Microbiota Stability and Gastrointestinal Tolerance in Response to a High-Protein Diet with and without a Prebiotic, Probiotic, and Synbiotic: A Randomized, Double-Blind, Placebo-Controlled Trial in Older Women

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ABSTRACT

Background Higher protein intakes may help reduce sarcopenia and facilitate recovery from illness and injury in older adults. However, high-protein diets (HPDs) including animal-sourced foods may negatively perturb the microbiota, and provision of probiotics and prebiotics may mitigate these effects.

Objective The aim of this study was to examine the effects of HPD, with and without a probiotic and/or prebiotic, on gut microbiota and wellness in older women.

Design We conducted an 18-week, double-blind, placebo-controlled, crossover study.

Participants/setting Participants were healthy, older women (mean age ± standard deviation = 73.7 ± 5.6 years; n = 26) recruited from Florida.

Intervention Participants received a weight-maintenance HPD for 2-week periods and the following, in random order: HPD alone (1.5 to 2.2 g/kg/day protein); HPD plus multistrain probiotic formulation (1.54 × 10⁹ Bifidobacterium bifidum HA-132, 4.62 × 10⁹ Bifidobacterium breve HA-129, 4.62 × 10⁹ Bifidobacterium longum HA-135, 4.62 × 10⁹ Lactobacillus acidophilus HA-122, and 4.62 × 10⁹ Lactobacillus plantarum HA-119), HPD plus prebiotic (5.6 g inulin), and HPD plus synbiotic (probiotic plus inulin), separated by 2-week washouts. Stools were collected per period for quantitative polymerase chain reaction (strain recovery) and 16S ribosomal RNA gene amplicon sequencing analyses (microbiota profile). Measures of gastrointestinal and general wellness were assessed.

Main outcome measures Microbiota composition and probiotic strain recovery were measured.

Statistical analyses Microbiota composition was analyzed by Wilcoxon signed-rank test and t test. Secondary outcomes were analyzing using generalized linear mixed models.

Results The microbiota profile demonstrated relative stability with the HPD; representation of Lactobacillus, Lactococcus, and Streptococcus were enhanced, whereas butyrate producers, Roseburia and Anaerostipes, were suppressed. Lactococcus was suppressed with synbiotic vs other HPD periods. Recovery was confirmed for all probiotic strains. Indicators of wellness were unchanged, with the exception of a minimal increase in gastrointestinal distress with inulin. Fat-free mass increased from baseline to study end.

Conclusions An HPD adhering to the recommended acceptable macronutrient distribution ranges maintains wellness in healthy older women and exerts minor perturbations to the microbiome profile, a group that may benefit from a higher protein intake.

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The Recommended Dietary Allowance (RDA) for protein (0.8 g/kg) is considered suboptimal for older adults due to age-related metabolic and physiologic changes. Higher protein intake is associated with reduced risk for sarcopenia, increased strength, and possibly improved bone health. To maintain muscle mass and offset sarcopenia, a daily protein intake of 1.0 to 1.2 g/kg of body weight is considered essential, and an even higher...
protein intake may benefit older adults who are physically active and those experiencing acute or chronic disease. However, research suggests that 10% to 12% of community-dwelling older adults, and particularly women, have an inadequate protein intake level when considering the Estimated Average Requirement of 0.66 g/kg (equivalent to an RDA of 0.8 g/kg) as a reference point. In addition, the number of older adults with inadequate protein intake levels rises to 23% to 27% when a proposed Estimated Average Requirement of 0.83 g/kg (corresponding to an RDA of 1.0 g/kg) is used as reference. In addition, physically active older adults, for whom a minimum protein intake of 1.2 g/kg/day is recommended, may not meet their needs.

For many adults, a protein intake corresponding to the RDA falls near or below the lower cut point of the acceptable macronutrient distribution range (AMDR) at 10% energy from protein. Conversely, in older women with lower energy requirements, higher protein diets (HPDs) may correspond closer to the higher cut point of the AMDR at 35% of energy from protein. Although the AMDR for protein is set to reduce the risk of disease while providing essential nutrients, little is known about the potential risks of consuming diets with >25% energy from protein. If higher-protein diets are to be broadly recommended to older adults, the potential adverse effects of such a dietary pattern warrant further study.

The relationships among macronutrient diet composition, gut microbiota, and health and disease have not been elucidated. Few studies have examined the effects of an HPD (25% to 35% energy from protein) on microbiota composition. David and colleagues compared a high-protein/very-high-fat, solely animal-sourced diet to a high-fiber, vegan diet. Predictably, the animal-sourced diet suppressed bacterial genera that hydrolyze plant polysaccharides, that is, Roseburia, Eubacterium rectale, and Ruminococcus bromii. Higher-protein diets intended for weight loss have also been shown to suppress Roseburia and E rectale, as well as Bifidobacterium, which are considered to be beneficial microbes. Although some have proposed that the negative impact of those diets may be mitigated by increasing the fiber content, others have noted that few studies have controlled for dietary variables known to significantly impact the microbiota, such as fiber content and energy intake, while manipulating protein intake.

Based on prospective cohort studies, Pedersen and colleagues suggested that an intake of 1.2 to 1.5 g/kg/day, representing approximately 15% to 20% energy from protein, could be considered safe for older adults. However, additional research is required to determine the potential adverse effects of higher protein intake. One of these adverse effects may be the dysbiosis through disruption of the commensal microbiota, a common condition in older adults. Specifically, a decrease in carbohydrate fermentation and an increase in proteolytic fermentation has been found in older adults. This fermentation shift may result in a decreased production of beneficial short-chain fatty acids (eg, butyrate), while increasing metabolites from protein fermentation, some of which are inflammatory in nature and are thought to increase the risk of renal and cardiovascular diseases. Increasing protein intake results in an increased amount of protein substrate reaching the colon, which subsequently increases proteolytic fermentation. Particularly in older adults with lower overall energy intake, consuming more protein-rich foods may occur at the expense of fiber-containing foods, which may reduce fiber intake further below recommendations and worsen dysbiosis. As many older adults already present with decreased microbial diversity and other deleterious, health-related changes in microbial balance, the impact of higher protein intake on gut microbiota in this population requires investigation. In addition, the potential role of probiotics and prebiotics in mitigating the possible protein-induced suppression of certain beneficial genera, such as Bifidobacterium, requires elucidation.

The primary aim of this study was to determine the effects of an HPD on fecal microbial communities in older women, as women are less likely to meet protein recommendations, and thus may benefit from a higher-protein diet. In addition, we aimed to investigate the viability of a multistrain probiotic, with and without a prebiotic, in the context of an HPD, as prebiotics, particularly fructans, have been shown to enhance Bifidobacterium, and multistrain probiotics have demonstrated efficacy in restoring microbiota balance after perturbations. It was hypothesized that consuming an HPD would produce undesirable changes in gut microbiota, including suppression of Bifidobacterium, whereas concomitant supplementation with a multistrain probiotic formulation and prebiotic may mitigate these effects. The impact of the HPD on the abundance of Akkermansia muciniphila and Faecalibacterium prausnitzii was also of interest, given they are inversely associated with disease.

Secondary aims were to determine the effects of the HPD on measures of wellness in older women.

