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Research paper

Integrative and quantitative bioenergetics: Design of a study to assess the impact of the gut microbiome on host energy balance

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ABSTRACT

The literature is replete with clinical studies that characterize the structure, diversity, and function of the gut microbiome and correlate the results to different disease states, including obesity. Whether the microbiome has a direct impact on obesity has not been established. To address this gap, we asked whether the gut microbiome and its bioenergetics quantitatively change host energy balance. This paper describes the design of a randomized crossover clinical trial that combines outpatient feeding with precisely controlled metabolic phenotyping in an inpatient metabolic ward. The target population was healthy, weight-stable individuals, age 18–45 and with a body mass index $\leq$30 kg/m^2. Our primary objective was to determine within-participant differences in energy balance after consuming a control Western Diet versus a Microbiome Enhancer Diet intervention specifically designed to optimize the gut microbiome for positive impacts on host energy balance. We assessed the complete energy-balance equation via whole-room calorimetry, quantified energy intake, fecal energy losses, and methane production. We implemented conditions of tight weight stability and balance between metabolizable energy intake and predicted energy expenditure. We explored key factors that modulate the balance between host and microbial nutrient accessibility by measuring enteroendocrine hormone profiles, appetite/satiety, gut transit and gastric emptying. By integrating these clinical measurements with future bioreactor experiments, gut microbial ecology analysis, and mathematical modeling, our goal is to describe initial cause-and-effect mechanisms of gut microbiome metabolism on host energy balance. Our innovative methods will enable subsequent studies on the interacting roles of diet, the gut microbiome, and human physiology.
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1. Introduction

1.1. Emerging evidence for the gut microbiome as a modulator of body weight

The global obesity epidemic has been influenced by a combination of biological and environmental factors [1,2]. Studies in animal models and humans have revealed a central role of the gut microbiome in modulating adiposity phenotypes [3–6]. When evaluating the impact of the gut microbiome on human health, the focus is specifically on the microbial community in the large intestine, which is the location of the vast majority of microorganisms in the human gastrointestinal tract [7].

Abbreviations: BMI, body mass index; COD, chemical oxygen demand; DEXA, dual energy x-ray absorptiometry; EB, energy balance; EE, energy expenditure; EI, energy intake; MFC, mass flow controller; NIST, national institute of standards technology; npRQ, non-protein RQ; PEG, polyethylene glycol; RMR, resting metabolic rate; RQ, respiratory quotient; SCFA, short chain fatty acid; TDEE, total daily energy expenditure; TEF, thermic effect of food; SEE, sleep energy expenditure; VAS, visual analog scale; VCH4, volume of methane produced; VCO2, volume of carbon dioxide produced; VO2, volume of oxygen consume.

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To understand the gut microbiome-human host relationship, fundamental questions need to be answered: Does the makeup of the gut microbiome matter in the development of obesity from the perspective of quantitative bioenergetics? How might we monitor and manipulate the microbiome to optimize its positive impact on the host? Answering these questions requires an understanding of both microbial and host bioenergetics-namely, the acquisition and transformation of energy to perform biological work [8].

Two key findings support the critical role of the gut microbiome on body weight and point us towards answers to our questions. First, animal models support the global hypothesis that the composition of the microbiome leads to obesity via multiple mechanisms, including more energy extraction from foods [6,9] and changes in energy expenditure (EE) [10]. Studies in humans are much more limited, but evidence supports the relationship between the gut microbiome and energy balance [11–13]. Furthermore, the literature suggests that host factors – namely, genetics and metabolic status – play important roles in host-microbiome interactions [14,15]. The overarching objective of our work is to understand how diet affects gut-microbial metabolism and, therefore, the host's energy balance.

1.2. Fundamental gap: insufficient data to claim causality

Published clinical studies have mainly catalogued a dictionary of microbiome diversity and functions in association with phenotypes of interest or a response to a particular intervention [4,5]. These studies have yielded limited data to decipher causality in humans. Previous studies showed that the intestinal microbiome can be rapidly changed (at least temporarily) through diet interventions [16]. However, these studies have not addressed, in a systematic and quantitative way, how these changes affect energy balance. Long-term prospective studies are needed to infer causality and integrate human and microbial bioenergetics. What is missing in the current literature is a quantitative approach that rigorously answers the question: Does the gut microbiome and its bioenergetics quantitatively change the absorption of nutrients, enteroendocrine secretions (affecting appetite, satiety, and therefore food intake), or energy expenditure? Because large clinical trials are expensive, one efficient approach is to employ the controls afforded in a metabolic ward combined with quantitative methodologies.

For such an approach to be successful for initial causal inferences, the following elements are required: a prospective design with comprehensively phenotyped participants, adequate dietary and environmental controls, and initial studies in healthy individuals to characterize expected responses. We describe the design of such an approach, along with quality control metrics and prospective statistical power. Using a novel microbial-bioenergetics model and two diets as tools to modify the gut microbiota, we aimed to understand in a quantitative way how gut microbial ecology, microbial bioenergetics, and microbial metabolites influence energy balance and ultimately body weight.

2. Study design and methods

2.1. Aims and hypotheses

Aim 1: Our first Aim was to create, test, and refine an integrated *in silico* model of energy balance in a metabolic ward setting using a typical Western Diet vs. a diet designed to enhance the activity of the microbial community in the large intestines (Microbiome Enhancer Diet), which consisted of whole foods and four key drivers: dietary fiber, large food particle size, resistant starch, and minimal processing. We hypothesized that switching between a typical Western Diet and a Microbiome Enhancer Diet would drastically alter the composition of the gut microbiome and its metabolic contributions to the host.

Aim 2: In Aim 2, we explored the effect of a Western Diet vs. the Microbiome Enhancer Diet on the gut microbial community, proximal and distal gut enteroendocrine hormones, gastric emptying, and small bowel transit time. We related these results to subjective hunger/satiety and measured food intake. We hypothesized that the changes in the microbiome associated with the Microbiome Enhancer Diet would lead to measurable and meaningful changes in host energy balance via changes in i) energy absorption, ii), gut hormone secretion, and iii) the host's energy expenditure.

2.2. Setting, participants and recruitment

The clinical trial was conducted at the AdventHealth Translational Research Institute (TRI) in Orlando, Florida. The study was approved by the AdventHealth Institutional Review Board. All participants were accurately and adequately informed. No study-related procedures were initiated prior to obtaining informed consent.

