofield equations [20] and the Goldberg 95% cut-offs [21]. Fecal samples and fasting-state blood samples were collected before and during the last 2 wk of the 3 3-wk dietary control periods, corresponding to study weeks 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, and 24 for participants consuming the 3 study diets. For female participants, samples were collected outside of their menstruation periods. The timing and testing during the baseline, intervention, and washout periods (Figure 1) were the same for the male and female participants. Therefore, before starting each diet (pre), blood and fecal samples were collected twice within 2 consecutive weeks (pre1 and pre2); during each intervention period (post), blood and fecal samples were collected once per week during the second week (post1) and the third week (post2).

Fecal sample collections

Whole fecal samples were collected by participants using specimen collection bowls with sealable lids. Gloves and transfer coolers were provided. Fecal samples were frozen immediately after collection at −20 °C and transferred on ice to the research laboratory by participants. Fecal samples were stored at −80 °C by the research team until analysis.

Bacterial 16S rRNA amplicon sequencing

Genomic DNA was extracted from fecal samples for analysis of gut microbiota composition using 16S rRNA sequencing. Before extraction, fecal samples were completely thawed at 4 °C overnight in closely sealed containers. Fecal bacterial DNA was extracted using the FastDNA SPIN Kit for Feces and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA), following manufacturer's instructions with modifications described by Yao et al. [22]. After extraction, the V4 region of the bacterial 16S rRNA was amplified using a dual-indexing approach (primer sets 515F-806R) as was described in the protocol by Kozich et al. [23] with modifications (sequences were prepared using KAPA HiFi HotStart ReadyMix-2x with 20 PCR cycles). Samples were sequenced using Illumina MiSeq 2x250 sequence platform at the Purdue Genomics Core, West Lafayette, IN.

Bacterial sequences were processed to improve sequence quality and prepared for downstream data analysis using the Mothur software package (version 1.44.3) following the Miseq Standard Operating Procedure (Miseq_SOP, https://mothur.org/wiki/miseq_sop/, most recently accessed in April 2022) (23). The Ribosomal Database Project (RDP) 16S rRNA database version 18 (https://mothur.org/wiki/rdp_reference_files/), and the SILVA reference alignment release 138 (https://mothur.org/wiki/silva_reference_files/) were used. Operational taxonomic units (OTUs) and phylotypes were generated using Mothur (version 1.44.3) for analyses of bacterial community composition, including alpha and beta diversity and bacterial abundances (see section Statistics).

Short- and branched-chain fatty acid extractions

SCFAs and branched-chain fatty acids (BCFAs) are produced from bacterial fermentation of indigestible carbohydrates and proteins. We extracted and analyzed SCFAs (acetate, propionate, and butyrate) and BCFAs (iso-butyrate and isovalerate) from fecal samples following a previously published protocol by Tucil et al. [24]. Specifically, frozen fecal samples were processed to extract approximately 0.2 g of frozen feces for SCFA and BCFA analysis. Each extraction of fecal sample was homogenized with 1000 μL of deionized water to make 5 times dilution, vortexed for 1 min, and centrifuged at 14,000 g for 10 min to remove cell debris. After centrifuging, 400 μL of supernatant was extracted and mixed with 100 μL of internal standard (4-methylvaleric acid, 85% phosphoric acid, and copper sulfate pentahydrate). Samples were saved at −80 °C and thawed for analysis on the same day. The SCFA and BCFA contents in supernatants were analyzed using gas chromatography (GC-FID 7890A, Santa Clara, CA) with injector temperature at 230 °C and oven temperature increasing from 100 °C to 200 °C. Each 25 mL of internal standard mixture was made with 157.5 μL of 4-metthyvaleric acid, 1.47 mL of 85% phosphoric acid, 39 mg copper (II) sulfate pentahydrate, and deionized water. Dilutions of acetic acid, propionic acid, butyric acid, iso-butyric acid, and isovaleric acid were used as external standards to construct standard curves. Samples were analyzed in triplicates, and averaged values were used for final analysis. After obtaining area under the curve data for external standards and samples, the concentration (mM/mL) of each SCFA and BCFA was calculated using standard curves. The fecal SCFA and BCFA concentrations (mM/g) were multiplied by a dilution factor of 1.25 (derived from the protocol) and divided by extracted frozen fecal weight.

Measurements of anthropometric parameters and cardiovascular diseases risk factors

Participants were scheduled for a 1-h in-house testing appointment between 7:00 am to 10:00 am on each test day.

Before each appointment, participants fasted for at least 10 h, during which only water consumption was allowed. Each participant's fasting-state body weight was measured using the same digital platform scale (model ES200L, Ohaus Corporation, Pine Brook, NJ) throughout the study. BMI (kg/m^2) was calculated from standing height and fasting-state body mass. Waist and hip circumferences were measured at the smallest point around the waist and the widest point around the hip, respectively. Waist-to-hip circumference ratio was calculated by dividing waist circumference by hip circumference. Sagittal abdominal diameter was measured between the lower back to the highest point of the abdomen in supine positions.

At each testing appointment, fasting blood samples were collected from an antecubital vein by a phlebotomist using standard clinical procedures. Blood samples were collected into serum separator tubes and centrifuged for 15 min at 4400 rpm and 4 °C. Separated fresh serum samples were shipped to the Mid America Clinical Laboratories on the same day (Indianapolis, IN) for analyses of lipid-lipoprotein profile (TC, triglycerides, HDL cholesterol, LDL cholesterol, and glucose concentrations) and comprehensive metabolic panel (e.g., kidney and liver functions, glucose and electrolytes concentrations). LDL cholesterol was calculated using the following equation: LDL cholesterol = TC – [HDL cholesterol + (triacylglycerol/5)]. The remaining serum and plasma samples were saved in aliquots at −80 °C. After participants rested in chairs for 15 min, fasting-state blood pressure in supine positions was measured manually 2 times and averaged (BP785, HEM-7222-Z, Omron Healthcare, Inc.).

