INTERVENTIONAL
RESEARCH PROTOCOL TEMPLATE

(HRP-503a)

STUDY INFORMATION

- **Title of Project**
  The Effect of Concord grape polyphenol-soy protein isolate complex (GP-SPI) on Gut Microbiota

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1.0 Research Design

1.1 Purpose/Specific Aims

The overall purpose of this study is to evaluate the effect of nutritional supplementation with a well-characterized preparation of Concord grape polyphenol-soy protein isolate (**GP-SPI**) on the composition of the gut microbiota.

A. Objectives

Primary objective:

- To evaluate the effect of nutritional supplementation with GP-SPI on gut microbiota composition.

Secondary objectives:

- To evaluate the effect of GP-SPI on kidney and liver health/function via comprehensive metabolic panel (**CMP**).
- Collect fecal, blood, and urine samples and store for a microbiome-wide association study (**MWAS,** funded by R01 AT010242-01).
- Obtain consent to enter de-identified study results into microbiome and metabolome nationwide data sharing database.

B. Hypotheses / Research Question(s)

We hypothesize that healthy subjects eating a diet supplemented with GP-SPI will demonstrate an increase in intestinal abundance of *Akkermansia* spp. within a short time period as well as changes in other genera relevant to promotion of intestinal/metabolic health. Such changes may explain, at least in part, how poorly absorbed dietary polyphenols from fruits and vegetables improve human resilience against chronic metabolic diseases.

1.2 Research Significance (500 words or less)

The proposed study will test the *hypothesis* that oral supplementation with GP-SPI complex can alter the human microbiome in a direction that is consistent with improved health and metabolic resiliency. Epidemiological, clinical, and preclinical data indicate that polyphenol compounds derived from food, such as berries, fruits, vegetables, and herbs have beneficial health effects in humans (1). The B-type proanthocyanidin (**PAC**) class of polyphenols contained in grape berries, especially skins and seeds, have been associated with health benefits (2; 3); however, PACs are poorly absorbed and reach high concentration only in the colon (4) raising questions about mechanism(s) of action. We and others have shown that dietary PACs from grape and cranberry alter the gut microbiota in association with metabolic resilience (5; 6). PACs are also biotransformed by gut bacteria to yield microbial metabolites (**MM**s) that may contribute to health benefits (3).

Sensitive to temperature and pH, polyphenols are often labile and lose effectiveness once extracted. Studies in the Raskin lab (7-11) have established methods to increase the stability of extracted dietary polyphenols through sorption to protein rich foods, such as SPI. The sorption process does not require any non-food additives. Dietary polyphenols extracted from blueberries and green tea were sorbed to SPI and this polyphenol-protein complex (dosed at 40 g/day for 17 days and delivering 2136 mg total polyphenols/day) was tested in human clinical studies (12; 13).

Biochemical properties of the GP-SPI food ingredient are well-documented (9; 10) and GP-SPI and SPI supplements have been tested extensively in mice (6; 9; 10; 14). The proposed study is a logical follow up to animal studies, which showed that compared to control mice fed a high-fat diet (HFD) supplemented with SPI alone, mice fed an isocaloric HFD supplemented with GP-SPI exhibited greater resistance to weight gain, adiposity, and glucose intolerance (6). These effects were accompanied by changes in murine gut microbiota composition, including increased abundance of the microbe *Akkermansia muciniphila*,

associated with metabolic resilience (6). Similar gut microbiota changes were observed in lean mice fed low-fat diet (LFD) supplemented with GP-SPI (14).

The proposed study will first investigate how short-term supplementation with GP-SPI may modify the gut microbial community in healthy participants while monitoring liver and kidney function/health. We will also perform a longitudinal, microbiome-wide association study (MWAS) to identify gut bacteria species/strains that are positively or negatively associated with GP-SPI supplementation. Metabolomics analysis will be performed on collected urine and blood samples to identify/quantify known and unknown metabolites. Shotgun metagenomic sequencing will be performed on fecal samples to generate high quality draft genomes for species/strain level identification. These dynamic data sets will serve as input for the MWAS to correlate increasing/decreasing levels of gut bacterial species/strains to increasing/decreasing metabolites in blood and urine. These bacteria-metabolite associations will then be used to infer cause-effect relationships that can be further tested *in vitro*. We expect that successful completion of these studies will contribute to mechanistic explanations for how dietary polyphenols such as grape PACs alter the gut microbiota and resulting MM to promote metabolic health.