The population was recruited from the Orlando, Florida area via a combination of external marketing and internal database and electronic medical record searches. Overall, the study included healthy, young men and women. By design, we enrolled approximately equal numbers of men and women (N = 9 and 8, respectively). Pre-menopausal women were studied during the same phase of the menstrual cycle due to the known impact of female sex steroid hormones [17,18]. Body mass index (BMI) was limited to $\leq$ 30 kg/m^2 because the literature suggests that people with higher BMIs have a different microbial ecology [19–21]. These microbiome differences are hypothesized to correlate with alterations in the hypothalamus that occur in obesity, including reactive gliosis and neuronal injury in regions of the brain that impact food control [22–25]. Therefore, it is possible that these factors might affect the planned food intake studies [26–28]. In addition, microbial metabolic activity is altered in the obese state and could affect interpretation of results [19,29]. Similarly, because gut microbial ecology changes with age [30], volunteers older than 45 were excluded. A screening medical history, physical exam, and laboratory work were used to exclude significant illness. Enrollment was based on the criteria shown below:

2.2.1. Inclusion criteria

1. Able to communicate meaningfully with the investigator and legally competent to provide informed written consent
2. Age 18–45 years, inclusive
3. Weight stable (±3 kg) during the 6 months prior to enrollment
4. BMI $\leq$30 kg/m^2

2.2.2. Exclusion criteria

Excluded were individuals having acute or chronic medical conditions or on medication that would contraindicate the participation in the research testing or could potentially affect metabolic function. This included, but was not limited to:

1. History or presence of cardiovascular disease (unstable angina, myocardial infarction or coronary revascularization within 6 months, presence of cardiac pacemaker, implanted cardiac defibrillator)
2. History of type 1 or type 2 diabetes
3. Bleeding disorders
4. Acute or chronic infections
5. Hepatitis and/or cirrhosis
6. Severe asthma or chronic obstructive pulmonary disease
7. Renal insufficiency or nephritis
8. Thyroid dysfunction (suppressed thyroid stimulating hormone (TSH), elevated TSH <10 μIU/ml if symptomatic or elevated TSH >10 μIU/ml if asymptomatic)
9. Uncontrolled hypertension (blood pressure >160 mmHg systolic or >100 mmHg diastolic)
10. Prior bariatric surgery

11. Gastrointestinal disorders including: inflammatory bowel disease or malabsorption, swallowing disorders, suspected or known strictures, fistulas or physiological/mechanical GI obstruction, history of gastrointestinal surgery, Crohn's disease or diverticulitis.
12. Participants with strict dietary concerns (e.g. vegetarian or kosher diet, multiple food allergies, or allergies to food provided during the study)
13. Current use of polyethylene glycol (e.g. Dulcolax, Miralax, Gavilax)
14. Cancer within the last 3 years (except non-melanoma skin cancer or treated cervical carcinoma in situ).
15. History of major depression within <5 years from screening visit or which, in the opinion of a medical investigator, will impact the participant's ability to complete the study.
16. History of eating disorders
17. Cushing's disease or syndrome
18. Untreated or inadequately controlled hypo- or hyperthyroidism
19. Active rheumatoid arthritis or other inflammatory rheumatic disorder
20. Pregnant or nursing females or females less than 6 months postpartum from the scheduled date of collection.
21. Tobacco use within the past 3 months
22. Metal implants (pacemaker, aneurysm clips) based on Investigator's judgment at Screening.
23. Unable to participate in magnetic resonance imaging assessments due to physical limitations of equipment tolerances (e.g. bore size) based on Investigator's judgment at Screening.
24. Unable to tolerate magnetic resonance imaging or claustrophobia.
25. Nickel allergy.
26. Had major surgery, donated or lost 1 unit of blood (approximately 500 mL) within 4 weeks prior to the pretrial (screening) visit.
27. Intolerance to acetaminophen use.
28. History of regular alcohol consumption exceeding 7 drinks/week for female participants or 14 drinks/week for male participants (1 drink = 5 ounces [150 mL] of wine or12 ounces [360 mL] of beer or 1.5 ounces [45 mL] of hard liquor) within 6 months before screening.
29. Anemia (hemoglobin <12 g/dl in men, <11 g/dl in women)

Likewise, participants were excluded if they were taking medications that include, but are not limited to:

1. Nitrate
2. Anti-diabetic agents
3. Oral, injected or chronic topical steroids (inhaled steroids for mild asthma are acceptable)
4. Chronic use of aspirin or other non-steroidal anti-inflammatory drugs, including COX-2 inhibitors (a single aspirin daily if prescribed for cardioprotection will be allowed as will occasional use of aspirin and other non-steroidal drugs, provided that they are used for < 3 consecutive days and not during the period of metabolic testing)
5. Antibiotics taken in the last three months.
6. Use of any medications known to influence glucose, fat and/or energy metabolism within the last 3 months (e.g., over the counter vitamins and supplements, growth hormone therapy, glucocorticoids [steroids], prescribed medications for weight loss, etc.)
7. Tricyclic antidepressants, atypical antipsychotics, or other psychiatric drugs with effects on body weight

2.3. Study design and randomization procedures

This was a randomized (to diet) within-participant, crossover clinical study that combined outpatient feeding and inpatient metabolic-ward feeding and testing. Randomization was determined by the TRI statistician. Although it was not possible to blind the participants or frontline staff to the components of the diet, as the food items could reveal which diet was being dispensed, the diet assignment was not tied to any data streams throughout the course of the studyFig. 1represents the overall study design.

After the screening period and initial determination of energy requirements, the first outpatient/inpatient period began with a 1-week measurement of habitual energy expenditure determined with accelerometry [31]. The participants were then randomized to a diet. The remaining aspects of the study were repeated with each diet: outpatient feeding (11 days), whole room calorimetry to measure energy expenditure and set kilocalories (kcals) for inpatient phase, and an inpatient metabolic ward phase (11-days) when all endpoints were measured and biological samples were collected. Throughout both phases, polyethylene glycol (PEG) was administered as a non-digestible marker of 24-h fecal production (described below). Study restrictions beyond exclusionary criteria included: avoidance of alcohol during feeding periods, no caffeine up to 72 h before admission, no artificial sweeteners, maintenance of usual physical activity with no strenuous physical activity 48 h prior to admission. The purpose of these restrictions was to minimize variability from these metabolism-influencing lifestyle factors. In addition, artificial sweeteners might impact the gut microbiome [32–34]. The post-study analytics will implement a multi-disciplinary integrative quantitative systems biology approach to analyze, interpret and model the data generated from the clinical trial.