Statistics

Reporting of this research followed the CONSORT reporting guidelines [25]. Our primary outcomes are the gut microbiota composition and fecal SCFAs and BCFAs. Secondary outcomes are cardiovascular disease risk factors. As reported in our trial registration (ClinicalTrials.gov, NCT03885544), we initially started the study with hypotheses about changes in specific bacterial groups (e.g., *Lactobacillus*, *Clostridium* XIVa, and *Erysipelotrichaceae*). The study sample size was conservatively determined based on previous research that compared a high-protein red meat-based diet versus a low-protein cereal-based diet in infants [13]. Sample sizes of 16 and 12 will provide 95% and 80% power, respectively, to detect a 20% between-group difference in changes of *Clostridium* XIVa relative abundances with 15% pooled SD (significance level at 5%). However, given the largely exploratory and multifaceted nature of gut microbiota outcomes (because of reasons such as a lack of prior research data and limitations in research reproducibility across different studies), a meaningful a priori power calculation was challenging. With the above considerations, instead of testing a priori hypothesis with a power-calculated sample size based on a selected bacterial abundance, we assessed the effects of consuming the LOV, URM, and PRM diets on changes in the overall gut microbiota composition at community and taxonomic levels.

Therefore, we conducted statistical analyses to measure: 1) the effects of adopting a prescribed diet (post versus pre); 2) the effects of consuming the LOV diet with or without unprocessed or processed lean red meats (post versus pre within the LOV, URM, and PRM diets), 3) any difference among the LOV, URM, and PRM diets using post-pre changes, post/pre fold changes, or cross-sectional post values; 4) any difference between pre1 and pre2 samples to determine if baseline data were reproduced between duplicate collections; 5) any difference between post1 and post2 samples to assess if changes occurred between the second and the third week of dietary intervention.

All data were checked for normality assumptions using Shapiro-Wilk tests and analyzed using either paired t-test or repeated-measures ANOVA when normality was achieved (with Box Cox transformation as appropriate) or using Kruskal-Wallis or pairwise Wilcoxon rank sum tests when normality was not achieved. Statistical outputs and figures were generated using Mothur (version 1.44.3), statistical software RStudio (version 2022.02.0+443), SAS (version 9.4), and GraphPad Prism (version 9).

The gut microbiota composition was analyzed using OTU- and phylotype-based taxonomic approaches. At the community level, alpha diversity for within-sample diversity (Chao1, ACE, Shannon, and Inverse Simpson indexes) and beta diversity matrices (Bray-Curtis, Jaccard, and Theta YC matrices) were calculated and compared between study timepoints and between study diets. For alpha diversity, Chao1 and abundance-based coverage estimator (ACE) richness indexes calculate the number of species, and Shannon and Inverse Simpson diversity indexes calculate both the richness and abundance of species. For beta diversity, which measures between-group differences, Bray-Curtis uses abundance, Jaccard uses richness, and Theta YC uses both abundance and richness. Using a combination of diversity measures provides a more comprehensive look into the gut microbiota composition at the community level. Data were then analyzed using the analysis of molecular variance (AMOVA) to assess the separation between the cloud clustering centroids of groups compared with within-group variation using beta diversity distance matrices. Differences between within-group variations were assessed using the homogeneity of molecular variance (HOMOVA). Next, at the taxonomic and OTU levels, the nonparametric linear discriminant analysis (LEfSe) with LDA effect sizes [26] was conducted to determine bacterial genera and OTUs that were differentially represented between groups. Zero-adjusted post/pre fold changes (delta values) of selected genera and OTUs were compared among study diets using Kruskal-Wallis or pairwise Wilcoxon rank sum tests. Data on gut microbiota composition were presented as means ± SD, with Benjamin & Hochberg adjusted p values for multiple comparisons and adjusted threshold of $p < 0.05$ for a 95% confidence level. It should be noted that the current names of 4 bacterial phyla have been updated with new names during the conduction of the study, including Firmicutes (now Bacillota), Proteobacteria (now Pseudomonadota), Actinobacteria (now Actinomycetota), and Bacteroidetes (now Bacteroidota) [27]. The names before updates were used in this study.

Data on SCFAs, BCFAs, and CVD risk factors were analyzed using SAS PROC MIXED procedure with 2-way ANOVA to assess the main effects of time and time by diet interaction. Averages of pre versus post values were used as no difference was detected between pre1 and pre2 or between post1 and post2 data, respectively. Data were adjusted for age, sex, and BMI and presented as adjusted least squares means ± standard errors (LS mean ± SE). Tukey-Kramer adjusted p values < 0.05 were considered to be statistically significant at a 95% confidence level. Missing data were accounted for using maximum likelihood in the SAS PROC MIXED procedure [28]. Additionally,

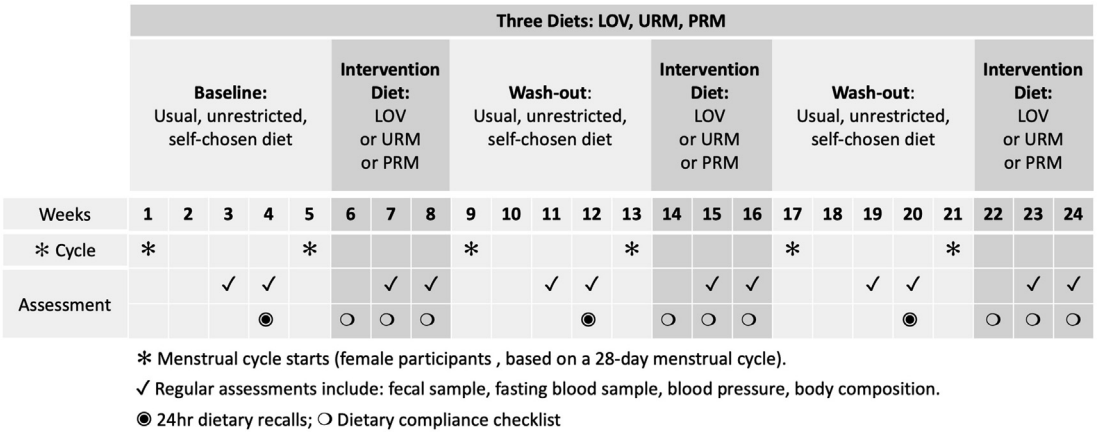

FIGURE 1. The schematics of study design and measurements. LOV: lacto-ovo vegetarian; URM: lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat; PRM: lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat.

Spearman's rank order correlations with Benjamin & Hochberg adjusted p values ($p < 0.05$) were conducted between bacterial outcomes (alpha diversity and significant OTUs or genera) and other health outcomes (SCFAs, BCFAs, and CVD risk factors).