1.3 Research Design and Methods

Overview

This is a pilot time course study to characterize the effect of GP-SPI on the gut (i.e. fecal) bacterial community of healthy subjects. Following screening, enrolled subjects will washout of PAC-rich foods for 5 days while also consuming SPI. Subjects will then consume GP-SPI over a 10-day supplementation period. Fecal, urine, and blood samples will be collected. Comprehensive metabolic panel (CMP) tests performed before and after the GP-SPI supplementation period will monitor liver and kidney health. Using deidentified fecal, urine, and blood samples collected from time course study a MWAS study (funded by R01 AT010242-01) will be performed.

Successful completion of this pilot study will yield data that will inform the design of a follow-up placebo-controlled intervention study, which will be described in a separate clinical protocol that will be submitted to IRB for approval.

A. Research Procedures

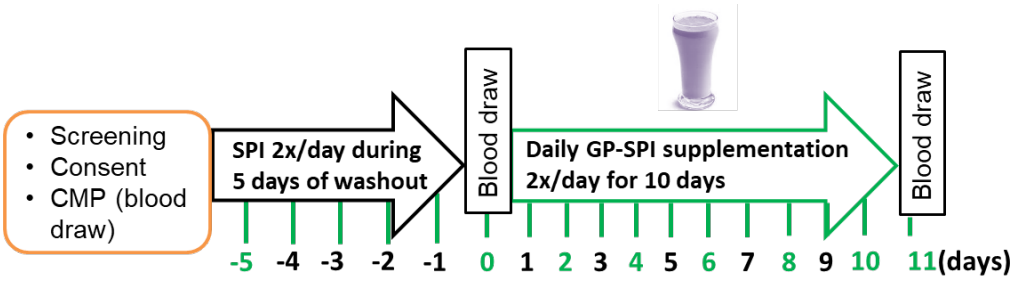

Figure 1. Clinical Study Design

Location

Screening and out-patient visits will take place in the Surgery Clinic in the Clinical Academic Building (CAB) or Surgery Department (MEB 5) and NJ Institute of Food Nutrition and Health (IFNH).

Recruitment

Prospective participants will be recruited through flyers posted locally on Rutgers University campuses and sent to university email lists (**Flyer** attached). Interested subjects will call in or email to receive further information about the study. A member of the study team will give a brief summary of the study. If the subject requests a screening appointment, the investigator will ask for verbal consent to proceed with a list

of questions (**Telephone Script** attached). Collecting this information at this time assists in expediting the paperwork during the visit and spares ineligible subjects the inconvenience of a futile appointment.

Eligibility Screening
At the screening visit (at IFNH), a member of the study team will explain the study in detail to the subject. Full explanation of study procedures and potential risks will be provided. The subject will have ample time to read the Consent and ask questions before signing and proceeding with screening procedures. A signed copy of Consent form (form attached) will be given to the subject.

After consent a participant ID will be assigned to subject. Demographics, medical history, height, and weight (**Demographics** and **Medical History** forms attached) will be recorded for each subject. Females will be given a urine-based pregnancy test onsite and result will be recorded. If inclusion criteria are met at this time, subject will be asked to schedule a visit at CAB with study nurses for a blood draw to perform CMP screening test. If CMP results are within normal range the subject will be enrolled and return to IFNH to receive study materials needed for participation in the study. If any CMP values are outside normal ranges subject will be advised that they do not fulfill all eligibility criteria, they will be given a copy of their CMP results, and if necessary, advised to see their primary care provider. All screened subjects will be documented on **Screening and Enrollment Log** (attached).

The final selection will be contingent upon the individuals meeting all eligibility criteria and deemed capable of complying with the study protocol.

Study Procedures

- Thirty subjects will be enrolled.

