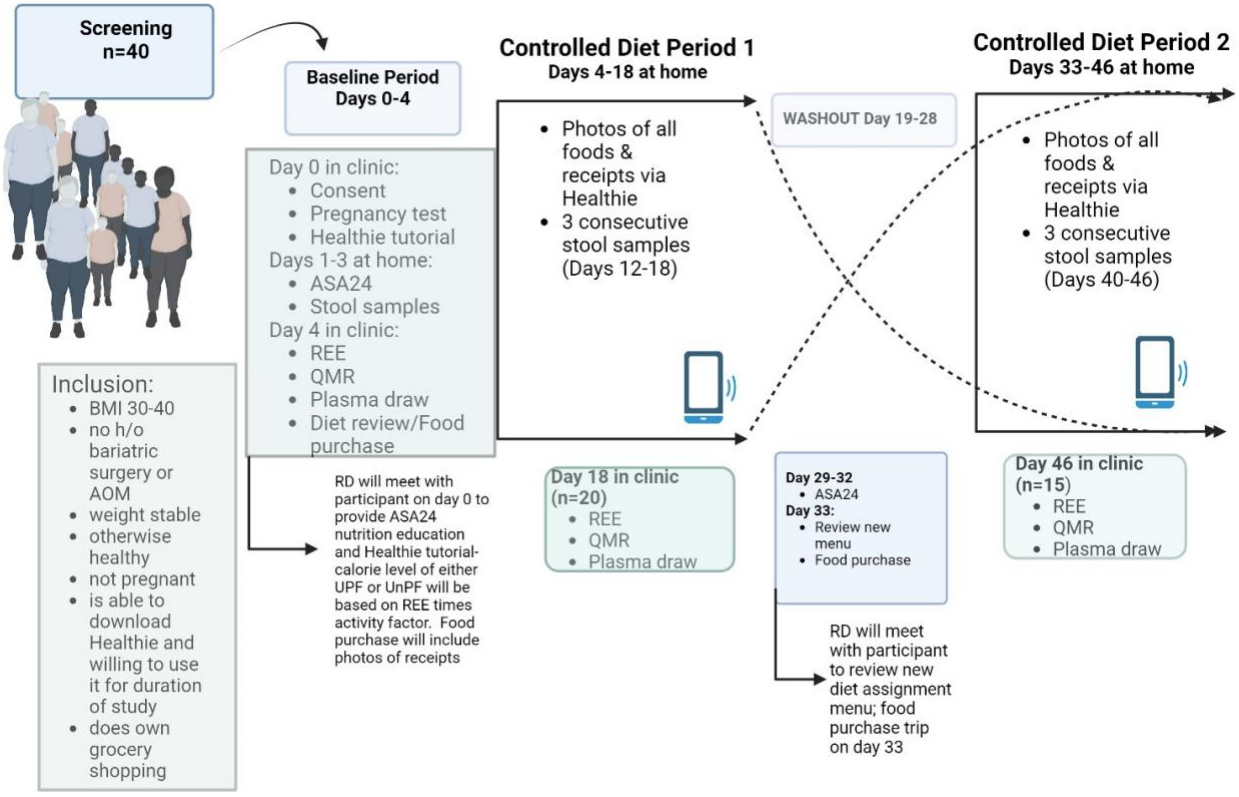
***Design overview:***

The proposed study will employ a randomized crossover-controlled feeding trial. Each participant will undergo two phases separated by a washout period. Each phase will represent one of two conditions: 80% ultraprocessed foods (UPFs) vs. 20% UPFs. Participants will consume isocaloric diets differing only in the level of processing, and the diets will be designed to match the participants' individual energy needs to maintain baseline body weight.

**Figure 1. Research design**




***Procedures:***

*Recruitment*

Healthy adults with obesity (30-40 kg/m$^2$) will be recruited by flyers posted in high traffic areas in the catchment areas of CUIMC/NYP and Columbia University's RecruitMe website. Additional recruitment methods involve in-person presentations to campus and community groups. Interested individuals will contact study staff for initial screening and review of eligibility criteria. Participants will be screened for any medical history contraindicated to metabolic studies (i.e., current or recent history of AOMs, other medications/supplements known to affect metabolism with no intention to change current medication/supplements for duration of study, recent antibiotic use, currently pregnant or intending to become pregnant), not actively trying to lose or gain weight, no known food allergies, and willing to commit to a 9 weeks study. Participants will also have to verbalize that they do their own grocery shopping. Participants who

successfully screen in will receive remuneration for their time and provided food for the two diet phases.