Results

Participant characteristics

Details of the study enrollment and conduction are described in Figure 2. Briefly, a total of 24 young and healthy participants (10 female [F] and 14 male [M]) were recruited and randomly assigned to the study to consume 3 study diets (LOV+URM+PRM). Five of the 24 participants dropped out of the study due to an inability to comply with the study sample collection schedule (1F and 1M) or to make the time commitment for the study (1F and 2M). Data from 19 participants (8F and 11M) who completed at least 1 of the 3 study diets were included in the final analysis. The baseline characteristics of all participants before random assignment are described in Table 1.

Dietary intakes

The nutrient composition of the unprocessed and processed lean red meats and the 3 study diets prescribed to participants are presented in Supplemental Table S2. Servings of Dietary Guidelines for Americans food groups consumed with the 3 study diets followed the 2015–2020 Dietary Guidelines for Americans recommendations (Supplemental Table S3). The 2015-HEI scores were 81 for the LOV diet, 82 for the URM diet, and 76 for the PRM diet, compared with an average score of 57 for participants' habitual diet at the first baseline (Supplemental

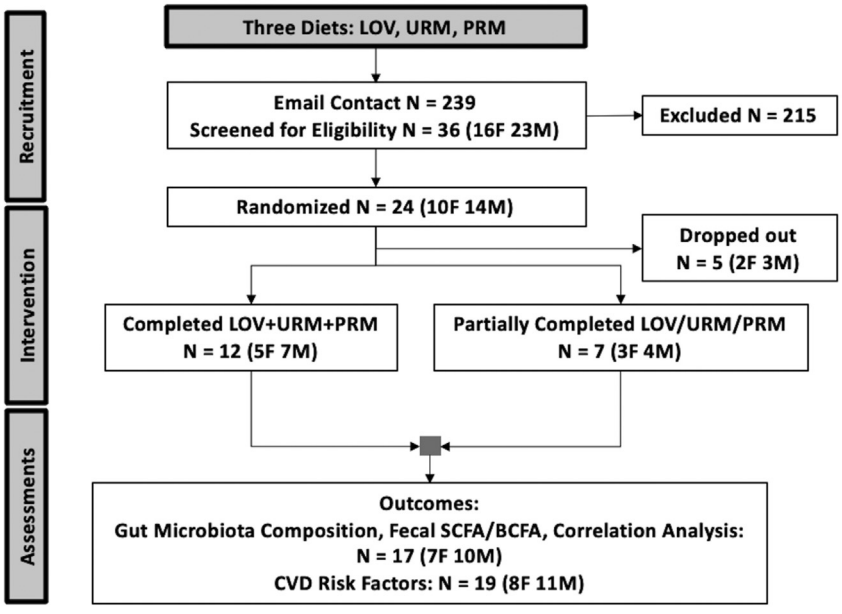

FIGURE 2. The CONSORT flow diagram of study enrollment. Participants with partial completion were discontinued in March 2020 due to the COVID-19 restrictions. BCFA, branched-chain fatty acid; F, female; LOV, lacto-ovo vegetarian; M, male; N, sample size; PRM, lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat; SCFA, short-chain fatty acid; URM, lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat.

TABLE 1
Characteristics of all participants at the first baseline

Outcomes	Gut microbiota, SCFA, and correlational analyses	CVD risk factors
Anthropometrics		
Sample size (N, F/M)	N = 17 (7/10)	N = 19 (8/11)
Age (y)	26 ± 4 (20–35)	26 ± 4 (20–35)
Weight (kg)	69 ± 15 (48–100)	69 ± 15 (48–100)
BMI (kg/m^2)	23 ± 3 (20–30)	23 ± 3 (20–30)
Waist-to-hip circumference ratio	0.87 ± 0.05 (0.81–0.96)	0.87 ± 0.05 (0.79–0.96)
Sagittal abdominal diameter (cm)	19.0 ± 2.4 (15.6–25.8)	19.0 ± 2.6 (15.6–25.8)
Systolic blood pressure (mm Hg)	109 ± 9.8 (89–121)	109 ± 10.1 (89–121)
Diastolic blood pressure (mm Hg)	68 ± 5.6 (59–80)	68 ± 6.1 (57–80)
Serum biomarkers		
Total cholesterol (mg/dL)	164 ± 23.9 (121–206)	164 ± 27.1 (121–213)
Triglycerides (mg/dL)	83 ± 32.5 (36–182)	81 ± 31.1 (36–182)
HDL-C (mg/dL)	53 ± 9.2 (40–77)	54 ± 12.1 (40–86)
LDL-C (mg/dL)	94 ± 23.8 (43–142)	94 ± 23.7 (43–142)
Total cholesterol/HDL-C ratio	3.2 ± 0.7 (2.2–4.9)	3.2 ± 0.7 (2.2–4.9)
Glucose (mg/dL)	86.2 ± 5.6 (75–94)	86.7 ± 5.8 (75–97)
Blood urea nitrogen (mg/dL)	13.1 ± 2.8 (8.5–18.5)	13.1 ± 2.7 (8.5–18.5)
Creatinine (mg/dL)	0.9 ± 0.1 (0.7–1.2)	0.9 ± 0.1 (0.7–1.2)

Notes: Results are presented as mean ± SD. Abbreviations: F, female; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; M, male; SCFA, short-chain fatty acids.

Table S3). As indicated by the 2015-HEI scores, our study diets effectively switched our participants from consuming a less healthy, averaged Western-style dietary pattern to a healthier dietary pattern. The average self-reported dietary adherence for the 3 study diets based on completed menu checkoff lists from 10 participants was 95.2%, which was comparable across the 3 study diets (LOV: 96.2%; URM: 94.8%; PRM: 94.3%). Not including plain black coffee and black tea, one of the 10 participants reported consuming unprescribed food items (2 times over the intervention period), and 3 participants consumed prescribed foods in extra amounts (median frequency, 4 times over the intervention period). Body weight did not change over time for any of the 3 intervention periods (mean change between post and pre ± SD; LOV: -0.9 to ± 0.9 kg, N = 15; URM: -0.7 to ± 1.0 kg, N = 15; PRM: -0.6 to ± 0.9 kg, N = 18; ANOVA $P = 0.6$).