2.3.1. Study activities

Fig. 2 depicts the daily procedures and key measurements. The study activities depicted in the figure are briefly described below with details on innovative methodologies in subsequent sections.

2.3.2. Baseline period (Days 1–9)

An armband accelerometer was placed to measure activity and estimate free-living energy expenditure (SenseWear Pro 3 Armband, BodyMedia Inc.) The accelerometer integrates motion sensor data with a variety of heat-related sensors to estimate the energy cost of free-living activity [35]. These data were used to estimate outpatient calorie requirements.

2.3.3. Outpatient/inpatient intervention period (Days 10–32 and 39–61)

Participants consumed either a typical Western Diet (control diet) or the Microbiome Enhancer Diet (test diet) on an outpatient basis for 11 days and an impatient basis for an additional 11 days. During the entire 22 day feeding period, PEG was administered at a dose of 0.5 g (in a gelatin capsule) three times per day with meals through the end of the test period. The 11-day period in the metabolic ward involved in-depth quantitative phenotyping including:

- body composition (lean and fat mass) measured with dual-energy X-ray absorptiometry
- whole room calorimetry block (6-days) for continuous measurement of volumes of oxygen (O_2) consumed and carbon dioxide (CO_2) and methane (CH_4) generated
- gut transit times determined with a SmartPill™, an oral radio transmitter that measured pH, temperature, and pressure
- urine and fecal matter collection over 24-h for the 6 days in the whole room calorimeter
- blood collection and specialized meals to evaluate enteroendocrine signaling
- visual analog scale to assess hunger, satiety, and appetite during a test meal

Once inside the whole room calorimeter, participants followed a set routine that matched the activity routine in the clinical research unit run-in. Rest, meals, treadmill walking, desk time, free time, and sleeping

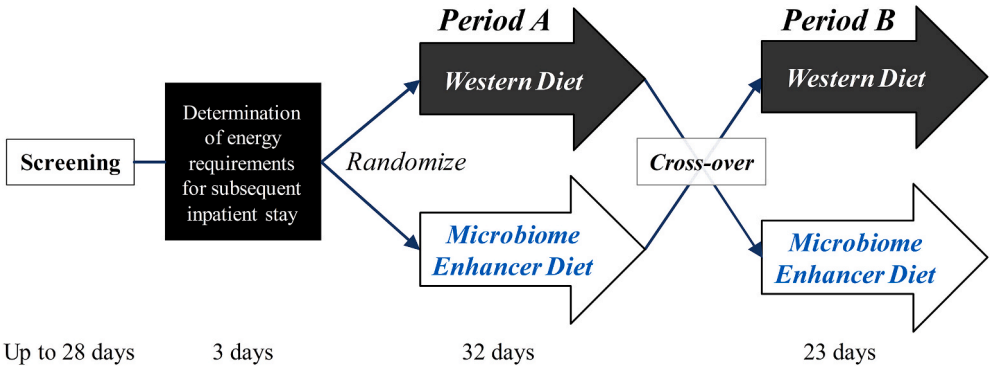

Fig. 1. Overall Study Design. This figure shows the screening period (up to 28 days), the initial period of activity monitoring (9 days) to determine initial energy requirements, randomization, and the two periods of outpatient and inpatient feeding (22 days) with diet assignment in random order.

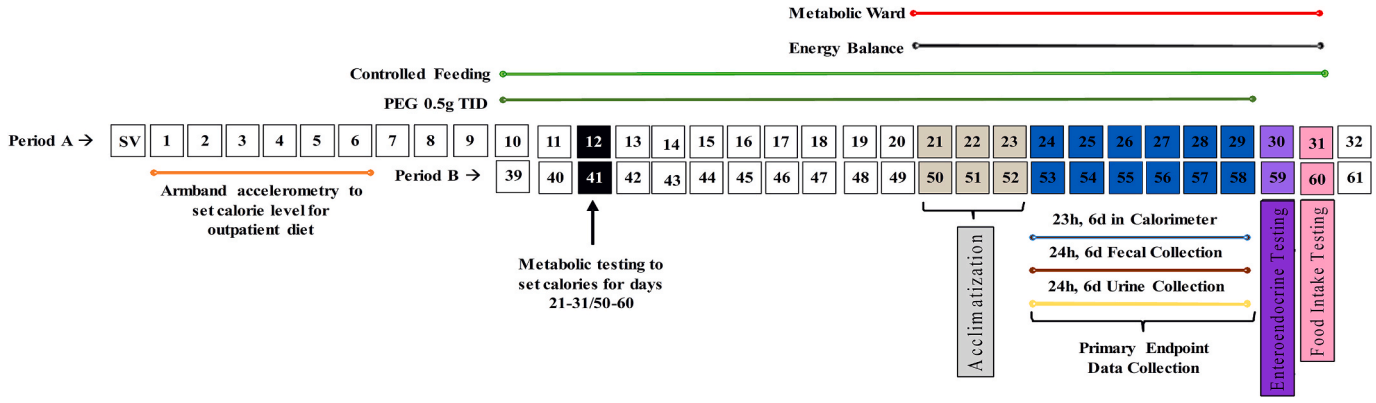

Fig. 2. Overview of Study Procedures and Endpoints. This figure shows the activities that occur during each study day. Abbreviations: D/C- discharge; SV-screening visit; PEG-polyethylene glycol; TID-three times daily.

were scripted to the minute and observed by the staff of the Clinical Research Unit (CRU) and the Energy Metabolism and Calorimetry Core through the calorimeter door and via camera [36].

2.4. Diet design and validation

2.4.1. Importance of the randomized crossover

Diet is one of the most potent modulators of the gut microbiome [37–41], although host factors such as genetics and geography also play a role [14,15,42]. In addition, the composition and diversity of each individual's microbiome influences response to diet [29,43]. Therefore, one of the challenges in understanding the physiological impact of the human gut microbiome is inter-person variability. We implemented a crossover design to allow us to see the effects of diet and changes in microbiome despite this variability by allowing each participant to serve as his/her own control.

2.4.2. Menu design and diet drivers

Menus were designed with the ProNutra research-diet design software (Version 3.5, Viocare, Inc, Princeton, NJ) with USDA Database Standard Reference 23. Menus were designed for each diet based on 2000 kcals per day, and the amounts were personalized to meet energy needs of participants by factoring the base menu to achieve the required kcal level. Once menus were assigned, any subsequent changes were made via the use of a "unit food". The unit food consisted of a beverage that matched the macronutrient and driver composition of the assigned diet. The beverage was included as part of each menu so it could be removed if kcals needed to be decreased. Additional volumes of the beverages were provided when kcal intake needed to be increased. During the whole room calorimetry days, changes were made based on predicted 24-h energy expenditure with a goal of achieving energy balance within ± 50 kcal/day to achieve a final energy balance of 50 kcals over the 6-day calorimetry period.