METHODS

Study Design

An 18-week randomized, double-blind, placebo-controlled, crossover study was conducted in Florida from May 2015 to December 2015. As suppression of Bifidobacterium by dietary means was unexplored, target sample size (n = 20) was based on previous trials demonstrating significant microbiome profile changes with dietary interventions, with the
expectation of a 25% dropout rate. Two-week periods of a controlled HPD were provided and, in random order, a multisstrain probiotic, prebiotic, and synbiotic (probiotic and prebiotic) were administered, separated by 2-week washouts during which the participants consumed their usual diet (Figure 1). During baseline, dietary intake was assessed using 3 days of 24-hour recalls by the multipass method. Portion-size aids were provided, including the US Department of Agriculture Food Model Booklets. Food intake was analyzed using ESHA Food Processor, version 11.0.117. To estimate energy expenditure and thus the requirement for weight maintenance, SenseWear Pro 2 armbands (Body Media multisensor devices) were worn over the triceps muscle for a 7-day period, a method validated previously in community-dwelling older adults by comparison to the doubly labeled water method. At randomization, weight (Seca 874 flat scale), height (Seca 217 portable stadiometer), hand-grip strength (JAMAR Plus+ digital dynamometer; Patterson Medical), and body composition by bioelectrical impedance analysis (HYDRA ECF/ICF Bio-Impedance Spectrum Analyzer, Model 4200; XITRON Technologies) were measured. Participants completed the Gastrointestinal Symptom Rating Scale (GSRS), Groningen Frailty Index, and SF-36v2 Health Survey. These measures were repeated on the last day of each 2-week period for the duration of the study, with the exception of bioelectrical impedance analysis, which was measured at baseline and 18 weeks only. Fasting venous blood was drawn at baseline and at the end of each HPD period. Participants completed daily questionnaires assessing stool frequency, stool form, general health, and compliance with the diet and supplementation. Single stool and 24-hour urine samples were collected per period for microbiota profile analysis and future exploratory metabolomics, respectively. Three-day, 24-hour recalls were administered during washout periods. The study was approved by the Institutional Review Board (IRB-01) at the University of Florida and was performed according to the guidelines established by the Declaration of Helsinki. All participants provided written informed consent. Participants received a prorated honorarium for participation in the 18-week study.

Randomization and Blinding
Participants were randomized using a sealed envelope method (prepared by an individual unaffiliated with the study), following a Latin square design with four intervention sequences. Participants, investigators, study staff, and the statistician were blinded to the sequence until the completion of the statistical analyses.

Participants
Healthy women 65 years or older with a usual diet providing <15% of daily energy from protein and <20 g/day of fiber were recruited. Exclusion criteria included current intervention for immune-modulating diseases (eg, human immunodeficiency virus/acquired immunodeficiency syndrome, autoimmune, hepatitis, and cancer); diabetes or chronic kidney disease;

Figure 1. Study design. An 18-week (randomized by Latin square), double-blinded, crossover study. Interventions include A=prebiotic, B=synbiotic, C=placebo, and D=probiotic.
vegetarianism; known food allergies or dietary restrictions; physician-diagnosed gastrointestinal disease or condition (such as ulcerative colitis, Crohn’s disease, gastroparesis, peptic ulcer disease, cancer, celiac disease, short bowel, and ileostomy); taking medication for constipation or diarrhea; antibiotic therapy during the preceding 2 months; current smoker; typically consume more than one alcoholic beverage per day; body mass index (calculated as kg/m²) > 30; or plans to lose/gain weight during the next 6 months.

HPD Intervention
A controlled HPD composed of preportioned, commercially available foods was provided to participants during the four intervention periods. A 4-day rotation, 1,600-kcal base diet (all meals, snacks, and calorie-containing beverages) was provided, plus additional study foods to meet each participant’s energy requirement while maintaining the AMDR for carbohydrate, protein, and fat. The HPD included fresh meat (cut, weighed, and packaged); fresh fruits and vegetables; frozen meals; pre-packaged foods, such as salads, protein drinks, breakfast foods; and an assortment of snacks. An example 1-day baseline menu is provided in Table 1 (available at www.jandonline.org). Shelf-stable foods were purchased in bulk before the study start to avoid changes in product formulation, and perishable products were ordered weekly. During the washout periods, participants were instructed to consume all food that was given to them during the HPD period. Participants were asked to return to their usual diet and were responsible for providing their own food. Participants were instructed to consume all food that was given to them during the HPD periods and to record any food that was not consumed in the daily questionnaire.

Supplements
The probiotic supplement contained *Bifidobacterium bifidum HA-132* (1.54 billion), *Bifidobacterium breve HA-129* (4.62 billion), *Bifidobacterium longum HA-135* (4.62 billion), *Lactobacillus acidophilus HA-122* (4.62 billion), and *Lactobacillus plantarum HA-119* (4.62 billion) (Lallemand Health Solutions Inc). Placebo capsules were sensorially identical and consisted of encapsulated potato starch and magnesium stearate. Participants were instructed to consume one capsule daily with a meal.

The probiotic, inulin (Sensus FRUTAFIT IQ), and the placebo supplement composed of maltodextrin (PenNovo MD10) were packaged in coded, silver, opaque sachets (5.6 g/sachet). Codes were generated using a random-number generator and assigned by an individual unaffiliated with the study. Participants were instructed to consume one sachet daily by thoroughly mixing its contents into water or another beverage.

Outcome Measures
**Fecal Microbiota Composition.** Fecal samples were collected during the last 3 days of each period. Coolers and commode collection kits (Fisher Scientific) were provided. Samples were mixed, aliquoted, and stored at −80°C within 6 hours of defecation. For DNA extraction, 5-mL aliquots of stool samples were thawed at 4°C. Genomic DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen) as per manufacturer’s protocol, with the following modifications: two washes with 50 mmol/L sodium phosphate buffer before the addition of InhibitEx, and a 0.1-mm zirconia/silica bead beating step (approximately 300 mg/tube, 4 m/s for 1 minute × 3) after incubation with InhibitEx. DNA concentrations were determined using a Nanodrop, and samples were stored at −20°C. Before quantitative polymerase chain reaction (qPCR) analysis, the samples were diluted fivefold in molecular biology–grade water.

The template DNA for standard curve analysis was obtained by spiking 10⁹ bacteria of the probiotic strain under investigation into approximately 250 mg of a fecal feces matrix, followed by DNA extraction, as described previously. Briefly, 1 g of lyophilized bacteria (*B. bifidum HA-132, B. breve HA-129, B. longum HA-135, L. acidophilus HA-122, or L. plantarum HA-119; Lallemand Health Solutions Inc*) was dissolved in 99 mL phosphate-buffered saline (HyClone) and the total bacterial cell count of the suspension was determined by flow cytometry (BD Biosciences Accuri C6 Flow Cytometer) with Fluorescence Nucleic acid stain SYTO 24 labeling (ThermoFisher Scientific) to calculate the volume required to spike with 10⁹ bacteria. Feces without the addition of bacterial suspension were used as negative control.

Absolute quantification of *B. bifidum HA-132, B. breve HA-129, B. longum HA-135, L. acidophilus HA-122, and L. plantarum HA-119* in fecal samples was performed by qPCR using the Viia 7 Real-Time PCR System (Thermo Fisher Scientific) and CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Standard curves for each strain were generated from feces spiked with 10⁹ *B. bifidum HA-132, B. breve HA-129, B. longum HA-135, L. acidophilus HA-122, and L. plantarum HA-119*, respectively. DNA recovered from spiked feces was serially diluted (10-fold) to generate templates for the standard curve ranging from 10⁹ to 10² bacteria for *L. acidophilus HA-122 and L. plantarum HA-119*, and 10⁹ to 10⁵ for *B. bifidum HA-132, B. breve HA-129, and B. longum HA-135*. The DNA samples to be quantified were diluted 5-fold in molecular biology–grade water before qPCR.