Gut microbial composition

16S rRNA amplicon sequencing. A total of 8,991,945 paired-end sequences merged in fastq files were included in the Mothur pipeline for quality screening of 175 fecal samples with technical and biological replicates from 17 participants. After sequence quality improvement, alignment, and processing, 70,714 unique sequences and a median length of 253 base pairs were used to prepare for OTU- and phylotype-based analyses. The OTU- and phylotype-based approaches classified sequences into 5,779 and 292 OTUs, respectively. A subsampling depth of 3,296 reads was chosen to rarefy and retain all viable samples to be included in the final analysis, leading to 2,152 OTUs for the OTU-based analysis. All sample coverages were above 97%, indicating effective sampling of the bacterial communities. Rarefaction curves generated from the OTUs are provided in Supplemental Figure S1.

Community level characteristics: consuming a healthy dietary pattern for 3 wk did not affect the overall gut microbiota structure. We first compared gut microbiota composition at the community level using alpha diversity measures for 1) pre versus post within each study diet, 2) among the 3 study diets at post, 3) changes from pre to post among the 3 study diets, 4) pre versus post with the 3 study diets combined, 5) among the 3 study diets at pre, 6) pre1 versus pre2 versus post1 versus post2 within each study diet, and 7) pre1 versus pre2 versus post1 versus post2 with the 3 study diets combined. The distributions of all 4 alpha diversity data did not follow a normality assumption (Shapiro-Wilk tests $P < 0.05$). No statistically significant difference for any of the comparisons was found using the 4 alpha diversity measures, Chao1, ACE, Shannon, and Inverse Simpson indexes (Supplemental Tables S4A-B).

Next, we assessed the between-group difference using beta diversity measures for the same comparisons, except for post-pre changes due to the high-dimensional nature of beta diversity. The Bray-Curtis diversity matrix was visualized using two-dimensional Principal Coordinates Analysis plots (Supplemental Figures S2-S4). Similarly, no statistically significant between-group difference was detected in the centroids and variations of group clustering (using AMOVA and HOMOVA, respectively) between pre and post within and among the 3 study diets, based on the 3 beta diversity measures, Bray-Curtis, Jaccard, and Theta YC matrices. (Supplemental Tables S5A-E). Therefore, adopting a healthy dietary pattern for 3 wk, independent of red meat, did not affect the gut microbiota structure at the community level.

Taxonomic- and OTU-level characteristics: adopting a healthy dietary pattern shifted the abundance of some genera and OTUs of gut microbiota, with changes specific to unprocessed or processed lean red meat intakes. Although we did not detect a global shift in gut microbiota community structure after adopting the study diets, we conducted analyses at the taxonomic and OTU levels to further identify bacterial at the genera and OTU levels that were differentially represented within comparison groups for (1) pre versus post within each study diet, (2) among the 3 study diets at post, (3) fold changes of post/pre among the 3 study diets, (4) pre versus post with the 3 study diets combined, and (5) among the 3 study diets at pre. A total of 292 genera based on phylotypes and 2152 OTUs were included for LEfSe analysis. The gut microbiota community composition at the genus level for

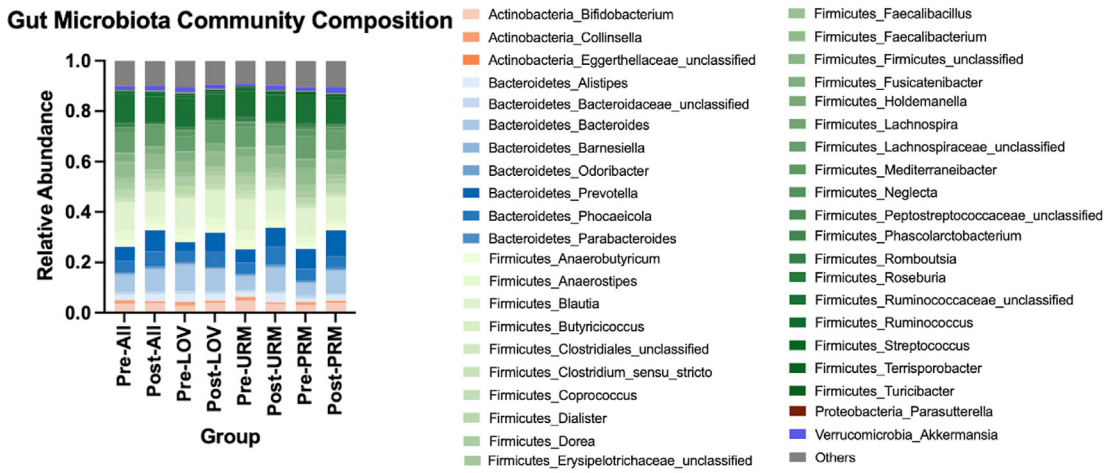

FIGURE 3. Gut microbiota community composition at the genus level for pre and postgroups with the 3 study diets combined and within each study diet. A total of 112 OTUs with at least 0.1% of abundance, representing 41 genera, are included and colored by phylum groups. The rest of OTUs with abundance lower than 0.1% are categorized into "Others". LOV, lacto-ovo vegetarian; OTU, operational taxonomic unit; PRM, lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat; URM, lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat.

TABLE 2
Total numbers of genera and OTUs.

Level	Genera		OTUs	
	Total	RA>=0.1%	Total	RA>=0.1%
Mothur output	292	49	2,152	90
LEfSe-Identified	32	20	55	42

Notes: LEfSe, nonparametric linear discriminant analysis; OTU, operational taxonomic unit; RA, relative abundance.

different groups is presented in Figure 3, Supplemental Tables S6, and Supplemental Figure S5. A total of 32 genera and 55 OTUs differed in at least one of the comparisons (Supplemental Tables S7 and S8). The LDA scores from LEfSe analyses at all taxonomic levels are visualized in Supplemental Figures S6–S8. To further identify OTUs with biological relevance, we included bacteria with at least 0.1% of relative abundance. Therefore, 20 genera and 42 OTUs were considered biologically relevant and included for interpretation (Table 2, Supplemental Tables S7–S8).