Based on the available literature, we designed the Microbiome Enhancer Diet as a tool to modify the gut microbiota by ensuring that more food reached the large intestine as compared to the Western Diet. We identified four key drivers that should increase the amount of food that enters the large intestine: (1) foods that are rich in dietary fibers; (2) foods that resist particle-size reduction; (3) foods that are high in resistant starch; and (4) minimally processed foods. The rationale for each driver is described briefly below.

Dietary fiber from whole grains, fruits, and vegetables is a critical influencer of gut microbial composition and metabolic state [38,41]. When considering chronic or lifelong exposures to fiber-rich foods, evidence suggests that under-consumption of fiber contributes to severe depletion of beneficial microbes [38,41,44,45]. Short-term (days to weeks) changes in fiber intake also alter gut microbiota composition and metabolic activity [16,38,46,47]. The Microbiome Enhancer Diet provided an average of 25.8 g of fiber/1000 kcals and the Western Diet provided an average of 6.5 g/1000 kcals.

Resistant starch is found in a subset of fiber-rich foods, such as whole grains (quinoa), legumes, and certain non-mature fruits [48]. Short-term (2-week) intake of a high level of resistant starch is associated with improved glucose metabolism, a change in bacterial community structure, and metabolomic alterations in lipid and bile acid metabolism [49]. Several other studies confirm the impact of short-term resistant starch intake on the gut microbiome [50–52]. The average resistant starch content of the Microbiome Enhancer Diet was estimated to be 10.3 g/1000 kcals and the Western Diet was 1.2 g/1000 kcals based on measured values-when available-merged with values from the literature

[53].

Foods with larger particle sizes are often lacking in the highly processed diets of modern societies [54]. Fine grinding of food particles increases energy bioavailability to the host for uptake prior to the colon [55]. The design of diets with larger particles is hypothesized to have beneficial effects on the human microbiota [54], but we are not aware of any clinical trials that have tested this directly. The microbiome enhancer was designed to focus on using whole foods with large particles. Similar foods were provided in the Western Diet but with small particles (i.e. whole nuts vs. nut butters).

Aside from having ingredients that are highly refined and small in particle size, diets high in processed foods have several "microbiome unfriendly" ingredients. Highly processed diets are high in simple sugars and fats, which have negative impacts on the microbiome [56]. In mice and humans, emulsifiers (additives used to promote suspensions, e.g. margarine) promote dysbiosis and a pro-inflammatory phenotype [57–59]. The Microbiome Enhancer Diet was devoid of any processed foods. Similar types of foods were selected for the Western Diet but in a processed version.

The control Western Diet mirrors typical US dietary intake [60], which is largely devoid of our microbiome enhancing drivers and contains nutrients that are known to have a negative impact on the microbiome (e.g., emulsifiers [57–59] and simple sugars [56]). This makes a diet based on population-level mean intakes an appropriate comparator diet to maximize within-participant shifts in microbiome and host phenotypes.

The western and Microbiome Enhancer Diet were matched in kcals and macronutrient composition. The average macronutrient composition of both diets was 17% protein, 35% fat and 52% carbohydrate. In addition, nutrients with known impacts on the gut microbiome, such as choline, betaine, and carnitine [61,62], were kept consistent in both diets. Foods were selected that were as similar as possible, with the main difference being in the drivers. For example: chunks of steak vs. ground beef, refined vs. whole grains, whole fruit vs. juice, and whole-grain breakfast foods vs. processed/prepackaged foods. Supplementary Table 1 contains a sample menu for each diet.

2.4.3. Length of diet

Although long-term dietary patterns are important for the establishment of the gut microbial communities that inhabit the human host [38], short-term diets (days-weeks) have profound effects [16,63–65]. We selected a combination of outpatient and inpatient controlled feeding periods that total 22 days. This length of time is appropriate for inducing microbiome changes [16,63–65]. Our crossover period was at least 14 days, which was sufficient to wash out the effect of the antecedent diet [16].

2.4.4. Validation, quality control and compliance

Once designed and tested for acceptability and palatability, sample meals were prepared, composited, and sent for chemical analysis of kcals, macronutrients (carbohydrates, protein and fat) and fiber (Covance, Inc). Resistant starch was measured only in high resistant starch foods as available assays have lower limits of detection that are well above the concentrations of resistant starch in our composited menus. Total resistant starch content was then estimated by combining measured values and tables available in the literature [53]. Revisions to the diets were made to achieve kcal and macronutrient targets that were balanced across the two diets. Quality control of prepared diets for each participant will be determined by sending a 6-day composite of duplicate meals consumed in the calorimeter for chemical analysis for each study participant. Chemical oxygen demand will also be measured as an additional metric of accuracy of total energy provided.

2.5. Run-in period (diet stabilization)

The outpatient diet period was intended to allow participants to become accustomed to the diet and for microbiome/metabolite profiles to reach stability prior to admission into our metabolic ward [16,66]). During this 11-day period, meals from the assigned diet were prepared based on kcal requirements determined from accelerometry data. Compliance with outpatient meals was monitored in several ways. Participants came to our center twice per week to pick up meals, bring back uneaten food (if any), and to consume one meal while in-house. During those visits, research staff evaluated and encouraged compliance. Weight was checked both at home and during these pickup times to model weight stability and as an indirect metric of compliance.

2.6. Metabolic ward setting, description

Our metabolic ward consists of an inpatient Clinical Research Unit, a Metabolic Kitchen, and all facilities needed for metabolic testing/phenotyping including whole room calorimetry, body composition, exercise testing, imaging, and a laboratory/biorepository. TRI developed algorithms to predict and achieve energy balance in a metabolic ward [67]. This capability is essential, as changes in energy balance can influence endocrinology and metabolism/substrate switching. In a recent study at TRI, we achieved excellent energy balance in the calorimeter as evidenced by net energy at 14 ± 12 kcal/day (0.56% of TDEE). We also established methods to predict and maintain energy balance in a whole-room calorimeter, for controlling the environment (temperature, activity, energy intake [EI]), for PEG administration to increase the precision of the fecal measurements, and to collect 24/7 fecal samples [67–69].