*Baseline (Day 0)*

At the start of their first study phase, accrued participants will complete a 5-day baseline period. On the first day of the baseline period (Day 0), participants will come to the laboratory to provide consent and receive a tutorial of the Healthie app, a HIPAA compliant dietetic practice app, that will be used as a compliance assessment tool and a communication tool. Healthie is a cloud-based practice management platform designed for nutrition and wellness professionals. The Healthie platform can be accessed from the web browser or through iOS or Android app. Participants will receive an assigned one time log in credential to access Healthie; this will be provided as Gmail alias accounts that will be administered through a main study Gmail account that will only be used for one time log-ins to the HIPAA compliant app at the Day 0 in person visit. There is no exchange of PHI/PII through the Gmail account. Participants will be provided with an email address and password to use that is appended to the main email account and follows the format of "email address+ParticipantIDnumber", which looks like an unique address but this way participants will not have to use their own personal email accounts, further protecting their anonymity with Healthie. Participants will be given an overview of the Healthie app, the NIH's Automated Self-Administered 24-h Diet Record tool and provided stool collection kits and instructions for use and shipping to the investigator. Additionally, pregnancy will be assessed by urine dipstick.

*Baseline (Days 1-3):*

Over the next 3 days, participants will electronically record all foods and beverages consumed each day using the NIH's Automated Self-Administered 24-h Diet Record tool. Participants will also be asked to collect and provide the date and time of at least 3 consecutive stool samples with date and time. The participants will ship the samples to CUIMC researchers. These samples will be deidentified and sent to UNC for microbiome analysis.

*Baseline (Day 4)*

On the fourth day of the of the baseline period (Day 4), participants will return to the clinic to have body composition, energy expenditure and cardiometabolic measures will be performed before randomization to their diet assignment order.

- *Body Composition* will be measured by quantitative magnetic resonance (QMR). QMR (Echo-MRI; Echo Medical Systems, Houston TX) is a non-imaging technique that uses a magnetic field to detect the hydrogen atoms of fat, lean tissue and water[4]. These protons have different spin characteristics according to their environment or tissue that are attached to that can be detected. The processed signal is obtained from the whole body at once. Participants will be scanned a total of three times for a total measurement time of no more than 10 minutes.

*Resting energy expenditure* (REE) will be measured using whole room indirect calorimetry[5]. Data collection will occur using a whole-room calorimeter consisting of an air-tight temperature-controlled room with pre-specified flow rate connected to a pull through calorimetry system (Sable Systems Intl, Las Vegas NV). By flowing a known amount of fresh air through the room,

respiratory gases from the study participant are sampled on the exhaust side of the system for measurement of oxygen and carbon dioxide concentrations. The respiratory gases are analyzed using fuel cell oxygen and near infrared carbon dioxide sensors (Model GA-3m2, Sable Systems International, Las Vegas, NV). All acquisition and analysis software for a SW-Promethium System is installed on a Sable Systems approved computer, with a Sable Systems Promethium Interface Module (Model IM-2). Data are recorded and processed on-line by the Sablesoftware programs Caloscreen and Expedata (Sable Systems International, Las Vegas, NV). During the 60-minute data collection period, the participant will be asked to remain motionless, awake and relaxed. The first 10 minutes of data collection and the final 10 minutes of data collection are discarded REE is estimated from the expiratory gases Participants will be asked to come in fasting (12 hours) and required not to exercise during the 24hr period before the measure. Participants will be monitored to ensure they remain awake during the measure.

- *Cardiometabolic markers:* Plasma will be collected after the REE measure. This includes fasting plasma glucose, insulin, c-peptide, serum lipid and lipoproteins, and inflammatory markers and metabolomics related to bacterial metabolism (e.g. LPS ).

*Diet Randomization Assignment*

On the final day of the baseline period, participants will meet with a registered dietitian nutritionist (RDN) to receive their diet prescription for the phase. The RDN will provide participants with detailed daily menus for each day of the phase along with a complete list of foods and beverages and amounts needed. Participants will be provided with a stipend and guidance on where to purchase food. They will be told to consume all foods and beverages listed on the respective day's menu and the specific times of day at which to consume meals. Participants will also be instructed not to consume any foods beyond those listed on the menus and to record any deviations from the protocol, including photos of any additional foods consumed and nutrition information. Compliance will further be assessed through 1) collection of receipts to verify food purchases, 2) participant provision of time-stamped photos of the empty food packages following consumption.

These procedures will be repeated for the second phase following a minimum 14-d washout period. Participants will be counseled to return to their usual diet as documented during the baseline phase (Days 1-3).

*Diet conditions (Days 4-18 and 33-46):*

Each diet period will be isocaloric and have similar energy densities, fiber content, micronutrient content, palatability, and CODEX Alimentarius annotated food additives to best isolate the effect of processing on intestinal energy harvest. Each diet period will mirror the other with the only difference being the level of UPF or unprocessed food. During each diet period, participants will be asked to photograph all their foods and upload to the HIPAA compliant app, Healthie, along with receipts, to help the investigator assess compliance to diet.