Overall, 3 wk of healthy dietary pattern consumption provided sufficient time for changes in gut microbiota to occur and converge to comparable composition compared to habitual intake. There were changes specific to the addition of unprocessed or processed lean red meats, as well as changes induced by the healthy dietary pattern independent of red meat intake (Table 3). In particular, genera *Collinsella* and *Mediterraneibacter* decreased within each study diet and with the 3 study diets combined. Genus *Roseburia* increased in the LOV diet, but not in the URM or PRM diets. Genus *Romboutsia* decreased in the LOV and URM diets and with the 3 study diets combined, but not in the PRM diet. Genus *Dorea* decreased in the URM and PRM diets and with the 3 study diets combined, but not in the LOV diet. Statistical test results of post/pre fold changes among the 3 study diets are presented in Supplemental Tables S9-S10. The post/pre fold changes were not different among the 3 study diets for any of the

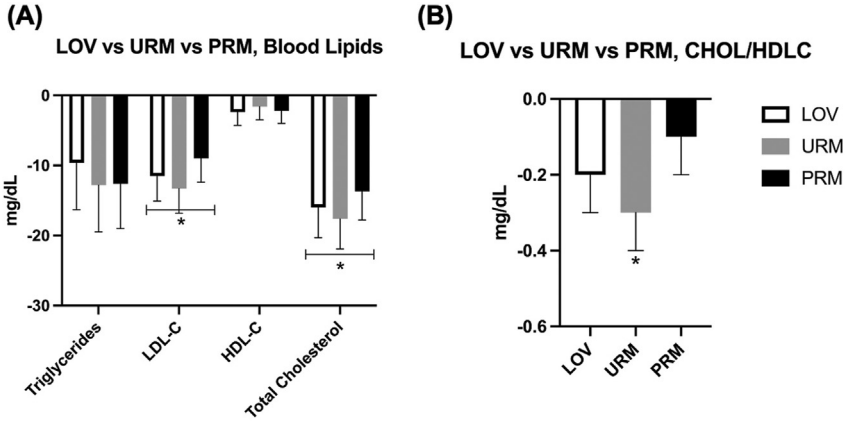

FIGURE 4. Comparisons of post-pre change values from blood CVD risk factors among the LOV (N = 15), URM (N = 15), and PRM diets (N = 18; A-B) [*change over time ($P < 0.05$); no difference between diet groups was detected (diet*time effect, $P > 0.05$); results presented as LS Means ± SE]. CHOL/HDL-C, total cholesterol to HDL-C ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LOV, lacto-ovo vegetarian; PRM, lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat; CHOL/HDL-C, total cholesterol to HDL-C ratio; URM, lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat.

TABLE 3
Bacterial genera and OTUs with significant changes or differences by study diets and associations with cardiovascular risk factors.

Comparison group	Increased over time	Association with CVD risk factors, r value (adjuted p value)					Decreased over time	Association with CVD risk factors, r value (adjuted p value)				
		TAG	LDL-C	TC	TC/ HDL-C	HDL-C		TAG	LDL-C	TC	TC/ HDL-C	HDL-C
LOV- Post vs Pre	Genus - *Roseburia*	0.11 (0.82)	−0.54* (0.001)	−0.51* (0.002)	−0.31 (0.26)	−0.05 (0.86)	[NS]					
	[NS]						Genera – *Romboutsia*, *Collinsella*, and *Mediterraneibacter*					
	OTU0071 - *Ruminococcaceae_unclassified*						OTU0035 - *Romboutsia*					
	OTU0066 - *Roseburia*						OTU0074 - *Firmicutes_unclassified*					
							OTU0088 - *Peptostreptococcaceae_unclassified*					
							OTU0026 - *Mediterraneibacter*					
URM- Post vs. Pre	[NS]						[NS]					
	Genera - *Lachnospira* and *Parabacteroides*						Genus – *Romboutsia*, *Collinsella*, *Mediterraneibacter*, and *Dorea*					
	OTU0076 - *Lachnospira*						OTU0008 - *Fusicatenibacter*					
							OTU0017 - *Dorea*					
							OTU0026 - *Mediterraneibacter*					
							OTU0035 - *Romboutsia*					
							OTU0057 - *Dorea*					
PRM- Post vs. Pre	[NS]						[NS]					
	OTU0058 - *Parabacteroides*						Genera - *Collinsella*, *Mediterraneibacter*, *Dorea*, and *Turicibacter*					
							OTU0017 - *Dorea*					
							OTU0026 - *Mediterraneibacter*					
							OTU0032 - *Mediterraneibacter*					
							OTU0056 - *Roseburia*					
Healthy dietary pattern (Three diets combined) – Post vs. Pre	Genus - *Coprococcus*	−0.02 (0.99)	−0.27* (0.007)	−0.26* (0.009)	−0.26* (0.009)	0.03 (0.98)	Genus - *Romboutsia*	−0.03 (0.89)	0.24* (0.01)	0.20* (0.03)	0.13 (0.16)	0.03 (0.87)
	OTU0034 - *Lachnospiraceae_unclassified*	0.13 (0.16)	−0.14 (0.15)	−0.17 (0.07)	0.02 (0.93)	−0.20* (0.03)	OTU0006 - *Blautia*	−0.18* (0.046)	−0.24* (0.008)	−0.22* (0.02)	−0.19* (0.04)	0.15 (0.11)
	OTU0061 - *Lachnospiraceae_unclassified*	−0.04 (0.80)	−0.17 (0.07)	−0.15 (0.12)	−0.19* (0.04)	−0.01 (0.94)	OTU0025 - *Collinsella*	−0.01 (0.94)	0.25* (0.007)	0.23* (0.01)	0.20* (0.03)	0.05 (0.76)
	[NS]						OTU0035 - *Romboutsia*	−0.03 (0.89)	0.24* (0.01)	0.20* (0.03)	0.13 (0.16)	0.03 (0.87)
	Genera - *Ruminococcus* and *Lachnospira*						OTU0037 - *Lachnospiraceae_unclassified*	−0.23* (0.01)	−0.13 (0.16)	-0.01 (0.94)	−0.27* (0.004)	0.39 (8E-06)
	OTU0043 - *Ruminococcaceae_unclassified*											
	OTU0058 - *Parabacteroides*											
	OTU0066 - *Roseburia*						OTU0057 - *Dorea*	0.07 (0.53)	0.24* (0.01)	0.24* (0.01)	0.18* (0.04)	0.01 (0.98)
	OTU0076 - *Lachnospira*						OTU0088 - *Peptostreptococcaceae_unclassified*	−0.26* (0.004)	−0.15 (0.12)	-0.16 (0.09)	−0.21* (0.02)	0.18* (0.045)
							[NS]					
							Genera - *Collinsella*, *Mediterraneibacter*, *Dorea*, and *Turicibacter*					
							OTU0017 - *Dorea*					
							OTU0026 - *Mediterraneibacter*					
							OTU0032 - *Mediterraneibacter*					

(*continued on next page*)

TABLE 3 (*continued*)