2.7. Energy balance procedures

2.7.1. Calorimetry: engineering/technical

Energy expenditure was assessed by indirect calorimetry from changes in O_2, CO_2, CH_4, and urinary nitrogen (UN) while participants were in a whole room respiratory chamber. The two "large room" TRI calorimeters (31,000 L) feature state-of-the-art technologies in a push – pull configuration. Inflow air consisted of dry medical air that was buffered through two compressed air tanks. Inflow and outflow O_2 and CO_2 were measured continuously using a paramagnetic O_2 sensor and CO_2 infrared analyzers (Siemens, Ultramat/Oxymat). Environmental conditions were controlled by an internal HVAC unit with a protocol specific temperature (22.5 °C) and humidity below 40%. The rate of chamber air flow in and out was adjusted by computer to maintain positive pressure and CO_2 below 0.4% inside the calorimeter via high accuracy mass flow controllers (MFCs). In parallel, we used a cavity ring-down analyzer (LGR, Los Gatos) paired with a multi inlet switch to allow us to sample chamber and inflow air to measure CH_4 concentration in inflow (medical air) and outflow (chamber air) and calculate volume of methane produced (VCH_4). Urinary Nitrogen (UN) was measured over 24 h for all days in the calorimeter. Stoichiometry equations were used to convert VO_2, VCO_2, VCH_4 and UN into energy expenditure units (kcal/day, Equation (1) [67]), non-protein RQ (npRQ, Equation (2) [70]) and substrate oxidation rates (g/day; Equations (3)–(5) [67]).

$$EE\cdot(\text{kcal/day})\cdot = \cdot 3.88\cdot VO_2\cdot(L) + \cdot 1.08\cdot VCO_2\cdot(L)\cdot - \cdot 1.57\cdot UN\cdot(g)\cdot - \cdot 0.642\ \ VCH_4\cdot(L)\cdot \quad (1)$$

$$npRQ = \frac{VCO2\ \ - (UN\ \ x\ \ 6.25\ \ x\ \ 0.774)}{VO2 - (UN\ \ x\ \ 6.25\ \ x\ \ 0.966)} \quad (2)$$

where, VCO_2 and VO_2 are given in l/min; (VCO_2); UN are grams of urinary nitrogen excreted by minute; 6.25 is the conversion factor to transform UN in oxidized protein; 0.774 and 0.966 are the liters of CO_2 produced and O_2 consumed per gram of protein oxidized.

$$\text{CarbOx} \cdot (\text{g}) \cdot = \cdot - \cdot 3.10 \cdots \text{VO}_2 \cdot (\text{L}) \cdot + \cdot 4.46 \cdots \text{VCO}_2 \cdot (\text{L}) - 3.86 \cdots \text{UN} \cdot (\text{g}) \quad (3)$$

$$\text{FatOx} \cdot (\text{g}) \cdot = \cdot 1.63 \cdots \text{VO}_2 \cdot (\text{L}) \cdot - \cdot 1.64 \cdots \text{VCO}_2 \cdot (\text{L}) \cdot - \cdot 1.77 \cdot \text{UN} \cdot (\text{g}) \quad (4)$$

$$\text{ProtOx} \cdot (\text{g}) \cdot = \cdot 6.25 \cdots \text{UN} \cdot (\text{g}) \quad (5)$$

The validation of this system relied on a gas blender (MEI Research, Ltd., Edina, MN) to infuse known amounts of nitrogen and CO_2 into the chamber to simulate O_2 consumption and CO_2 production. The blender consisted of four MFCs of different ranges allowing for an unlimited number of infusion profiles, all of which were traceable to National Institute of Standards and Technology (NIST). This setup allowed for periodic validation using an instrument calibrated to NIST standards. By using this technology, our respiratory chambers had accuracy of 0.48% (VO2) and −0.62% (VCO2). Overall performance characteristics generated for this study are presented in Table 1.

2.7.2. Prediction of energy needs

We followed methods similar to our published paper to predict energy needs [71], but utilized in-house calorimetry data to generate our own regression models. Briefly, free-living energy needs were predicted after a one-week assessment of energy expenditure with a multi-sensor device (described in section 2.8.2). An algorithm based on body composition from a DEXA was used to calculate an initial prediction for the single Day 12/41 chamber stay. The measured energy expenditure from the Day 12/41 chamber stay determined the base kcal level for the 6-day run of calorimeter stays (Days 24–29/53–58).

2.7.3. Maintenance of energy balance

Energy balance was established via currently known principles of metabolizable energy [72], which do not account directly for the contribution of microbial metabolism on ingested food or fecal energy losses. To achieve energy balance, a portion of the menu consisted of a macronutrient and driver-balanced beverage (See section 2.4.2 for additional details) that allowed for minor adjustments from day to day. Precise amounts of this beverage were removed or added based on predicted energy needs. Day 24/53 intake was exactly what was measured on Day 12/41. Each subsequent day's EI was based on the previous day's measured EE for the remaining 5 chamber days and on collaborative pattern analysis of various components of energy expenditure by our team.

Poor compliance with following the prescribed schedule, changes in free time activity, and change in sleep patterns can result in large deviations from the established EE baseline. In these cases, the team was able to adjust the intake level to compensate for these unexpected changes in calorimeter EE. This method combined with the consecutive days of controlled activity allowed us to achieve our intended energy balance within an average of 50 kcals/day over the 6 calorimeter days. Specifically, energy balance during Period A was −4.2 ± 26.7 kcal/day and during Period B was −1.78 ± 21.3 kcal/day. Energy balance was not different by period (F value = 1.38, P = 0.263 for time × period interaction) (Fig. 3). A second metric for energy balance was weight stability. We calculated weight stability by determining the daily percent change in weight as compared to the baseline weight (Day 12 for Period A and Day 41 for Period B) for each of the 6 inpatient calorimetry days (Days 24–29 for Period A and Days 53–58 for period B). During both Period A (Fig. 4A) and Period B (Fig. 4B) the slope % change in weight per day was quite small (−0.043% and −0.101%, respectively), which demonstrates weight was stable.