- **Food List**: Participants will maintain their usual diet except for a provided list of PAC-rich foods that they will be asked to abstain from for a 5-day wash-out period and during the 10-day intervention. The Food List is provided below. The goal is to have GP-SPI as the main/only source of PAC in the diet for the study period.

- **Wash-out and SPI (Day -5 through -1):** On day -5 (pre-baseline) before any supplementation, each subject will collect their fecal and urine sample. Each subject will then consume SPI twice a day (provided as pre-weighed packets of 20 g) during a 5-day wash-out period (days -5, -4, -3, -2, and -1). Subjects will be instructed to mix each packet of SPI in 250 mL of water or in a smoothie (recipe example below will be provided) using allowed foods as detailed in instructions and consume once in the morning before breakfast and once in the evening before dinner.

- **Day 0:** On the following day each subject will collect their baseline (day 0) stool and urine samples (Fig. 1). They will visit the CAB building to have a blood sample (2 tablespoons or 30 mL) drawn by a study nurse. Blood will be used for CMP test and prepared serum will be aliquoted and stored at -80 °C until processing for marker analysis and metabolomics. Analysis of day 0 samples should help isolate any effects due to SPI alone from subsequent samples collected during GP-SPI intervention. On day 0 subjects will drop off urine and stool samples at IFNH and collect GP-SPI packets. On Day 0 participants will also take a break from consuming SPI and will start GP-SPI supplementation on morning of Day 1.

- **GP-SPI (Day 1 – 10):** Each subject will receive twenty pre-weighed 20 g packets of GP-SPI. On day 1 subjects will be instructed to mix each packet of GP-SPI in 250 mL of water or in smoothie (recipe example will be provided, please see below) using allowed foods as detailed in instructions and consume the GP-SPI mix once in the morning before breakfast and once in the evening before dinner for 10 consecutive days (Fig. 1).

- Subjects will be provided with a personal blender for smoothie preparation (value ~ $25) that they can keep.

- **Digital food diary**: Participants will be asked to take photos of all their food and drink (except water) including the study supplements with their personal mobile computing device (e.g. smart phone, iPad, or similar). Participants will be required to download the free mobile app WhatsApp to send photos with a brief description of the food items. Photos and food description may also be sent to an email address that will be created for this study (food4microbes@gmail.com). The food diary must be kept over the 5-day SPI and wash-out period, day 0 (break day), the 10-day GP-SPI intervention period, and up until the final blood draw on day 11 (17 days total).

- **Stool samples**: Subjects will be provided with Omnigene®Gut OMR-200 kits (DNA genotek, Ottawa, Canada) or tubes containing 95% ethanol and/or tubes containing 50% glycerol (50% water) along with paper toilet accessories for easy self-collection of fecal samples; each participant will be instructed on use of stool collection materials. The Omnigene®Gut OMR-200 kit allows transport and storage of stabilized DNA at ambient temperature for 60 days. Fecal samples collected in 95% ethanol are stable at ambient temperature for 8 weeks (15). Subjects will collect samples on days 2, 4, 6, 8, and 10 and be asked to bring their samples to the IFNH laboratory as soon as possible, within 1-3 days of collection, for processing. Fecal samples will be aliquoted and stored at -80 °C until extraction. Extraction of fecal samples collected with the OMR-200 kit or 95% ethanol yields high quality DNA (as tested in Roopchand lab) suitable for 16S rRNA microbiome profiling, shotgun metagenomic sequencing, qPCR, and arrays. Collection of fecal samples in 95% ethanol has additional benefit that sample may be used for metabolomics analysis (16). Collection of fecal samples in 50% glycerol and freezing will allow culturing of gut bacteria for *in vitro* experiments.