Comparison group	Increased over time	Association with CVD risk factors, r value (adjuted p value)					Decreased over time	Association with CVD risk factors, r value (adjuted p value)				
		TAG	LDL-C	TC	TC/ HDL-C	HDL-C		TAG	LDL-C	TC	TC/ HDL-C	HDL-C
At Post	OTU0056 - *Roseburia* (higher in URM than LOV)	−0.11 (0.54)	−0.32* (0.04)	−0.29* (0.04)	−0.25 (0.09)	0.02 (0.95)	[NS]					
	OTU0063 - *Parabacteroides* (higher in URM than LOV)	0.29* (0.04)	0.09 (0.61)	0.06 (0.77)	0.34* (0.03)	−0.29* (0.04)	Genus - *Anaerostipes* (higher in LOV than PRM or URM)					
							OTU0010 - *Anaerostipes* (higher in LOV than PRM or URM)					
At Pre	OTU0069 - *Ruminococcus* (higher in PRM than URM)	0.12 (0.46)	0.32* (0.02)	0.21 (0.14)	0.29* (0.04)	−0.21 (0.14)	[NS]					
	[NS]						Genus - *Firmicutes_unclassified* (higher in LOV than PRM)					
	Genera – *Roseburia* and *Lachnospiraceae_unclassified* (higher in PRM than LOV or URM)						Genus - *Bacteroides* (higher in LOV than URM)					
	Genus - *Bifidobacterium* (higher in URM than LOV or PRM)						Genus - *Clostridiales_Incertae_Sedis_XIII_unclassified* (higher in LOV than URM)					
	OTU0007 - *Bifidobacterium* (higher in URM than PRM or LOV)											
	OTU0045 - *Ruminococcus* (higher in PRM than URM)											
	OTU0056 - *Roseburia* (higher in PRM than PRM or LOV)											

Notes: Correlation analyses were conducted using the corresponding subset of data from the LOV diet, URM diet, PRM diet, and healthy dietary pattern with the 3 study diets combined (preintervention and postintervention), data from preintervention timepoint with the 3 study diets combined, and data from postintervention timepoint with the 3 study diets combined. The number of tests controlled by Benjamin & Hochberg adjustment for correlation analyses was 80 between selected genera and CVD risk factors and 120 between selected OTUs and CVD risk factors.
Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LOV, lacto-ovo vegetarian; OTU, operational taxonomic unit; PRM, lacto-ovo vegetarian plus 3 oz/d of cooked processed lean red meat; TAG, triglycerides; TC, total cholesterol; TC/HDL-C, total cholesterol to HDL-C ratio; URM, lacto-ovo vegetarian plus 3 oz/d of cooked unprocessed lean red meat.

* Significant association; NS, no significant associations were detected between the genus or OTU with any of the selected CVD risk factors.

identified genera or OTUs with biological relevance (Kruskal-Wallis or pairwise Wilcoxon rank sum tests $P > 0.05$).

Notably, more genera and OTUs differed at pre when participants were on their habitual diets than at post after receiving 3-wk dietary control. Great individuality was observed among participants in post/pre fold changes within the 3 study diets for genera with some extreme values (Supplemental Figure S9) and OTUs (Supplemental Figure S10). Such individuality was also observed in individual shifts of gut microbiota community over time from pre1 to post2 using Bray-Curtis 2-dimensional Principal Coordinates Analysis plots (Supplemental Figure S11).

Fecal SCFA and BCFA levels were not affected by our 3-wk dietary interventions

No difference in fecal SCFA and BCFA levels was found between pre and post within each study diet or with the 3 study diets combined (Supplemental Table S11). Neither was any difference found among diets at pre or at post in total fecal fatty acids, total SCFA, and any SCFA levels. The PRM diet led to higher total BCFA, isobutyrate, and isovalerate levels than the LOV and URM diets at post, though the post-pre change was not significant. Elevated fecal BCFA levels are indicative of increased proteolysis from branched-chain amino acids (e.g., valine, leucine) in the human colon. Unadjusted raw means and SD of SCFA results are presented in Supplemental Table S12.

Adopting a healthy dietary pattern improved blood lipid profile, which was not influenced by consumption of lean red meat

As a main effect of time, consuming all study diets reduced blood TC and LDL-C concentrations (time, $P < 0.05$, Figure 4A). CHOL/HDL ratio was reduced by consuming the URM diet ($P < 0.05$), but not by the LOV or PRM diets (Figure 4B). Waist-to-hip circumference ratio, sagittal abdominal diameter, systolic and diastolic blood pressure levels, or blood glucose and triglycerides concentrations did not change over time between pre and post on any study diet (Supplemental Table S13). No difference in post-pre change among the 3 study diets was observed (time by diet, $P > 0.05$). When red meat was consumed, the URM and PRM diets led to higher blood urea nitrogen concentrations than the LOV diet (time by diet, $P = 0.04$). The clinically normal range identified by Mid America Clinical Laboratories (Indianapolis, IN) was 10 to 20 mg/dL. The group mean blood urea nitrogen concentrations before and after each study diet were within the clinically normal range. The adjusted LS means with SE and raw (unadjusted) means with SD for CVD outcomes at pre and post for each study diet are presented in Supplemental Tables S13 and S14, respectively. Overall, adopting a healthy dietary pattern independent of red meat improved CVD risk factors. Adding unprocessed or processed lean red meats did not influence the improvements induced by the healthy dietary pattern.

Correlation analyses

Results of correlation analyses suggested associations between selected OTUs or genera and other health indexes, including anthropometric, CVD, and SCFA outcomes (Supplemental Figures S12-S15). As reductions by study diets were observed in TC and LDL-C concentrations and CHOL/HDL-C ratio, associations between selected bacterial genera and blood lipids are emphasized. At the first baseline (Supplemental Figures S12-S13), no association between bacteria and blood lipids was found. In contrast, based on the overall correlation analysis with data from the 3 study diets combined (Supplemental Figures S14-S15), genus *Bacteroides* was negatively associated with LDL-C concentration. Genera *Roseburia* and *Coprococcus* were negatively associated with TC and LDL-C concentrations and CHOL/HDL-C ratio. On the contrary, genus *Romboutsia* was positively associated with TC and LDL-C concentrations, and *Clostridiales_Incertae_Sedis_XIII_unclassified* was also positively associated with TC concentration.