2.7.4. Polyethylene glycol and fecal energy losses

Precise measurements of fecal energy and calculated dietary energy extraction/digestion require knowledge of gut transit time, precise fecal collection procedures and careful chemical analysis. Traditional methods for determining gut transit time, which include non-digestible fecal markers, such as carmine red and charcoal [73], are subject to substantial error due to mixing of the intestinal contents during transit and uncertainty in identifying pre-vs. post-marker fecal material. An alternative is continuous marking, which uses ingestion of a constant amount of a non-absorbable and non-digestible substance, such as PEG [68,69,74]. Using this methodology, any analyte measured in the feces can be normalized to the PEG concentration to assess total production per day, as opposed to per gram of feces. For this study, the purpose of normalization with PEG is to extrapolate fecal energy losses over 24-h. Energy losses will be assessed by measuring fecal chemical oxygen demand (COD). Microbial ecology models use COD as a measure of electron equivalents in organic substances, because electrons are a conserved quantity in biochemical and microbiological reactions. COD is the amount of oxygen required to completely oxidize a unit weight of sample [75]. For example, hydrolysis and anaerobic fermentation

Table 1
Calorimetry variable performance characteristics by period. The coefficient of variation (CV) is presented for the 6-day calorimetry inpatient block for each study period. A total of 17 people completed both periods of calorimetry. * paired sample *t*-test between periods A and B for individual components of CV ($[(\Sigma^K M)^2/K)]-[\Sigma^K M^2]$, where K is the number of days in chamber; M is the absolute value of each day). Abbreviations: 24-h RQ = 24-h respiratory quotient; REE = Resting Energy Expenditure; RMR = Resting Metabolic Rate; $RQ_{Resting}$ = Respiratory Quotient During Rest; $RQ_{Sleeping}$ = Respiratory Quotient During Sleep; SEE = Sleeping Energy Expenditure; TDEE = Total Daily Energy Expenditure (24 h).

Variable		Period A		Period B			
		(n = 17)		*(n = 17)*		*Paired t-test**	
		CV (6-day)		CV (6-day)			
		Absolute	%	Absolute	%	t-Ratio	*P*
TDEE	*kcal/day*	39	1.9%	50	2.5%	1.449	0.167
RMR	*kcal/day*	76	4.7%	69	4.4%	−0.809	0.431
SEE	*kcal/day*	36	2.6%	44	3.2%	0.837	0.415
24-h RQ		0.010	1.2%	0.010	1.1%	−0.998	0.333
RQ_{Rest}		0.021	2.4%	0.020	2.3%	−0.497	0.626
RQ_{Sleep}		0.013	1.6%	0.012	1.5%	−0.599	0.557

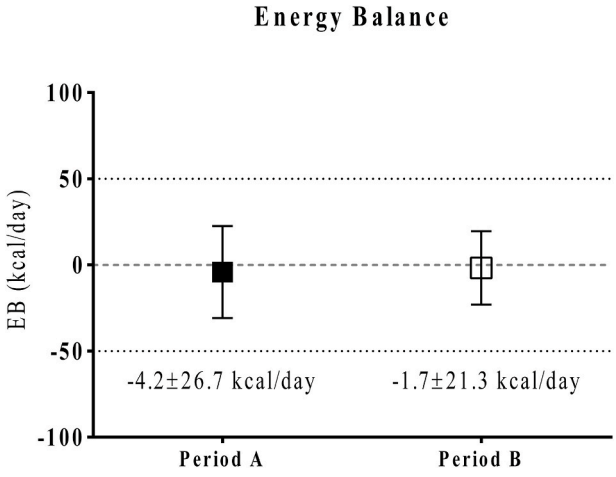

Fig. 3. Energy Balance. This figure shows the overall estimated energy balance within each 6-day calorimetry block (N = 17). Energy balance is defined by energy expended vs. kcals consumed. The mean energy balance per day for each 6-day calorimetry block was: Period A (mean±standard deviation) = −4.2 ± 26.7 kcal/day; Period B = −1.78 ± 21.3 kcal/day. There was no difference in energy balance between periods (F value = 1.38, P = 0.263 for time × period interaction).

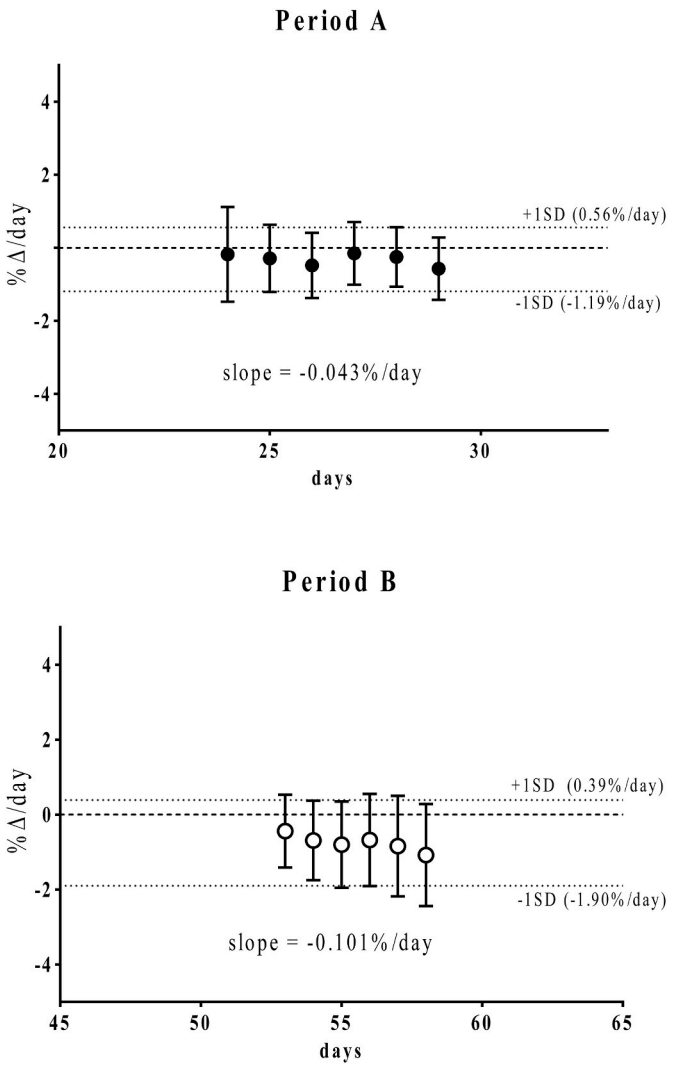

Fig. 4. Weight Stability. This figure shows weight stability (N = 17) during all inpatient calorimetry assessments. Weight stability was calculated by determining the percent change in weight for each calorimetry day as compared to baseline. A slope was calculated to quantify the weight change trend, which was indicative of weight stability. A) Period A slope % weight change trend was −0.043% (Baseline Day 12 compared to Calorimetry Days 24–29). B) Period B slope % weight change trend was −0.101% (Baseline Day 41 compared to Calorimetry Days 53–58).

reactions do not change the COD. By measuring COD in the diet and subtracting the COD in the stool, in gas streams (e.g., CH_4), and captured by reduced inorganic products (e.g., H_2S), we can compute the COD absorbed by the human. The room calorimeter then offers the complementary means to directly measure energy utilization by the human.