The qPCR reaction mixture (25 μL) consisted of 300 nM of the appropriate primers, 1X SYBR Select Master Mix (Thermo Fisher Scientific, cat# 4472908) and 2.5 μL diluted DNA when assays were run on Viia 7 Real-Time PCR System in a 96-well format. Volumes were downscaled when assays were run on the CFX384 Touch Real-Time PCR Detection System to a final reaction volume of 10 μL, including 1 μL diluted DNA. Primer sequences are given in Table 2 (available at www.jandonline.org). Standard curve samples were analyzed in duplicate and unknown samples in triplicate. Cycling conditions consisted of initial incubations at 50°C and 95°C for 2 minutes each, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A dissociation curve analysis (60°C to 95°C) was also performed to ensure primer specificity.

Relative quantification was carried out for *A. muciniphila, F. prausnitzii*, and total *Bifidobacterium* spp. DNA was normalized using 16S ribosomal DNA Universal Bacterial primers and the 2⁻ΔΔCt method to determine the relative fold gene expression when comparing baseline to each intervention period. Primer sequences and assay conditions for *A. muciniphila* and *F. prausnitzii* were obtained from Colado and colleagues and Lopez-Siles and colleagues, respectively. The *Bifidobacterium* spp primers were designed in-house (forward: TGG AAG GTC TCG ATG GAG GT and reverse: CTG GAC AAG CCG TTC CTG AT) and use the same assay and cycling conditions as listed for the primers used in strain quantification. A dissociation curve analysis (60°C to 95°C) was also performed to ensure primer specificity for all
assays. All qPCR reactions were prepared using the epMotion 5075tc liquid handling robot and Select SYBR Mastermix (Thermo Fisher Scientific) and analyzed on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories).

DNA was amplified using gene-specific primers for the V4 hypervariable region of the 16S ribosomal RNA gene and tagged with unique identifiers. As described in MacPherson and colleagues, Illumina’s “16S Metagenomic Sequencing Library Preparation” guide (part #15044223 Rev. B) was used to prepare the bacterial 16S ribosomal RNA gene libraries. The Qiagen HotStarTaq MasterMix was used for the first PCR (amplicon PCR) at 25 cycles with annealing temperatures of 55°C. For the second PCR (index PCR) only 50% of the reagent volumes were used. The template-specific primers, 515f (5'—GTGCCACCMGCGCGGTAA-3') and 806R (5'—GGACTACHVGGGTWTCTAAT-3'), flanked with appropriate overlap adapter sequences, were used. Before loading on an Illumina MiSeq, samples were diluted and pooled, and sequenced using a 500-cycle MiSeq Reagent Kit v3.

The demultiplexed amplicons were processed with the quality filter software encapsulated in QIME2 (Quantitative Insight Into Microbial Ecology—2). After visual inspection of the imported dataset and assessment of the high-quality reads throughout the length of the amplicon, the reads were trimmed at 240 bp and quality controlled with “quality-filter q-score” using the default parameters. The quality-controlled reads were clustered into amplicon sequence variants (ASVs), functionally equivalent to the more widely used operational taxonomic units, with the denoising algorithm Deblur, implemented in the QIME2 suite. The resulting ASVs (and their relative abundances) were used to generate basic metrics (α-diversity per treatment), principal component analysis (with popular distance algorithms, like Bray-Curtis, Jaccard, Weighted UniFrac). The resulting ASVs were then attributed to the closest known taxa with QIME2’s “feature classifier,” classifying using the sklearn machine learning classification module.

The taxonomically attributed tables (tables containing known taxa and their relative abundance in each sample) were exported from the QIME2 framework for further analysis (table collapsed at the genus level, L=6). The relative abundances profiles were compared between treatment groups using linear discriminant analysis effect size (LEfSe). This package is designed for the discovery of biomarkers from relative abundance microbiome tables. For ease of use (because of its high number of software dependencies), the formatted tables have been uploaded to the Huttenhower galaxy online platform (http://huttenhower.sph.harvard.edu/galaxy/) and LEfSe was run from this implementation.

**Wellness**

Participants were asked to record in a daily questionnaire, their fasting weight, hours of sleep, level of hunger, supplement and diet compliance, antibiotic use, doctor visits, changes in physical activity, medication usage, stool frequency, and stool form using the Bristol Stool Form Scale (1 to 7: 1 = difficulty, 7 = easy passage). Daily symptoms were monitored using a 7-point Likert-like scale (0 = none, 3 = moderate, 6 = severe) and included constipation, diarrhea, and fatigue. Syndromes included gastrointestinal distress (bloating, flatulence, cramping, and abdominal noises); psychological (feeling anxious, depressed, or stressed); cephalic (headache and dizziness); and emetic (nausea and vomiting) using the same 7-point scale.

Participants completed the GSRS, SF-36v2 Health Survey, and Groningen Frailty Indicator every 2 weeks at study visits. Scores of the SF-36v2 Health Survey (originally reported on a 0 to 100 scale) were normalized to the 2009 US population using a linear transformation with a mean ± standard deviation (SD) score = 50 ± 10 (QualityMetric Health Outcomes Scoring software, version 4.5). The Groningen Frailty Indicator was scored using guidelines outlined by Peters and colleagues. Hand-grip strength was assessed during each period using the Jamar Plus+ digital dynamometer (Patterson Medical) as described by Flood and colleagues.

Fasting blood was collected at baseline and during each HPD period. Lipid panel, comprehensive metabolic profile, and C-reactive protein were analyzed by accepted clinical laboratory methods. Insulin-like growth factor-1 was measured in serum samples using Human IGF-1 Milliplex Map Kit (cat# HIGMFAG-52K; Millipore Corp) according to manufacturer’s directions. The beads were analyzed on a Luminex 200 instrument (Luminex Corp) with xPONENT software, version 3.1.

**Compliance**

Two-week supplies of the sachets and capsules were provided during each intervention period. Participants were asked to return any unconsumed supplements at the end of each period and to record their intake compliance of the probiotic, prebiotic, and diet included in their daily questionnaire.

**Statistical Analyses of Wellness Outcomes**

All wellness data were analyzed on an intent-to-treat basis (n=26). Unless noted otherwise, data represent the least squares mean ± standard error of mean and significance at a P value <0.05. For body weight, equivalence testing was performed using a tolerance level set at 1 kg. Bioelectrical impedance analysis data were analyzed using a paired Student’s t test. Sleep was compared using a quasi-binomial distribution of daily category modeling using mean daily proportion of people with <7 hours of sleep. Nonparametric analysis, estimates, and least squares mean difference Tukey’s honestly significant difference were used to analyze symptoms and syndromes recorded in the daily questionnaires. Biweekly means of stool frequency and Bristol Stool Form Scale (slow transit, types 1 and 2; normal transit, types 3 to 5; fast transit, types 6 and 7) and GSRS were log-transformed and analyzed using generalized linear mixed models with a random effect of subject and a fixed effect of intervention. As no sequence, week, or washout/carryover effects were seen, all washouts were combined. For log constipation, an effect of sequence and its interaction with intervention was added. A pairwise comparison of the sequences was then conducted. For Groningen Frailty Indicator, a binomial model with a random effect of subject was used, and for the SF-36v2, the scored norm-based data were analyzed with a generalized linear mixed model fitted with intervention as a fixed effect and a random-subject effect. Serum markers were analyzed using a general linear mixed model with a random subject effect and a fixed effect of treatment or intervention and log transformation where appropriate. Kruskal-Wallis one-way analysis of variance was...
used to compare qPCR data and Shannon α-diversity index with α set to 0.05.  