Group-specific correction analyses were also conducted between genera or OTUs that were impacted by diet and CVD outcomes using data from each study diet (preintervention and postintervention), data from preintervention with the 3 study diets combined, and data from postintervention with the 3 study diets combined (Table 3). Within each study diet, the LOV diet increased *Roseburia* abundance, which was associated with lower TC and LDL-C concentrations; changes in bacterial abundances in the URM and PRM diets were not associated with the selected CVD outcomes.

Discussion

Our study uniquely assesses the effects of consuming unprocessed or processed lean red meats within a Healthy U.S.-Style Dietary Pattern on gut microbiota and CVD risk factors. The basal diet for this study is the Healthy U.S.-Style Vegetarian Dietary Pattern as recommended by the 2015–2020 Dietary Guidelines for Americans [15]. Importantly, participants consumed unprocessed or processed lean red meats in addition to the basal diet without substitution of other foods. The nonmeat and nonnutrient dietary components in a healthy dietary pattern have considerable contributions to the dietary effects on gut microbiota and CVD risk [29–31]. This study design provides a novel opportunity to assess the effect of red meat on gut microbiota without being confounded by other sources of flesh foods or altering the amounts and sources of the basal diet.

Our results showed that independent of lean red meat, the healthy dietary pattern changed gut microbiota profile, including reductions in *Collinsella* and *Mediterraneibacter* abundances which were also observed within each study diet. Although abundances of multiple bacteria changed over time within each study diet, we did not observe any difference in post/pre fold changes among study diets with the addition of lean red meat. Results of a systematic review of literature on meat intakes and gut microbiota [32] identified only 2 studies that specifically addressed the effects of consuming red meat in healthy adults without modifying or substituting the basal diet. One crossover RCT compared protein sources with high versus low saturated fat concentrations; the effects of saturated fat from dairy and butter outweighed the effects of protein sources (red meat, white meat, or plant-based foods) on gut microbiota [33]. Another study compared the effects of consuming pork versus chicken in a healthy dietary pattern recommended by the Dietary Guidelines for Americans for 10 d among older adults aged at least 50 y [34]. The healthy dietary pattern shifted the gut microbiota composition by reducing phyla Bacteroidetes and family *Bacteriodaceae* but increasing family *Christensenellaceae*, independent of pork or poultry intakes. However, besides the comparable shifts in the top 20 most abundant genera between

pork and poultry intakes, the authors did not report changes or differences in abundances for the less abundant genera, which may potentially miss important distinct effects at this lower taxonomic level [34]. Other studies assessing the effects of consuming unprocessed red meat on gut microbiota either compared red meat with mushroom [35] or whole grains [12] without controlling the basal diet, or compared higher versus lower red meat intakes with cointervention of resistance training [36]. Contrary to our results, these previous findings suggested lower *Parabacteroides* but higher *Dorea* abundances after red meat consumption compared with mushroom [35]; increased gut microbial diversity after consuming whole grain products but not red meat [12]; or changes in genera [36] that were not observed in our study. The gut microbiota results observed in these studies may not be attributed to red meat intake without controlled full-feeding basal diets. Our findings provide novel insights into studying the effects of red meat intake on gut microbiota using a crossover RCT with a fully controlled basal diet which was exactly the same for all 3 intervention periods. Notably, when assessing the effects of consuming meat of all types, rather than focusing on red meat, our results overlapped with findings from the systematic review for several bacterial genera [32], including *Anaerostipes*, which decreased following higher meat consumption, and *Roseburia,* which increased following higher meat consumption. However, our study was designed from a whole foods perspective, where the effects of red meat should be evaluated considering its whole food matrices and level of processing and not to be attributed to its nutrients [37]. A different experiment would be needed to assess the influence of a particular nutrient within a food on these outcomes.

Our results also show that consuming the healthy dietary pattern for 3 wk, independent of unprocessed or processed lean red meats, improved blood TC and LDL-C concentrations in healthy young adults. Our findings support and extend previous findings on the beneficial CVD effects of adopting a healthy dietary pattern for at least 4 wk with varying amounts of red meat intakes in individuals with elevated CVD risk [4,5,38–40]. While higher blood urea nitrogen concentrations were observed after the URM and PRM diets, these short-term changes in blood urea nitrogen concentrations are within the range of clinical normalcy and documented in the literature [41]. Importantly, changes over time in bacterial abundance were associated with changes in blood lipids induced by our study diets. For example, the associations between changes in *Roseburia* or *Romboutsia* abundances and indices of blood lipids and lipoprotein concentrations may implicate the ability of gut microbiota in reducing CVD risk upon adopting a healthy dietary pattern. The addition of lean red meat to the vegetarian-style healthy dietary pattern did not differentially affect changes in bacterial abundance or improvements in CVD risk factors after consuming the healthy dietary pattern. In previous research studying the effects of lean red meat intake on gut microbiota, only one study correlated bacterial taxa abundance with CVD outcomes [36]. Inconsistent with our findings, although associations were found in the other study between bacterial taxa and CVD risk factors, the CVD outcomes were not affected by the higher versus lower red meat intakes without a controlled basal diet [36]. Overall, our results align with previous research on CVD improvements from adopting a healthy dietary pattern [4,5,38–40] and document associations between bacterial changes and CVD improvements. Although gut microbiota has been shown to modulate CVD outcomes in preclinical models [42], clinical research in humans supporting causal effects is lacking [37].

Our observation that adopting a healthy dietary pattern, independent of lean red meat, did not affect fecal SCFAs is consistent with previous findings in which consuming lean beef versus mushroom for 10 d [35] or consuming lean red meat versus whole grain for 3 wk [12] within habitual diets did not affect fecal SCFA levels in healthy adults. In contrast, we observed higher fecal BCFA levels (isobutyrate and isovalerate) after consuming the processed lean red meat for 3 wk. Our findings are consistent with results from 2 previous crossover RCTs, in which consuming lean red meat versus mushroom for 10 d led to higher fecal BCFA isovalerate [35], or consuming an animal-based diet high in processed (cured) meats versus a plant-based diet for 5 d increased BCFAs isobutyrate and isovalerate [9]. Increased fecal BCFA levels from the addition of red meat intake were expected because, unlike SCFAs (acetate, butyrate, and propionate) that are produced through carbohydrate fermentation in the proximal and distal colon in humans [43], BCFAs (isovalerate and isobutyrate) are primarily produced from fermenting branched-chain amino acids leucine and valine [44]. BCFAs and SCFAs are also produced by fermenting fats and peptides [45–47]. As red meat is a significant source of leucine and valine, it is explicable to observe increased fecal BCFA levels from increased red meat consumption. However, the fecal BCFA levels trended higher at baseline before participants consumed the PRM diet, which might bias their higher levels at post. As a result, findings on changes in fecal BCFA levels should be interpreted with caution.