We chose PEG because of (i) its availability (over the counter) and safety, (ii) precise and accurate methods for measuring PEG [69], (iii) excellent tolerability at the dose used (which does not induce osmotic stress, a known microbiome modulator [76]), and (iv) decreased variability when compared to traditional fecal marker methods. The PEG method enables "a metabolic study as short as 6–7 days rather than the customary 12–16 days" [68]. Given that several days are needed to stabilize fecal PEG content, we administered PEG for 14 days prior to the collection period. A study at TRI confirmed the utility of this approach (NCT01967563). PEG capsules (500 mg each) were made in a compounding pharmacy with quality control data provided on the weight of each capsule (weights were accurate within 2.13%). In addition, we did a second round of QC by re-weighing 100 capsules and we found the weights to be accurate within 2.80%.

2.7.5. Gut transit time

Diet [16,40,41] and gut transit [77] have a strong effect on the gut microbiota, which collectively impact host health [78]. In addition to assessing gut transit as a function of total 24-h fecal collection, we directly measured it. While in the calorimeter, participants orally ingested a small, single-use, nondigestible capsule (SmartPill™ Motility Testing System, Medtronic) that sent data wirelessly to a sensor worn by the participant to record internal temperature, pressure, and pH. These data will be used to determine whole gut transit and transit within specific segments of the gut: gastric emptying, small bowel transit time, and colonic transit time. The capsule normally remains in the body for 24–72 h [79]. Fecal samples were collected until the capsule was retrieved. Fecal collection continued for all 6 chamber days even if we confirmed pill exit before completion of calorimetry testing.

2.7.6. Gastric emptying

Orally administered acetaminophen has been used extensively to assess gastric emptying in humans [80]. Orally administrated acetaminophen is rapidly absorbed by the small intestine, but not by the stomach. The acetaminophen method correlates well with scintigraphic measurements without radiation exposure [80]. We assessed gastric emptying via acetaminophen appearance after a test meal administered during the enteroendocrine day. Participants were provided with a dose of acetaminophen of 1.5 g at nominal time point zero. Blood sampling for determining acetaminophen concentrations was performed at each of the pre-defined nominal time points for the breakfast meal: −30, −15, +30, +60, +120, and +180 min.

2.7.7. Food intake testing

To evaluate hunger and satiety, we employed a visual analog scale (VAS) methodology, which is a reproducible and valid method to evaluate these parameters [81]. Participants were fed a 500-kcal breakfast of the same foods from their assigned diet. For lunch and dinner, they were presented with a buffet of foods from the assigned diet (1.5 X the energy content of their energy balanced diet) and instructed to eat as much or as little as they liked. During the meal testing, participants were monitored. Participants were asked to complete a survey to identify their perception of hunger on a line with qualifying statements such as "Not at all"/"The least I can possibly" and "Extremely"/"The most I can possibly" anchoring the line on the extreme left and right side, respectively. In response to each question, participants were asked to draw a vertical mark on the horizontal line to represent the magnitude of their response to the question. A value for each response was quantified by measuring the distance of their mark (in mm) relative to the left end of the line. Therefore, the values (or "scores") for each question ranged from 0 to 100. Before the food intake questions, participants answered two unrelated questions to reduce the impact of list placement on low agreement with the first question [82]. VAS scales were administered at −30, −15, +30, +60, +120, and +180 min pre/post each meal. Unconsumed foods were weighed on electronic balance scales to the nearest gram. Calorie and macronutrient consumption were calculated using ProNutra (Version 3.5, Viocare, Inc, Princeton, NJ) with USDA Database Standard Reference 23.

2.7.8. Magnetic resonance imaging and spectroscopy

We utilized magnetic resonance imaging and spectroscopy (MRI/S) to evaluate host factors that could impact overall energy homeostasis, namely hepatic fat [83–85] and organ size [86,87]. To achieve this, we assessed lipid content, volumetric fat and organ volume quantitation using an Acheiva 3T (Philips, Amsterdam, the Netherlands). Intrahepatic Lipid (IHL) content was measured using both imaging and spectroscopy. Volumetric measurement of fat, muscle, organs and bone was completed across the whole body.

2.8. Primary and secondary endpoints

2.8.1. Primary endpoint

The primary endpoint for the protocol was the within-participant difference in 24-h fecal energy, as measured by COD, normalized to the total daily energy intake and to the non-metabolizable marker PEG [COD (mg/g stool)/PEG (g)]. We were testing the hypothesis that fecal COD will be higher on the Microbiome Enhancer Diet vs. the Western Diet.

2.8.2. Principal secondary endpoints

The principal secondary endpoints tested hypotheses about how changes in the gut microbiota might change enteroendocrine hormone secretion, hunger/satiety, and food intake.

2.9. Biological samples and analytes

2.9.1. Blood

We collected blood samples for measurements of bacterial and host metabolites, enteroendocrine hormones, acetaminophen, and other exploratory endpoints. Samples were processed via standard clinical laboratory procedures.

2.9.2. Urine

Urine was collected for each 24-h period over the 6-day chamber stay. Nitrogen/creatinine were measured for the calculation of substrate (fat, carbohydrates and protein) oxidation rates as well as npRQ. Urinary Creatinine (mg/24 h collection) was used to monitor the completeness of the 24-h urine collection and was also used to adjust 24-h total urea nitrogen to account for day-to-day variation in the collections due to incomplete voiding and/or incomplete or missing urine sample [36].

2.9.3. Stool

All fecal matter produced was collected over each of the two 6-day periods in the calorimeter. The stool was either aliquoted daily for or composited over 6 days to measure fecal energy losses and PEG. Samples were processed in an anaerobic chamber within 1 h of being produced. These samples will be used to quantify COD and to analyze the microbial community structure and function. Briefly, we will quantify the effects of the two diets on microbial community in three ways. First, we will characterize the *alpha*- and *beta*-diversity of bacteria and archaea, and taxonomic changes to understand how the Microbiome Enhancer Diet changes the richness, diversity, and composition of those microorganisms in the gut. The second is by combining two measurements on the stool samples collected and homogenized over the 6-day period: 1) total bacteria via quantitative PCR (qPCR) 2) the measurement of PEG. This combination of measurements will give us an estimate of microbial enhancement in terms of total bacterial 16s rRNA gene copies per day. Third, we will analyze metabolites in the samples and link important metabolites with microbial pathways and with specific microbes. A major innovation that will be achieved by the data generated in this study is the ability to calculate the impact of food on the host by accounting for the role of gut microbes on energy extraction.