RESULTS  
The study flow diagram is presented in Figure 2. Participant characteristics at baseline (n=26; mean±SD—age 73.7±5.6 years) are shown in Table 3. Twenty-one participants completed the entire study. No study-related adverse events were reported during the study. Estimated energy requirement for participants at baseline was 1,839±39 kcal/day (mean±SD). HPD provided 1,792±36 kcal/day (mean±SD), individualized within ±50 kcal/day of each participant’s estimated energy requirement. The base HPD provided 30% energy from protein, 46% from carbohydrate, and 27% from fat. With adjustments for energy (primarily carbohydrate), protein provision was 1.8 g/kg/day (range 1.5 to 2.2 g/kg/day) and 27% of energy. Fiber was provided at 15.5±0.4 g/day (mean±SD). Energy intake assessed by 24-hour recall during baseline was 1,455±506 kcal/day (mean±SD) and washouts were 1,667±615 kcal/day (mean±SD). Compliance of participants was 99.5% for the stool protocol, 96.6% for probiotic consumption based on capsule counts, 95.0% for the prebiotic based on packet use, 99.8% for completion of the daily questionnaires, and most study foods were consumed.

Strain Recovery and Microbiota Composition
Absolute quantification by qPCR of five probiotic strains from stools, from participants with complete collections, confirmed strain recovery in the context of the HPD (n=20) (Figure 3). Probiotic strains were recovered in fecal samples after probiotic ingestion during the probiotic and synbiotic periods, with minimal strain detection during the prebiotic and placebo periods. All five strains were recovered during probiotic and synbiotic periods for 18 of 20 participants. One participant showed no detection of B. breve HA-129 after both probiotic and synbiotic interventions and another showed no detection of B. breve HA-129, B. longum HA-135, and L. acidophilus HA-122 during the synbiotic intervention. As washouts before the probiotic interventions did not detect any of the strains, detection of the remaining four of five and two of five strains, respectively, confirmed supplement compliance in the 20 participants. No significant differences were seen between interventions for the relative quantification by real-time PCR of A. muciniphila, F. prausnitzii, and Bifidobacterium spp (Figure 4). Shannon α-diversity index was not statistically different among HPD periods compared to baseline and washouts (Figure 5; available at www.jandonline.org) or when comparing pooled HPD periods to baseline and washout (Figure 6; available at www.jandonline.org). Using principal coordinate analysis based on the UniFrac metric, no clustering by treatment was detected (Figure 7; available at www.jandonline.org). Figure 8 (available at www.jandonline.org) shows the UniFrac analysis for the individual participants and the four intervention orders of the Latin design. Although there were some differentiated ASVs when separated out by intervention, the differences were small and not meaningful, given the small sample sizes of the subgroups.

Figure 2. Flow diagram of participant recruitment, allocation, and analysis. Interventions include A=prebiotic, B=synbiotic, C=placebo, and D=probiotic.
Table 3. Baseline characteristics of older women participating in an evaluation of a high-protein diet, with and without prebiotic and probiotic supplements, on microbiota composition and general wellness (n=26)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male/female, n</td>
<td>0/26</td>
</tr>
<tr>
<td>Age, y, mean±SD*</td>
<td>73.7±5.6</td>
</tr>
<tr>
<td>Race/ethnicity, n (%): White</td>
<td>23 (88.5)</td>
</tr>
<tr>
<td>Race/ethnicity, n (%): Black</td>
<td>2 (7.7)</td>
</tr>
<tr>
<td>Race/ethnicity, n (%): Amer Indian</td>
<td>1 (3.8)</td>
</tr>
<tr>
<td>Not Hispanic or Latino</td>
<td>26 (100)</td>
</tr>
<tr>
<td>Body mass index, mean±SD (range)</td>
<td>25.7±3.1 (20.0 to 29.9)</td>
</tr>
<tr>
<td>Normal weight, n (%)</td>
<td>12 (46.2)</td>
</tr>
<tr>
<td>Overweight, n (%)</td>
<td>14 (53.8)</td>
</tr>
<tr>
<td>Groningen Frailty Indicator score, b mean±SD</td>
<td>1.6±1.7</td>
</tr>
<tr>
<td>SF-36v2, mean±SD</td>
<td></td>
</tr>
<tr>
<td>Mental Component Summary</td>
<td>57.8±10.0</td>
</tr>
<tr>
<td>Physical Component Summary</td>
<td>55.6±5.8</td>
</tr>
<tr>
<td>Hand-grip strength, kg, mean±SD</td>
<td>20.3±1.0</td>
</tr>
<tr>
<td>Laboratory values, mean±SD</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL (mmol/L)</td>
<td>197.9±7.4 (51.3±1.9)</td>
</tr>
<tr>
<td>Triglycerides, mg/dL (mmol/L)</td>
<td>92.3±6.9 (1.04±0.08)</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mg/dL (mmol/L)</td>
<td>68.9±3.3 (17.8±0.9)</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol, mg/dL (mmol/L)</td>
<td>110.6±6.6 (28.6±1.7)</td>
</tr>
<tr>
<td>Very-low-density lipoprotein cholesterol, mg/dL (mmol/L)</td>
<td>18.5±1.4 (4.8±0.4)</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL (mmol/L)</td>
<td>92.1±1.8 (5.1±0.1)</td>
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<tr>
<td>Blood urea nitrogen, mg/dL (mmol/L)</td>
<td>16.7±1.2 (6.0±0.4)</td>
</tr>
<tr>
<td>Albumin, g/dL (g/L)</td>
<td>4.3±0.1 (43.1±1)</td>
</tr>
<tr>
<td>Total protein, g/dL (g/L)</td>
<td>7.0±0.1 (70±1)</td>
</tr>
<tr>
<td>Creatinine, mg/dL (µmol/L)</td>
<td>0.73±0.02 (65.2)</td>
</tr>
<tr>
<td>C-reactive protein, mg/dL (mg/L)</td>
<td>0.50±0.13 (5.0±1.3)*</td>
</tr>
<tr>
<td>Alanine transaminase, U/L</td>
<td>16.6±1.3</td>
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<tr>
<td>Alanine aminotransferase, U/L</td>
<td>21.7±1.0</td>
</tr>
<tr>
<td>Alkaline phosphatase, U/L</td>
<td>72.1±3.5</td>
</tr>
</tbody>
</table>

*SD=standard deviation.
*Score >4 indicates frailty.
*Maximum strength of dominant hand.
*Values reported as <0.3 mg/dl were assumed to be 0.3 mg/dl.

Figure 9 depicts the microbiome profile (relative abundance of bacteria observed) at three different taxonomic levels, at baseline, and during each HPD and washout period for each participant. The same profiles are presented at three different taxonomic levels, with detail level increasing from left to right. Using LEfSe to compare the relative bacterial proportions between the HPD vs washouts demonstrated enrichment of Roseburia and Anaerostipes during washouts and enrichment of Lactobacillus, Lactococcus, and Streptococcus in the HPD periods (pooled) (Figure 10A). Relative abundance by period of Roseburia and Anaerostipes are shown in Figure 11 (available at www.jandonline.org) and Lactococcus and Streptococcus in Figure 12 (available at www.jandonline.org). Lactobacillus was also higher during baseline vs washouts (Figure 13; available at www.jandonline.org). There were no significant differences between placebo vs probiotic or placebo vs prebiotic interventions. However, for the placebo vs synbiotic comparison, the placebo group was higher in Lactococcus or inversely, these taxa were suppressed in the synbiotic intervention (Figure 10B). Washouts were compared to baseline to determine whether the intermittent provision of the HPD produced any carryover effect with time. Pooled washouts vs baseline exhibited enhanced Actinomyces at baseline (Figure 14A; available at www.jandonline.org). There was no evidence of a difference between baseline and washout 1. However, washout 2 showed minor changes in unknown taxa (Figure 14B; available at www.jandonline.org), washout 3 showed increased Bacteroidetes and Actinobacteria at all taxonomic levels (and is most similar to baseline, probably responsible for most of the difference in the “pooled washouts vs baseline” comparison) (Figure 14C; available at www.jandonline.org), and washout 4 was enriched in Fusobacteria compared with baseline (Figure 14D; available at www.jandonline.org).