- **Bristol stool scale form**: Subjects will be provided with the Bristol stool scale (BSS) form and asked to complete it for stool samples they collect on days -5, 0, 2, 4, 6, 8, and 10 of the study. Stool consistency has been shown to strongly correlate with gut microbiota richness and composition, enterotypes, and bacterial growth rates (17). Self-assessed stool consistency as categorized by the BSS is used in clinical studies as a proxy for colonic transit rate. The BSS classifies human feces into 7 consistency categories. The highest scores (6 - 7) correspond to loose stools and fast transit time (i.e. diarrhea), while lower scores (1 - 2) correspond to hard stools and longer colon transit time (i.e. constipation). Scores between 3 – 5 are reflective of easily passed stools (i.e. normal bowel movement) (18; 19). Each consistency category reflects differences in moisture. Decreased water is associated with longer transit time and less microbial growth due to lower nutrient mobility and enzyme activity (17). Bowel movement frequency in healthy adults (n= 124) was reported to range from 3 times per week to 3 times per day (19). Subjects that report at least one bowel movement per day will be recruited for ease of compliance with study protocol. In addition, the BSS form contains an extra column to capture information about menstruation during time of sample collections as this variable could impact metabolite or bacteria profiles .

- **Urine samples**: Subjects will be provided with sterile collection containers for collection of urine samples on days 2, 4, 6, 8, 10 and asked to keep samples in 4 °C fridge until transport to the IFNH laboratory to maintain metabolite stability (20). Subjects will be asked to bring their samples to the IFNH laboratory as soon as possible, within 2-3 days of collection, for processing. Urine samples will be aliquoted and stored at -80 °C until processing for metabolomics studies.

- **Blood sample**: On day 11 subjects will go to CAB to visit study nurses for a final blood sample draw (2 tablespoons or 30 mL). Blood will be used for CMP test and prepared serum will be aliquoted and stored at -80 °C until processing for marker analysis and metabolomics.

Table 1. Schedule of events and sample collections

Days	Screen	-5	0	2	4	6	8	10	11
Informed Consent	X								
Incl/Excl Criteria	X								
Med. History	X								
Height &Weight	X								
Clinic Visit	X		X						X
Blood Draw	X		X						X
Urine Sample		X	X	X	X	X	X	X	
Stool Sample		1	2	1	1	1	1	2	
Urine Pregnancy	X								
SPI		**Twice a day for 5 days**	**Break**						
GP-SPI				**Twice a day on days 1 - 10**					
Food Diary		**Daily up until blood draw on day 11**							

Food List

To ensure that GP-SPI ingredient is the main source of PAC consumed, participants will be given the below list of PAC-rich foods that they will be asked to abstain from during the washout and study period as well as a list of allowed foods. The food list is based on published quantification of PACs in commonly consumed food items (21; 22). In addition, participants will be provided with the following example smoothie recipe.

Smoothie Recipe:

- 1 banana (or another fruit from the allowed list of foods)
- 175 - 200 mL water or milk (add more or less liquid depending on preference; may also use milk substitutes like soy, almond, or rice milk)
- 1 - 2 tablespoons ice cream or yogurt (may also use frozen yogurt or non-dairy ice cream)
- 1 packet of GP-SPI
- 1 or more cubes of ice - *optional*
- Combine ingredients and blend

Plant Foods to abstain from: Study participants should abstain from the below list of plant foods for 5 days before (i.e. washout period), during the 10-day study intervention period, and until after the final blood draw on day 11. These foods contain high levels of naturally occurring proanthocyanidin compounds, major bioactives in GP-SPI ingredient, and will interfere with interpretation of results.

- Berry fruits (green grapes, red grapes, blueberries, cranberries, choke berries, strawberries, black berries, bilberry, black currant, aronia, hawthorn, rosehip, sea buckthorn) and all berry products (i.e. juices, preserves, jams, etc.)
- Plums
- Apples

- Apricots
- Cherries

- Sorghum
- Barley
- Pinto beans
- Red kidney beans
- Small red beans
- Black-eyed peas

- Almonds
- Hazel nuts
- Pecans
- Pistachios
- Walnuts
- Peanuts with skins

- Olives
- Olive oil
- All vinegars

- Dark chocolate
- Milk chocolate

- Red wine
- White wine
- Green tea
- Black tea

- Cinnamon
- Curry powder

Plant Foods allowed during study period: The below list of plant foods have undetectable levels of proanthocyanidin compounds and may be freely consumed. Vegetables are not a significant source of proanthocyanidins and those not listed below may also be freely consumed.