2.10. Power analysis

Approach and assumptions in the power analysis (Primary endpoint, Fecal COD): Source data for this power analysis came from replicate samples from 10 participants having within- and between-participant variances, as well as technical replicates. Replicate variability of the COD method was approximately 3.0%. These variances were in line with those reported for fecal calcium [88]. Our power analysis indicates that a sample size of $n = 14$ (completers) is needed to observe an effect size of roughly 80 kcal/day at 80% power. Our model predicted a delta of approximately 110 kcal/day in fecal COD between the two dietary interventions, thus we were sufficiently powered with $n = 18$ to observe the predicted difference in fecal energy in this study. From reviews of published reports [68,69], using PEG administration to normalize fecal energy measurements will decrease variability from 18% to 3%. Six consecutive calorimeter stays and measuring a composite of six days of feces and urine further increased our power. Thus, we anticipated more power than illustrated by our initial power analysis.

For energy expenditure, we used data from a previous study at our facility (NCT01967563 [67]) as the basis for many of the procedures employed in this study: strict control of diet energy content (metabolic kitchen), the environment (CRU), and prescribed/observed physical activity. Based on test-retest stability using analogous clinical procedures, we were well powered to detect changes in total daily energy expenditure - and sleeping EE - down to <6% (approximately 120 kcal/day). We assumed no meaningful changes in body composition over 6 days. Lastly, these power analyses were constructed based on two (paired) days in the calorimeter. Six consecutive calorimeter days and measuring a composite of six days of feces and urine further increased our power. Indeed, upon completion of all calorimetry measurements on 17 completers, we determined that 26.5 kcal/day is the minimum detectable difference we can expect to at 80% power with 6 repeated measures. Therefore, the study was closed with 17 participants rather than the 18 that were in the original power estimate.

2.11. Statistical analysis plan

We will use repeated measures and a within-participant model (SAS PROC MIXED with diet and participant as factors in the model) to compare the integrated six-day energy balance for the control vs. Microbiome Enhancer Diet [89]. Secondary analyses/endpoints, using the same statistical analysis approach, include energy/nutrient absorption (as a % of the ingested kcals) for the Western Diet vs. Microbiome Enhancer Diet. Once the mechanistic *in silico* model is well developed, we will compare model outputs (predictions) to directly measured (observed) energy absorption using our state-of-the-art metabolic-ward techniques. For enteroendocrine hormones, the primary analysis will be the $AUC_{24\ h}$ of enteroendocrine secretions. Each hormone will be analyzed independently. Once again, we will use SAS PROC MIXED with diet and participant as factors in the model. Additional analyses will include diet effects on gastric emptying, bowel transit time, and measured food intake/VAS.

3. Discussion

3.1. Advantages of the design and procedures

We implemented an integrated approach to understand how the composition of the diet plays a pivotal role for the gut microbiota and, consequently, the host's energy balance. In the clinic, we controlled diet and environmental conditions as we collected key clinical data (e.g., gut transit time, gastric emptying, enteroendocrine hormones, appetite, weight and body composition, energy balance, and methane production). Because the crossover nature and clinically controlled conditions reduce noise and the impact of interindividual variability, we expect to generate high quality data that is amenable to quantitative interrogation. The data generated in the clinical study will also help us develop and refine an *in silico* model that integrates human energy balance with the microbial ecology of the intestines. Ultimately, the model will allow us to quantify the microbial contribution to energy balance.

3.2. Limitations

There are some disadvantages to tightly controlled trials in comparison to "real-world" settings. While tight control is ideal for establishing new mechanistic paradigms, it may limit generalizability. For example, if the control diet led to significant alterations in an individual's microbiome, our results might not be reflective of the true

impact of a Microbiome Enhancer Diet on that person's metabolism. The specific environmental conditions in our local area and our metabolic ward are likely to affect the results. Additional, multi-center studies will be needed in diverse geographical regions to establish broader translatability.

The Microbiome Enhancer Diet was designed to yield a maximum delta in the microbiome and not as a therapeutic diet intended to be implemented broadly. Future studies will be needed to identify the specific drivers mediating the effects and scalable approaches to convert our discoveries into dietary recommendations.

Finally, we selected generally healthy individuals within a reasonable range of ages and body sizes to reduce variability. However, the long-term impact of factors such as the genome, epigenome, usual dietary habits, medication use, and other environmental influences could confound our results in ways that are impossible to fully control. We established a biobank that will allow us to evaluate some of these factors to generate additional hypotheses and mechanistic insights. The fact that we restricted our population to generally healthy people also limits our ability to understand the impact of disease states such as obesity, cancer, diabetes and non-alcoholic fatty liver disease. Follow-up studies in various disease states are needed.

3.3. Future directions

Our ultimate goal is to build a mathematical model to reveal initial cause-and-effect links between environmental parameters (i.e., diets of different composition) with quantitative measurements of human metabolism and detailed laboratory measurements of the gut microbial ecology, including stool constituents (chemical and microbiological). To achieve this, we built a prototype mathematical model and will continue to refine an integrated mathematical framework that establishes mass and energy balances in human participants and their colonic microbial communities. In addition, we will accomplish our mathematical framework by measuring COD to establish the electron balance on each participant.

This clinical trial will generate data to support quantitative advancements in understanding the interactions between diet, gut microbiome, and host. The methods and insights gained from this study will allow future investigations aimed at defining the role of the microbiome on human metabolic disease with a goal of moving from simple associations to causal understanding. In addition, the mathematical models we will build have the potential to inform clinical decision making [90] and therapeutics (nutritional and pharmaceutical) [91,92]. This is highly relevant given the physiological interconnectedness of the microbiome and human host.

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Declaration of competing interest

Rosa Krajmalnik-Brown and Bruce Rittman declare the following patent as a potential conflict of interest: Reducing short-chain fatty acids and energy uptake in obese humans by managing their intestinal microbial communities; Patent number: 9,549,955. All other authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.conctc.2020.100646.

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