Wellness
Participants’ metabolic panel and liver function tests were normal at baseline (Table 3) and remained unchanged throughout the study, with the exception of blood urea nitrogen. Mean±SD blood urea nitrogen increased during all HPD periods as follows: prebiotic 22.0±1.3 mg/dL (7.9±0.5 mmol/L); synbiotic 23.8±1.3 mg/dL (8.5±0.5 mmol/L); placebo 22.6±1.3 mg/dL (8.1±0.5 mmol/L); and probiotic 21.9±1.3 mg/dL (7.8±0.5 mmol/L), compared to the baseline mean±SD of 16.7±1.2 mg/dL (6.0±0.4 mmol/L) (P<0.001). A significant increase of 0.21 ng/mL in serum insulin-like growth factor-1 (baseline vs all HPD periods) was seen, with no differences among HPD periods (mean±SD=4.24±0.08, 4.25±0.08, 4.25±0.08, 4.25±0.08 ng/mL; not significant). Assessment of body composition showed an increase of fat-free mass from mean±SD of 41.1±1.1 kg at baseline to 43.2±1.2 kg at study end (P=0.03). In addition, a trend (defined as P<0.10) was observed for decreased fat mass (P=0.05) and percent fat (P=0.05). Intracelular (P=0.03) and total body water (P=0.03) were also significantly increased compared to baseline. SF-36v2 scores remained unchanged with interventions, with four participants exhibiting frailty (Figure 15; available at www.jandonline.org).

As the dataset of the daily questionnaire contained a large proportion of “no reported symptoms” for diarrhea, constipation, and for the psychological, cephalic, and emetic
Figure 3. Absolute quantification by real-time polymerase chain reaction of the five probiotic strains (Bifidobacterium bifidum HA-132, Bifidobacterium breve HA-129, Bifidobacterium longum HA-135, Lactobacillus acidophilus HA-122, and Lactobacillus plantarum HA-119) (n=20). Assay’s limit of detection is indicated by the dotted line. Solid black lines indicate the median absolute quantification value for each strain during each intervention.

Figure 4. Relative quantification by real-time polymerase chain reaction of Akkermansia muciniphila, Faecalibacterium prausnitzii, and Bifidobacterium spp (n=20). Mean \( \log_2 \) fold-change from baseline are represented by solid black lines (\( P<0.05 \)).
Figure 9. Fecal microbiota profile. Phyla, family, and genus levels for the four intervention periods (placebo, probiotic, prebiotic, and symbiotic) and washouts. The same profiles are presented at three different taxonomic levels, with detail level increasing from left to right.
Figure 10. Linear discriminant analysis (LDA) and cladogram analyses of fecal bacteria during high-protein diet (HPD) interventions. (A) LDA score represents log changes in relative bacterial representation between pooled HPD interventions and washout periods. The cladogram shows the phylogenetic relationship among the bacterial genus, families, and orders that were different between the pooled HPD diet interventions and washout periods. (B) LDA score represents log changes in relative bacterial representation between the placebo and synbiotic HPD periods. The cladogram shows the phylogenetic relationship between the bacterial genus, families, and orders that were different between the placebo and synbiotic HPD periods (threshold = 2.0, α = .05).
syndromes scores, statistical analyses were precluded. No differences were seen for fatigue or sleep; however, level of hunger was lower during HPD and washout periods compared to baseline ($P<0.001$) (Table 4). Daily symptom reporting indicated that gastrointestinal distress was very low, but higher for the prebiotic and synbiotic periods compared to baseline ($P<0.001$) (Table 4). Mean±SD stool frequency at baseline (1.38±0.10 stools/day) and Bristol Stool Form Scale (3.84±0.21; normal transit) were unchanged with interventions.

For the GSRS, mean syndrome scores were all <2 (slight discomfort). Abdominal pain was higher with the prebiotic ($P<0.05$), synbiotic ($P<0.01$), and placebo ($P<0.01$), but not the probiotic when compared to baseline plus washout. However, when compared to baseline alone, there were no differences in abdominal pain. Indigestion syndrome was higher when participants received the prebiotic ($P<0.001$), synbiotic ($P<0.001$), and probiotic ($P<0.05$), but not the placebo compared to baseline plus washout. However, when compared to baseline only, indigestion syndrome was significantly higher only for the prebiotic ($P<0.01$) and synbiotic ($P<0.001$). Results of the GSRS questionnaire are shown in Table 5.

**DISCUSSION**

The primary aim of this study was to determine whether an HPD exerted undesirable changes on fecal microbial communities, focusing on diversity and suppression of abundance of health-enhancing taxa *Bifidobacterium* spp, *A. muciniphila*, and *F. prausnitzii*. There was no evidence that diversity was affected by study interventions. These findings are in agreement with Beaumont and colleagues, who found no significant changes in diversity with a higher-protein diet in overweight adults. In the present study, there was no evidence that quantitative *Bifidobacterium* abundance was affected by HPD interventions. Although an age-related decline in *Bifidobacterium* has been reported in frail older adults, only four participants were assessed as frail based on the Groningen Frailty Indicator. *Bifidobacterium* levels observed were similar to what has been reported in an adult population. In contrast, Zhu and colleagues found a low level of abundance for *Bifidobacterium* in 375 older adults residing in China. Similarly, there was no evidence that quantitative abundance of *A. muciniphila* or *F. prausnitzii*, a noted butyrate producer, was affected by the HPD interventions. Abundance of *F. prausnitzii* is reported to be inversely correlated with age; however, the sample size of the present study precludes such analysis.

An HPD consisting solely of animal-sourced foods was shown to suppress the bacterial genera *Roseburia*, *E. rectale*, and *R. bromii*. In the present study, the relative representation of *Roseburia* and *Anaerostipes* were suppressed with the HPD, but no changes were seen in *Ruminococcus* and *Eubacterium*. The provision of fiber from plant-sourced foods may have maintained *Ruminococcus* and *Eubacterium*, as these genera respond to the consumption of vegetarian diets. The butyrate producers *Roseburia* and *Anaerostipes* are considered beneficial constituents of the microbiota, as butyrate is essential for the maintenance of intestinal barrier function, among other critical roles as a mediator of inflammation. Suppression of these taxa may negatively affect colonic butyrate production; however, this was not measured. Inulin provided in the prebiotic and synbiotic periods did not help to sustain the relative abundance

### Table 4. Participant wellness outcomes reported by the study participants during baseline, each of the four 2-week high-protein diet intervention periods, and washout periods