- Cantaloupe
- Medjool dates
- Grape fruit
- Orange
- Tangerine
- Honeydew
- Watermelon
- Pineapple
- Figs
- Tomato

- Coffee

- Artichoke
- Asparagus
- Broccoli

- Broccoli Raab
- Cabbage
- Carrot
- Celery
- Cucumber
- Eggplant
- Lettuce
- Onion
- Pepper
- Potato
- Radish
- Sweet potato
- Zucchini
- Beets

- Corn meal
- Tortilla chips
- Wheat
- Oats
- Rice
- Soybeans
- Navy beans
- Chick peas
- Macadamia nuts
- Pine nuts
- Brazil nuts

- White chocolate

- Basil
- Oregano
- Parsley
- Paprika
- Black pepper
- Mustard seeds
- Poppy seeds
- Garlic powder
- Ginger
- Onion powder
- Tumeric
- Cloves
- Chili powder

The following plant foods have low levels of proanthocyanidin compounds and may be consumed in moderation during the study period, meaning <u>just 1 serving per day of any food from the list below</u>.

- Pear (1 fruit)
- Nectarine (1 fruit)
- Cherries (10 cherries)
- Apricot (1 fruit)

- Kiwi (1 fruit)
- Avocado (1 fruit)
- Mango (1 fruit or 1 cup of sliced fruit)
- Banana (1 fruit)
- Persimmon (1 fruit)
- Pomegranate (1/4 cup kernels)

- Indian Squash (1 ounce or 28 g)
- Black beans (1 ounce or 28 g)
- Lentils (1 ounce or 28 g)

- Roasted peanuts (40 pieces = 1 ounce or 28 g)
- Peanut butter (1 tablespoon)
- Cashews (18 pieces = 1 ounce or 28 g)
- Chestnuts (8-10 kernels)

- Beer (1 can/bottle = 12 ounces)
- Apple juice (1 cup or 230 mL)
- Chocolate milk (1 cup or 230 mL)

A. Data Points

Data elements to be collected include: medical history, demographics, digital and written food diaries, adverse events, metabolomics data from blood and urine samples, microbiome data from fecal samples

B. Study Duration

It will take 2 years to complete this study. Each subject will participate for 17 days.

C. Endpoints

Primary endpoint:

1) Evaluate the effect of nutritional supplementation with GP-SPI on gut microbiota composition using 16S rRNA gene sequencing.

Secondary endpoints:

1) To evaluate the effect of GP-SPI on kidney and liver health/function (safety) via comprehensive metabolic panel (CMP).
2) Collect fecal, blood, and urine samples and store for a microbiome-wide association study (MWAS, funded by R01 AT010242-01).

Microbiome wide association study (MWAS)

Overview: Using the deidentified fecal, urine and blood samples from Part 1 of above-described parent study, we will perform a MWAS. Healthy humans have unique gut microbial communities; however, we will investigate whether supplementation with GP will promote elements of similarity in the gut microbiota and resulting microbial metabolites (MM), including PAC-derived MM and host-derived MM such as secondary bile acids (BA), that could be linked to healthy states and metabolic resilience. Such data may reveal common ecological or functional rules existing in microbiotas of healthy individuals as well as GP- or host-derived MM biomarkers with diagnostic potential. Below is an example of a MWAS work flow.

Shotgun Metagenomics Workflow

gDNA will be extracted from deidentified fecal samples. Libraries will be constructed followed by high-throughput sequencing on an Illumina HiSeq instrument (Genewiz). Cluster generation, template hybridization, isothermal amplification, linearization, and blocking, denaturing and hybridization of the sequencing primer will be performed according to the workflow specified by service provider. Prinseq (23) will be used to trim and de-duplicate raw reads. Reads that align to the murine/human genome (24) will be removed.

De novo non-redundant metagenomic gene-catalogue construction and gene abundance-profile calculations: High-quality paired-end reads from each sample will be used for de novo assembly with IDBA_UD (25) into contigs of at least 500 bp. Genes will be predicted using MetaGeneMark (26). A non-redundant gene catalogue of microbial genes will be constructed with CD-HIT. Quality reads will mapped onto the gene catalogue using SOAPaligner (27). Aligned results will be sampled and downsized to an appropriate number of reads per sample.