Another novelty of our study is the addition of processed lean red meat in a Healthy U.S.-Style Vegetarian Dietary Pattern. We observed several potentially differential effects of consuming processed versus unprocessed lean red meats on gut microbiota that were not statistically supported when comparing post/pre fold changes among study diets (Table 3). Most of the changes specific to unprocessed or processed red meats align with CVD improvements observed in our study. Exceptions were OTU0008-*Fusicatenibacter* (reduced by the URM diet) and OTU0056-*Roseburia* (reduced by the PRM diet) that were negatively associated with changes in blood lipids. With all data combined, the OTU0008-*Fusicatenibacter* was negatively associated with blood triglycerides and LDL-C concentrations and the OTU0056-*Roseburia* was negatively associated with blood TC and LDL-C concentrations (Supplementary Figure S14B). There seems to be a paucity of research assessing the effect of consuming processed red meat on gut microbiota in humans, although processed red meat comprises nearly a quarter of meat intake in the US [48]. One study in healthy human adults added nitrates in water but not specifically processed them into red meat [49]. One study in rats formulated beef-based chow with or without nitrate and assessed gut microbiota in the ileal and jejunal regions of the intestine [50]. Their results showed that consuming nitrate-containing versus unprocessed beef increased bacterial levels of bacterial families *Lachnospiraceae* and *Erysipelotrichales* in the rat's small intestine. Yet, the results have limited comparability with our study as the majority of gut microbiota in humans resides in the large intestine. These findings underscore the importance of cautious evaluation of microbial results.

Notably, we observed more differences in bacterial abundance among the 3 study diets at pre than at post. This may be

attributable to greater dietary variances among participants while they were consuming their self-chosen, unrestricted diets. This possibility is supported by findings from a previous study with reduced intraindividual and even interindividual variances from consuming a standardized healthy diet for 2 wk [51] or a homogenous Western diet for 7 d [52]. Without a controlled, standardized, baseline diet before each intervention, the observations of different bacterial abundances between study diets at pre warrant additional research to establish reproducibility, potentially by adding dietary control with diets that are comparable with participants' usual dietary patterns before the intervention diets.

Because of these differential bacterial profiles at baseline, we also assessed the effects of consuming red meat by cross-sectionally comparing bacterial profiles among study diets at post. Most of the bacterial profiles were not differentially abundant after consuming the healthy dietary pattern without or with red meat in our study. However, to our knowledge, limited research has been done or reported on the reproducibility of dietary effects on bacterial taxa using a randomized controlled, crossover design. In addition, given that diet rapidly alters gut microbiota [6,9], we recognize the challenge of returning the abundances of all gut bacteria to the same baseline when participants were consuming unrestricted, self-chosen diets. Despite the differences at pre, our study design with controlled intervention diets for 3 wk is considered a strength for investigating the effects of consuming lean red meat on gut microbiota [51]. Independent of lean red meat, our study diets effectively shifted and converged participant gut microbiota profiles into comparable compositions at the taxonomic levels after 3 wk.

We are also mindful that adding lean red meat to the LOV diet without substitution shifted energy balance by about 200 kcal/d, which theoretically would have changed body weight by about 1 lb over 3 wk (assuming 500 kcal/d of energy deficit for 1 lb/wk of body weight). But our measurements of body weight showed no difference or change over time on a group basis. Using 16S rRNA sequencing on the V4 region restricted us from reliably assessing bacterial profile at the species level. We comprehensively analyzed the gut microbiota structure at all available levels, from the community level using alpha and beta diversity measures to all available taxonomic levels (down to the genus and OTU levels). In addition, we conducted LEfSe analyses for all OTUs and reported results in Supplemental Tables S7-S8, which avoids neglecting important signals from low-abundance taxa. We then screened and included the genera and OTUs with at least 0.1% of relative abundance, which is a commonly used cut-off in the microbial research [53], to include abundant taxa for biologically meaningful findings. On the other hand, in line with previous research findings on the individuality in human gut microbiota [51,52,54], we observed variations within and among individuals in bacterial responses to dietary interventions. Relatedly, the challenge of having a meaningful a priori power calculation for studies such as ours investigating untargeted gut microbiota outcomes should also be noted. A post hoc sample size calculation using post/pre fold changes of *Roseburia* suggested a sample size of 41 to provide 80% power with an effect size of 0.45 between the LOV and URM diets and a sample size of 48 for the LOV diet versus the PRM diet with an effect size of 0.42 (significance level at 5%).

The full lists of bacterial taxa and OTUs with statistical significance are available in the supplemental tables, which can serve as pilot data for power calculations for future studies to reproduce and extend the findings. The differences in bacterial responses to the unprocessed versus processed lean red meat intakes observed in our study warrant further investigation. Independent of red meat intake, the associations between changes in gut microbiota and CVD improvements induced by the healthy dietary pattern suggest the potential utility of gut microbial outcomes as clinical biomarkers for assessing and predicting CVD risk [55].

In conclusion, independent of lean red meat intake, consuming the Healthy U.S.-Style Dietary Pattern shifted the gut microbial structure in a favored direction of the improved blood lipid profile. Healthy young adults are therefore encouraged to adopt a healthy dietary pattern that may be vegetarian or omnivorous, including lean red meat.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.03.013.

Author contribution

The authors' responsibilities were as follows – YW, SRL, T-WLC, MT, WWC: designed research; YW, CMC: conducted research; YW: analyzed samples and data; YW, WWC, SRL, T-WLC, MT: wrote the paper; WWC: had primary responsibility for final content; and all authors read and approved the final manuscript.

Data Availability

Data described in the manuscript, code book, and analytic code will be available upon request, pending application and approval.

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Author disclosures

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honoraria for scientific presentations, or consulting services from the following organizations: U.S. National Institutes of Health, U.S. Department of Agriculture, National Science Foundation, Showalter Research Trust, Grain Foods Foundation, CP Kelco US, OLIPOP, Inc, Council for Responsible Nutrition. YW, T-WLC, MT, and CMC declare no conflict of interest. The funder and these other organizations had no role in the design and conduct of the study, analysis, interpretation of the results, and writing of the manuscript.

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