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fatigue $^{ab}$</th>
<th>Hunger $^{ac}$</th>
<th>Gastrointestinal distress $^{d}$</th>
<th>Sleep $^{e}$</th>
<th>Stool Frequency $^{f}$</th>
<th>Bristol Stool Scale Score $^{g}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.39±0.20</td>
<td>1.76±0.22</td>
<td>0.31±0.07</td>
<td>0.40±0.17</td>
<td>1.38±0.10</td>
<td>3.84±0.21</td>
</tr>
<tr>
<td>High-protein diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prebiotic+placebo</td>
<td>1.40±0.20</td>
<td>1.20±0.22</td>
<td>0.58±0.08</td>
<td>0.32±0.16</td>
<td>1.38±0.10</td>
<td>3.71±0.21</td>
</tr>
<tr>
<td>Prebiotic+probiotic</td>
<td>1.73±0.21</td>
<td>1.23±0.23</td>
<td>0.62±0.08</td>
<td>0.36±0.16</td>
<td>1.43±0.10</td>
<td>3.79±0.22</td>
</tr>
<tr>
<td>Control+placebo</td>
<td>1.56±0.20</td>
<td>1.20±0.22</td>
<td>0.36±0.08</td>
<td>0.33±0.16</td>
<td>1.39±0.10</td>
<td>3.78±0.21</td>
</tr>
<tr>
<td>Control+probiotic</td>
<td>1.34±0.21</td>
<td>1.01±0.22</td>
<td>0.39±0.08</td>
<td>0.36±0.16</td>
<td>1.39±0.10</td>
<td>3.58±0.21</td>
</tr>
<tr>
<td>Washout period</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.60±0.21</td>
<td>1.31±0.22</td>
<td>0.31±0.08</td>
<td>0.40±0.17</td>
<td>1.42±0.10</td>
<td>3.75±0.21</td>
</tr>
<tr>
<td>2</td>
<td>1.54±0.21</td>
<td>1.32±0.22</td>
<td>0.32±0.08</td>
<td>0.32±0.16</td>
<td>1.38±0.10</td>
<td>3.74±0.21</td>
</tr>
<tr>
<td>3</td>
<td>1.41±0.22</td>
<td>1.10±0.23</td>
<td>0.29±0.08</td>
<td>0.31±0.15</td>
<td>1.36±0.11</td>
<td>3.77±0.22</td>
</tr>
</tbody>
</table>

*Severity of symptoms from 0 (none) to 6 (very severe). Fatigue=mean of nonzero reports; individuals with zero reporting removed (n=18) (not significant). Hunger=mean of nonzero reports (n=26) (P<0.001). Gastrointestinal distress=mean of bloating, flatulence, abdominal cramping, and abdominal noises scored on a scale of 0 (no symptoms present) to 6 (very severe) (n=26) (P<0.001). Sleep categories: <5 h, 5 to 6 h, 6 to 7 h, 7 to 8 h, 8 to 9 h, >9 h. A quasi-binomial distribution of daily category (<7 h or >7 h) modeling mean daily proportion of participants with sleep <7 h (n=26) (not significant). Stool frequency as mean number of stools per day (n=26) (not significant). Bristol Stool Form Scale responses grouped as slow transit (types 1 and 2), normal transit (types 3 to 5), and fast transit (types 6 and 7) (n=26) (not significant). *P<0.05.
of *Anaerostipes*, which has been shown to utilize this substrate.\(^{33}\) Of note, higher abundance of *Roseburia* is associated with whole-grain intake\(^{30}\) and higher diet quality,\(^{77}\) whereas lower abundance has been associated with inflammation in kidney disease\(^{16}\) and inflammatory bowel disease,\(^{79}\) further evidence that suppression may be unfavorable.

As demonstrated by the linear discriminant analysis—LEfSe analysis, increases in relative abundance of *Lactobacillus*, *Lactococcus*, and *Streptococcus* were seen with the HPD. This is in contrast to the study by David and colleagues,\(^{12}\) which demonstrated increased abundances of *Alistipes*, *Bilophila*, and *Bacteroides* with an animal-sourced diet providing similar energy from protein. As these genera are considered resistant to the bactericidal effects of bile acids, their increase was logically due to the very-high-fat provision in the experimental animal-based diet provided vs the higher protein. In the present study, the higher relative abundance of *Lactobacillus* in the HPD vs washout period may have been due to the provision of yogurt containing *Lactobacillus delbrueckii* subsp *bulgaricus* during the controlled HPD; however, levels were only higher in the placebo and probiotic HPD periods. Not surprisingly, the linear discriminant analysis—LEfSe analysis did not demonstrate enrichment in *Bifidobacterium* or *Lactobacillus* with the provision of probiotic strains. This may be due to the stability of the microbiome in the absence of dysbiosis, as has been reported previously,\(^{80,81}\) the dose amount in relation to the microbiome, and the possibility of displacement. The increase in relative abundance of *Streptococcus* may be unfavorable. Higher relative abundance of *Streptococcus* has been associated with major depressive disorder\(^ {82} \) and atherosclerotic cardiovascular disease;\(^ {83} \) however, the microbiome—metabolite—disease interrelationships and potential causation have yet to be elucidated. Although human research is lacking, a recent animal study similarly showed significantly increased *Streptococcus* with an HPD.\(^ {84} \) Interestingly, the relative abundance of *Streptococcus* during the HPD periods was lowest during the synbiotic period, suggesting mitigation of the HPD effect. Ho and colleagues recently noted that *Streptococcus* was lower with prebiotic treatment vs placebo in children with type 1 diabetes, providing support for this premise. Increased abundance of other proteolytic taxa, predominantly from the phylum Bacteroidetes,\(^ {85} \) expected to respond to the HPD, did not change. This may be due to the provision of fiber from a variety of plant sources, supporting saccharolytic vs proteolytic fermentation.\(^ {86} \)

In addition, although the HPD contained daily meat, plant proteins contributed a significant amount of the total protein. It has previously been suggested that the microbiota response to plant-sourced proteins may differ from that of meat and thus, may have modulated the impact of the HPD.

We examined the effects of a multistain probiotic supplement, prebiotic, and synbiotic on strain recovery in the context of a higher-protein diet. The presence of the five strains was consistently detected during administration and strain recovery was not impacted by the HPD or prebiotic administration. Provision of the multistain probiotic did not increase the abundance of *Bifidobacterium* or *Lactobacillus* spp, as is most often seen when probiotics are provided to individuals without dysbiosis.\(^ {86} \) The strains administered may have temporarily replaced resident strains. It is also possible that HPD may have blunted the expected increase in *Bifidobacterium*. Supplementation with 5 g of fiber from inulin did not enhance *Bifidobacterium* spp abundance, although this dose has been previously shown to exert such an effect.\(^ {87} \) Alternatively, as both naturally occurring and added fructans and other oligosaccharides are widespread in the food supply, the dose of inulin administered may not have been sufficient to elicit an additional increase in *Bifidobacterium*, especially considering that dose—response to inulin is thought to be limited.\(^ {87} \) An increase in flatulence, which drove the change in gastrointestinal distress during the prebiotic and synbiotic periods, confirmed fermentation of this substrate, suggesting that the inulin was fermented by resident gas-producing bacteria, such as from *Clostridium* cluster IV, which includes *F prausnitzii*.\(^ {88} \) However, there was no evidence that the absolute abundance of *F prausnitzii* was affected by the HPD intervention or the supplements. The inulin may have induced changes in

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**Table 5. Gastrointestinal Symptom Rating Scale syndrome\(^ {8} \) reported weekly by the study participants during baseline and each of the 2-week high-protein diet intervention periods**

<table>
<thead>
<tr>
<th>Period</th>
<th>Abdominal pain(^ {b} )</th>
<th>Constipation(^ {c} )</th>
<th>Diarrhea(^ {d} )</th>
<th>Indigestion(^ {e} )</th>
<th>Reflux(^ {f} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.17±0.04</td>
<td>1.38±0.07</td>
<td>1.16±0.03</td>
<td>1.37±0.05</td>
<td>1.19±0.06</td>
</tr>
<tr>
<td>High-protein diet</td>
<td>Prebiotic+placebo</td>
<td>1.24±0.06</td>
<td>1.52±0.15</td>
<td>1.24±0.11</td>
<td>1.63±0.10**</td>
</tr>
<tr>
<td>Prebiotic+probiotic</td>
<td>1.33±0.11</td>
<td>1.54±0.15</td>
<td>1.19±0.06</td>
<td>1.78±0.14***</td>
<td>1.27±0.11</td>
</tr>
<tr>
<td>Control+placebo</td>
<td>1.29±0.06</td>
<td>1.64±0.16</td>
<td>1.07±0.03</td>
<td>1.47±0.08</td>
<td>1.46±0.16</td>
</tr>
<tr>
<td>Control+probiotic</td>
<td>1.15±0.04</td>
<td>1.78±0.24</td>
<td>1.09±0.04</td>
<td>1.45±0.06</td>
<td>1.24±0.07</td>
</tr>
</tbody>
</table>

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\( ^{8} \)*Syndromes are means of individual symptom ratings of 1 (no discomfort at all) to 7 (very severe discomfort).