Co-abundance gene groups (CAGs): Individual genes will be binned in to co-abundance gene groups (CAGs) with a canopy-based algorithm (28). Raw CAGs will be removed in the subsequent analyses if they have fewer than 3 genes or 90% of the total canopy profile is distributed in no more than three samples. CAGs with >700 genes will be regarded as bacterial CAGs for further analyses. The principal component analyses of the bacterial CAGs based on the Bray-Curtis distance and Procrustes will be performed with QIIME (29). Abundance of genes identified per sample will be determined to follow changes in gene richness over time course of GP-SPI supplementation.

Assembly and taxonomic assignment of bacterial CAGs: De novo assembly will be performed for each of the prevalent bacterial CAGs which are share by more than 20% of the samples as previously described (30). The six criteria for high-quality draft genome assembly will be used from the Human Microbiome Project (http://www.hmpdacc.org/reference_genomes/finishing.php) and checkM (31) to assess the quality of the assemblies. We will identify phylogenetic taxonomy of CAGs and assign them to species or genus level using previously described methods (14).

Profiling the metagenomics of PAC-active enzymes: Genes encoding PAC-active enzymes will be identified using a modified HMMscan pipeline (https://www.ebi.ac.uk/Tools/hmmer/) and the best-hit alignment will be retained. Abundances of genes belonging to the same enzyme family will be summed together.

Functional Annotation: Metagenomic data will be metabolically profiled using HUMAnN (32). All high-quality reads will be aligned with Bowtie2 to the KEGG database 2014 (33), from which sequences of eukaryotes were excluded. Alignments will be transformed into bam format with SAMtools and entered into HUMAnN to obtain the abundance of pathways. The non-redundant gene catalogue and the open reading frames will be predicted from high-quality draft genomes with Prodigal and aligned to sequences using BLASTP.

Metabolomics Workflow

1) Sample preparation

a) Blood samples will be collected, centrifugated at 3000 g at 4 °C for 10 min, and serum supernatant will be aliquoted and stored frozen. Serum will be subjected to deproteinization as follows (34): 20 µL ascorbic acid (4 mg/mL) and taxifolin (internal standard; 0.25 µM) will be added to 380 µL of sample (final concentration of 1 mM). The samples will be combined with 1 mL hexane, homogenized and centrifuged for 10 min at 17 000 g. The aqueous phase will be recovered and added dropwise to 1200 ml acetonitrile (ACN), vortexed for 2 min and centrifuged for 10 min at 17 000 g. The supernatant will be recovered, and the pellet reconstituted with 400 µL ACN. After centrifugation, ACN supernatants will be combined, dried

and stored at - 80°C until analysis. Samples will be reconstituted with 100 µL of water and filtered before analysis.

b) Urine samples (100 µL aliquots) will be centrifuged (10,000 g for 10 min at 4 °C) to remove particulates followed by dilution with water (1:1 to 1:3 vol/vol). Samples (10 µl) will be added to 10 ml of ACN and centrifuged at 3000 g for 15 min. The supernatant will be collected, dried, reconstituted in 500 µl of ethanol (50 % v/v) containing 0.1 % ascorbic acid and (w/v) and transferred to HPLC vials for untargeted analyte detection by high resolution MS (20; 35).

To deplete common matrix interferences such as phospholipids, salts, and proteins and optimize sample preparation each sample supernatant produced in a) and b) may also be loaded to Oasis Prime HLB Cartridges (Waters). Sample preparation methods will be tested.

2) Chromatographic separation and high-resolution mass spectrometry (MS): Triplicates (i.e. technical replicates) of each serum or urine sample will be separated on a Dionex UltiMate 3000 Binary Rapid Separation UHPLC with Diode Array Detector with a reverse phase (RP) C18 column for retention of medium polar and non-polar analytes as well as a hydrophilic interaction chromatography (HILIC) column for retention of highly polar metabolites (35). Separation of samples on RP and HILIC columns prior to MS will provide more comprehensive metabolome coverage. To detect the greatest number of metabolites, gradient elution protocols will be optimized separately for serum and urine samples. Chromatographic separations will be followed by MS using a Thermo Scientific Q Exactive Plus Orbitrap high resolution mass spectrometer. The orbitrap provides high mass accuracy (1–2 p.p.m.), resolution up to 100,000 and dynamic range of 5,000. Data will be collected in both positive and negative ESI modes (mass range of 100–2,000 m/z).