*Measures during diet periods 1 and 2:* During each diet period, participants will be asked to provide *3 consecutive stool samples* in the same fashion as the baseline period. At the end of each diet phase, participants will be asked to return to the clinic for a repeated measure of QMR, REE and cardiometabolic markers.

*Outcome and process assessments*: All energy intake, blood, energy expenditure and body

composition data will be collected and analyzed at the Human Phenotyping Core (HPC), NYNORC, along with dietary counseling. All microbiome data will be shipped to and analyzed at the Microbiome Laboratory, UNC NORC. Fecal samples will be saved in sterile plastic containers and frozen within 24 hours of collection until final processing and analysis.

*Stool sample collection and storage:* Samples will be collected according to UNC published protocols[1,3,10,7] . Briefly, stool samples will be collected by participants at home using stool colletion kits. The participants will be provided supplies to record date and time and immediately same day ship each sample with cold packs to the investigator. Recorded data will be checked by the investigator for completeness and samples will be sent on ice to the UNC Microbiome Laboratory where the sample will be weighed and then mechanically homogenized and partitioned into as many 2 mL cryotubes as possible. Aliquots of fecal samples will be stored in a -80ºC freezer.

*Bomb calorimetry*: Frozen fecal samples will be used to assess stool calorie loss (a proxy for the degree of energy harvest from the intestine). An aliquot of stool will be mixed with distilled water equal to total weight of the fecal sample. This mixture will then be homogenized and dehydrated (incubated at 65˚C for 48 hours)[2]. Next, the dried fecal pellets will be used to determine the energy content of stool samples via bomb calorimetry using the Parr 6200 Calorimeter with a Parr 6510 water handling system (PARR Instrument Co). Weighed Samples will be run in duplicate, and results averaged. If duplicate samples are >40 kcal apart, calorimetry will be repeated until duplicate samples are <40 kcal apart or the mean of four consecutive readings will be used. Benzoic acid standards will be used following 10 samples for standardization. The calorie loss from stool will be calculated as

(i) kcal per g of sample (weight of the entire stool sample is recorded at the time of homogenization), and
(ii) relative energy content (# of kcal in stool sample divided by the number of kcal consumed the previous day).

*16S rRNA gene sequencing*: Genomic microbial DNA from fecal samples will be isolated[11]. Fecal microbiotas will be characterized by creating sequencing libraries from the variable 4 (V4) region of the 16S rRNA gene (515 bp-806 bp) using PCR and sequencing on the Illumina MiSeq platform at the High-Throughput Sequencing Facility in the Carolina Center for Genome Sciences at UNC[6,11]. The DADA2 pipeline will generate SVs at a 100% identity threshold. Read counts will be normalized prior to any statistical analysis[9]. Taxonomic classification will be performed using DADA2-formatted reference databases (such as silva) with assignTaxonom and addSpecies functions in DADA2. This approach will generate taxonomic abundances (phylum-genus level) and diversity measures (α- and β-diversity) of gut microbiotas in mice. Gut microbiotas will also be characterized by 2 × 150 bp paired-end whole genome sequencing of fecal microbial DNA using the Illumina NovaSeq 6000 platform at the same high throughput sequencing facility. Sequences will be taxonomically classified using the Kraken2 pipeline 20. Metabolic pathways will be profiled using the HUMAnN2 pipeline and the MetaCyc database 4,8. This approach will generate taxonomic abundances (phylum-species level), α- and β-diversity measures, and metabolic pathway abundances.

## ***Statistical analysis***

The primary outcomes are fecal energy harvest differences in the two diet periods compared to baseline and changes in microbial taxa between baseline and diet period 1 and baseline and diet

period 2. Statistics associated with diet treatment will be performed using linear models implemented in Stata. A mixed effect model will be used to include a random intercept term. Exploratory path analysis with multiple mediators will be utilized to understand mechanisms of change and estimate effect sizes. Covariates will be included to control for differences, if any. Links between additive exposures and study outcomes will be explored.

***Anticipated participation***

We anticipate screening n=40 to obtain our n=20 participants for enrollment. We have budgeted for n=15 study completers.

***Additional Information***

All measurements collected have strict manuals in place and will be conducted by individuals certified to perform them. Measures of weight and height and body composition by QMR, and REE will be performed by certified staff in the respective HPC labs. Rigorous quality control procedures are in place for instrument calibration, collection of measures, analysis of data, data storage and transfer to PI. All data are deidentified at time of collection and transmitted via secured channels This proposal will be carried out at the NYNORC by Dr. Whyte and the UNC-CH NORC by Dr. Carroll.

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