\( ^{b} \)Abdominal pain scale (abdominal pain, hunger pains and nausea symptoms) (P=0.001).

\( ^{c} \)Constipation scale (constipation, hard stools, and feeling of incomplete evacuation symptoms) (P=0.12).

\( ^{d} \)Diarrhea (diarrhea, loose stools, and urgent need for defecation symptoms) (P=0.11).

\( ^{e} \)Indigestion (stomach rumbling, bloating, burping, and increased flatus symptoms) (P<0.001).

\( ^{f} \)Reflux scale (heartburn and acid regurgitation symptoms) (P=0.15).

\( **P<0.01.\)

\( ***P<0.001.\)
fermentation and metabolite production without impacting relative abundance of specific genera.

A secondary aim of this research was to explore the effects of HPD on wellness, with and without probiotics and prebiotics. An increase in abdominal pain was observed with the HPD and mitigated by the probiotic. In comparison, Skov and colleagues reported no difference in gastrointestinal discomfort or gastrointestinal symptoms with ad libitum consumption of a low-fat HPD (25% energy) for weight loss. While abdominal pain and indigestion were reported with the prebiotic, the average scoring level was below clinical significance. This finding is in accordance with Saarinan and colleagues, who reported increased gastrointestinal symptoms with a prebiotic-supplemented fermented milk with 4 g/day inulin vs without. In young women, daily consumption of 8 g oligofructose resulted in an increased probability of reporting at least one gastrointestinal symptom (primarily flatulence), whereas a 16-g dose produced an even higher probability of reporting gastrointestinal symptoms in young men and women. Pedersen and colleagues23 supplemented a low-fat diet with 14 g inulin in healthy young women and reported that self-reported gastrointestinal symptoms were higher with inulin, findings that are in accordance with the present study. Adaptation to the inulin, a decrease in symptom frequency and intensity over time, was not observed, and this is in agreement with other trials.24 Suppression of hunger level was observed with the HPD and persisted during all washout periods, although participants resumed their usual protein intake. However, energy intake was lower than the participants’ average requirement during baseline, and thus may explain this finding. Hunger levels and energy intakes were similar in the HPD and washout periods, suggesting no specific effect of the HPD. Previous research on higher-protein diets and their enhanced effect on satiety or hunger suppression have been primarily limited to weight-loss studies, and thus this effect of protein may be more apparent with energy restriction.

Aging is associated with a decline in lean mass and an increase in fat mass, and high levels of mortality and impairment are associated with sarcopenia in older adults. Data from the present study suggest that older women on an intermittent HPD (every other 2-week period) over 18 weeks may exhibit improved body composition, specifically by increasing lean body mass. This was an unexpected result, given that resistance exercises, which have been deemed required for a short-term effect, were not included in the intervention. Instead, participants were asked to maintain their usual physical activity level. Weight stability suggested that participants were compliant. However, dietary protein intake has been associated with decreased loss of lean mass over longer time frames.

Notable limitations of this trial were the lengths of the intervention and washout periods. It is possible that if an HPD is administered for longer periods of time, further modulation of the microbiota may occur. The qPCR data suggest that after probiotic intake, strains were detected in a few individuals during washout periods. Carryover effects were seen for L. plantarum HA-119 (four subjects), B. bifidum HA-132 (seven subjects), and L. acidophilus HA-122 (one subject), indicating that a 2-week washout period was not adequate for all participants, and strains may have persisted into the following treatment period for some participants. Typical crossover studies with probiotics include a washout period of variable duration, lasting 2 to 5 weeks between intervention periods. However, a recent study found that probiotic persistence after end of intervention appears to be strain-specific, suggesting that the adequate washout period for crossover trials will likely vary according to the strains administered. Therefore, more studies on probiotic strain-specific persistence behaviors in human populations are required. Alternatively, adaptive designs could be useful in that regard; qPCR could be performed after the 2-week washout period and negative results would validate passing onto the next period, or prolongation of the washout in the case of positive results. In addition, in future studies, participant stratification based on their baseline microbiome profiles may help decrease interindividual variation among older adults, which in turn may allow identification of characteristics distinguishing dietary or probiotic intervention responders from nonresponders. Examining a sample of only 20 women was an additional limitation. Findings may differ in older men and younger cohorts and thus, the generalizability of the results may be limited. Further, given individual variation, microbiota findings may differ with geographical region and subgroup selection.

**CONCLUSIONS**

Provision of a higher-protein diet, compliant with the AMDRs for macronutrient contents, elicited no change in self-reported wellness or interference with probiotic strains recovery in older women. Modulation of microbiota profile by

**What Is the Current Knowledge on This Topic?**

Studies investigating gut microbiome, health, and disease often disregard diet, although plant-based foods, diet quality, and macronutrient intakes modulate the microbiota and its activity. Few studies have explored the effects of an HPD on microbiota profile and evaluation of tolerance to an HPD has been underexplored.

**How Does This Research Add to Knowledge on This Topic?**

Provision of an HPD, AMDR-compliant for macronutrient contents, maintained general wellness and increased lean body mass in older women. The HPD suppressed butyrate-producing bacteria, but did not affect probiotic recovery, and thus their efficacy.

**How Might This Knowledge Impact Current Dietetics Practice?**

The findings of this study suggest that attention to dietary quality, such as the inclusion of whole grains, may be needed to help maintain levels of certain microbes and their beneficial metabolic activities while consuming an HPD.

**PRACTICE IMPLICATIONS**

How Might This Knowledge Impact Current Dietetics Practice?
the HPD, specifically the suppression of butyrate-producing organisms, suggests a need to consider dietary quality, such as inclusion of whole grains, to help maintain these taxa and their beneficial metabolic activities. Supplementing the HPD with a multistrain probiotic may benefit gastrointestinal wellness even in healthy individuals reporting a high level of wellness. Intermittent HPD provision over 18 weeks suggests benefit for lean mass. However, the controlled diet made it possible to demonstrate mild gastrointestinal symptoms with as little as 5 g of inulin per day, confirming fermentation without an increase in Bifidobacterium and suggesting that the HPD may have suppressed this effect. Given the high interindividual variation observed among older women, future research should consider stratification by microbiome phenotype when testing effects of dietary interventions.

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70. nPOINT [computer program]. Version 3.1. Austin, TX: Luminex; 2009.


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STATEMENT OF POTENTIAL CONFLICT OF INTEREST

V. Nagulesapillai, A. Piano, J. Auger, S.-A. Girard, and T. A. Tompkins are employees of the Rosell Institute for Microbiome and Probiotics, the research group of Lallemand Health Solutions Inc. No potential conflict of interest was reported by the remaining authors.

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ClinicalTrials.gov ID: NCT #02445560.

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AUTHOR CONTRIBUTIONS

A. L. Ford, W. J. Dahl, T.A. Tompkins, and S.-A. Girard designed the trial. A. L. Ford and W. J. Dahl conducted the clinical trial. M. Christman carried out the statistical analysis of wellness outcomes and J. Auger, the microbiota. V. Nagulesapillai, A. Piano, and A. L. Ford carried out the microbiota and persistence analyses. A. L. Ford, W. J. Dahl, V. Nagulesapillai, A. Piano, J. Auger, and M. Christman wrote the manuscript. All authors reviewed the final manuscript.
Table 1. An example 1-day menu of the 1,600-kcal/day base high-protein diet provided to the study participants during the four 2-week high-protein diet interventions

<table>
<thead>
<tr>
<th>Meal</th>
<th>Item</th>
<th>Energy, kcal</th>
<th>Protein, g</th>
<th>Carbohydrate, g</th>
<th>Fiber, g</th>
<th>Fat, g</th>
<th>Sugar, g</th>
<th>Sodium, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td>Egg Beaters Smart Cups&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Thomas’ Double Protein English Muffins&lt;sup&gt;b&lt;/sup&gt;</td>
<td>150</td>
<td>7</td>
<td>26</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>Smucker’s Concord Grape Jelly&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Snack</td>
<td>Friendship Fit to Go 1% low-fat cottage cheese,&lt;sup&gt;d&lt;/sup&gt; 5 oz</td>
<td>110</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>Kellogg’s Special K Protein Meal Bar&lt;sup&gt;e&lt;/sup&gt; (honey almond)</td>
<td>180</td>
<td>10</td>
<td>22</td>
<td>5</td>
<td>6</td>
<td>12</td>
<td>160</td>
</tr>
<tr>
<td>Lunch</td>
<td>Artisan Bistro Wild Alaskan Salmon Bake&lt;sup&gt;f&lt;/sup&gt;</td>
<td>240</td>
<td>16</td>
<td>30</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>Dannon Light and Fit Greek Raspberry Chocolate nonfat yogurt&lt;sup&gt;g&lt;/sup&gt;</td>
<td>80</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>Dinner</td>
<td>Beef cubed steak, 5 oz</td>
<td>203</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Libby’s Vegetable Cups&lt;sup&gt;h&lt;/sup&gt; (cut green beans, 4 oz)</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Potato, flesh and skin, 170 g</td>
<td>131</td>
<td>3</td>
<td>30</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Snack</td>
<td>Justin’s Peanut Butter&lt;sup&gt;i&lt;/sup&gt; (1.15 oz squeeze pack)</td>
<td>190</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>16</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Saltines Original Fresh Stacks&lt;sup&gt;j&lt;/sup&gt;</td>
<td>220</td>
<td>4</td>
<td>39</td>
<td>1</td>
<td>5</td>
<td>1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>440</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>1,609</td>
<td>120</td>
<td>181</td>
<td>&lt;16</td>
<td>46</td>
<td>39</td>
<td>2,141</td>
</tr>
</tbody>
</table>

<sup>a</sup>Conagra Brands.  
<sup>b</sup>Bimbo Bakeries.  
<sup>c</sup>The J.M. Smucker Company.  
<sup>d</sup>Saputo Dairy Foods USA Inc.  
<sup>e</sup>Kellogg NA Co.  
<sup>f</sup>Ruz Food Products.  
<sup>g</sup>Danone North America.  
<sup>h</sup>Libby’s Brand Holding.  
<sup>i</sup>Justin’s.  
<sup>j</sup>Mondelēz Global LLC.

Table 2. Primer sequences used to quantify, by quantitative polymerase chain reaction (qPCR), the strains provided in the probiotic formulation during two of the high-protein diet periods

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Gene target</th>
<th>Annealing temperature, °C</th>
<th>Amplicon size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium</em>&lt;sup&gt;bi&lt;/sup&gt; <em>bifidum</em> HA-132</td>
<td>HA-132_GB_NC2_F</td>
<td>AAGTGTGAGCCGGTGATAGC</td>
<td>Noncoding sequence</td>
<td>60</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>HA-132_GB_NC2_R</td>
<td>CAGTACGTCGGCCGTTACAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium</em>&lt;sup&gt;breve&lt;/sup&gt; HA-129</td>
<td>HA-129_225-F2</td>
<td>CGACCCTAATGACGTGGAGG</td>
<td>Hypothetical protein</td>
<td>60</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>HA-129_225-R2</td>
<td>CATTTCAGCCAGTACGTGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium</em>&lt;sup&gt;longum&lt;/sup&gt; HA-135</td>
<td>HA-135_AP_HP10_F</td>
<td>GTCGCCACATTTCATCGCAA</td>
<td>Hypothetical protein</td>
<td>60</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>HA-135_AP_HP10_R</td>
<td>GAGAGCTTCGATTGGCGAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus</em>&lt;sup&gt;acidophilus&lt;/sup&gt; HA-122</td>
<td>HA-122- F1</td>
<td>AGAATCAAGCAGAGACTGGCTACG</td>
<td>An ORF&lt;sup&gt;a&lt;/sup&gt; in plasmid</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>HA-122- R1</td>
<td>GGACCGGATTGTAGTAGGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus</em>&lt;sup&gt;plantarum&lt;/sup&gt; HA-119</td>
<td>HA119_GB_PP_F2</td>
<td>AGGTTGACATGGGACACTGGCT</td>
<td>Phage protein</td>
<td>60</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>HA119_GB_PP_R2</td>
<td>CCCGTTGCTAAACAACCGAA</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>ORF = open reading frame.
Figure 5. Pirateplot showing the distribution of Shannon α-diversity index for participants (intent-to-treat) during baseline, each high-protein diet period (placebo, prebiotic, probiotic, and synbiotic) and washouts ($P=0.36$) (bar/line=median, band/box=highest-density interval).
Figure 6. Pirateplot showing the distribution of Shannon $\alpha$-diversity index for participants (intent-to-treat) during baseline, high-protein diet intervention periods combined, and washouts ($P=0.34$) (bar/line=median; band/box=highest-density interval).
Figure 7. Principal coordinate analysis based on the weighted UniFrac metric from fecal samples of all participants (intent-to-treat) during baseline, high-protein diet interventions (prebiotic, synbiotic, placebo, and probiotic), and washouts (pooled), each indicated by a different color.
Figure 8. Principal coordinate analysis based on the weighted UniFrac metric from fecal samples of all participants (intent-to-treat) at all intervention periods. (A) Each individual is indicated by a different color and (B) each intervention order of the Latin design (A=prebiotic, B=synbiotic, C=placebo, D=probiotic).
Figure 11. Relative abundance of (A) *Roseburia* and (B) *Anaerostipes* for participants (n=20) at baseline and during the high-protein diet periods and washouts.
Figure 12. Relative abundance of genera (A) Lactococcus (B) Streptococcus for participants (n=20) at baseline and during the high-protein diet periods and washouts.
Figure 13. Relative abundance of *Lactobacillus* for participants (n=20) at baseline and during the high-protein diet periods and washouts.
Figure 14. Linear discriminant analysis (LDA) of fecal bacteria of participants (n=20) during baseline vs washout periods. (A) LDA score represents log changes in relative bacterial representation between baseline and washouts. (B) LDA score represents log changes in relative bacterial representation between baseline and washout 2. (Note: Taxonomy is not determined at the genus level). (C) LDA score represents log changes in relative bacterial representation between baseline and washout 3. (D) LDA score represents log changes in relative bacterial representation between baseline and washout 4 (threshold=2.0, α=.05).
Figure 15. Radar chart of the SF-36v2 health domains, Mental Component Summary (MCS) and Physical Component Summary (PCS) of participants (n=26) for baseline and each high-protein diet treatment period. No differences were observed for SF-36v2 for eight health domains: physical functioning (PF), role-physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional (RE), and mental health (MH), MCS or PCS scores. A = prebiotic; B = synbiotic; C = placebo; D = probiotic; NBS = norm-based scoring.