Quality control (QC): Three kinds of QC samples will be implemented: 1) A standard mixture solution of PAC-derived MM and/or bile acids at a concentration of 1 mg/L; 2) A bulk QC sample made by pooling equal aliquots of each urine or serum sample; 3) Bulk urine or serum QC sample spiked with standard mix. These QC controls will help evaluate retention time stability, peak shape, detector response and mass accuracy. To avoid freeze-thaws QC controls will be prepared, aliquoted, and stored at -80 °C. QC samples will be injected 5 times at the beginning of the run to ensure system equilibration, and then once every 10 samples to monitor stability of analysis. Samples will be randomized to reduce systematic error associated with measurement variability (35).

3) Data processing and metabolite identification: XCMS Online software (https://xcmsonline.scripps.edu) will be used to identify and line up MS peaks/metabolites. Background noise will be filtered. A 0.2 min retention time and 0.002 Da mass tolerance window will be applied. Metabolites will be identified using online databases such as Chemspider, METLIN, (https://metlin.scripps.edu/index.php), Human Metabolome Database (HMDB) (http://www.hmdb.ca/), MZedDB (http://maltese.dbs.aber.ac.uk:8888/hrmet/index.html) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). Metabolite profile and identification will be achieved by comparing mass, RT, MS/MS fragmentation patterns in secondary LC-MS/MS analysis, and/or against standard compounds for aglycones. We will look for particular signatures of metabolites that are significantly increased in blood after GP intake (humans) or PAC intake (mice). As many metabolites are likely to be conjugated to glucuronide, sulphate and methyl groups, enzymatic treatments will be applied to release the aglycone for quantification purposes. After enzymatic treatment, samples will be profiled and compared to untreated samples. After deconjugation an increase in the signal of particular aglycones (previously quantified) is expected. The relative increase in these signals coupled with the decrease/disappearance of the conjugated compound will be used to quantify the concentration of these metabolites.

4) Statistical analysis: To find the bacteria that respond to GP either positively or negatively (i.e. GP responders) we will omit those strains that are found to change with only SPI supplementation. To further understand how the GP responders affect host metabolic health as a group, we will derive a GP Responder

Index (GRI) based on the abundance and diversity [Heip evenness] of the identified bacteria (36). Data will be subject to two-way repeated measures ANOVA to decipher main effects and interactions between GP and SPI. $P < 0.05$ will be considered significant.

To detect significant differences in metabolites MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca/) will be used. Principal components analysis (PCA) will transform original data variables into a smaller number orthogonal variables built from linear combinations to explain most of the data variance and cluster samples from each time-point or group to be analyzed. Significant difference ($p < 0.05$) will be determined within subject at different time-points, within group, and between groups. Variable importance in projection (VIP) scores will be estimated to select metabolites biomarkers from a partial least squares-discriminant analysis (PLS-DA). VIP scores are a weighted sum of PLS weights for each variable and measure the contribution of each predictor variable to the PLS-DA model. Compounds with VIP score > 4 represent metabolites with greatest change, which will be chosen for proof of identity. Before carrying out PCA and PLS-DA analyses, peak intensity will be controlled by a logarithmic transformation, and monitored by Pareto scaling. Methods for data preprocessing and statistical analysis will be optimized and additional corrections and tools will be applied as needed (37; 38).

Co-inertia analysis: To aid interpretation of the relationship between the bacterial responders and metabolomics datasets, they will be integrated using a multivariate method known as co-inertia analysis (CIA) (39). We expect to uncover bacterial species/strain level increases and decreases in GP-SPI-supplemented subjects that correlate with a concomitant increase and decrease in production of their metabolites.

1.4 Preliminary Data

The proposed study is a logical follow up to animal studies, which showed that compared to control mice fed a high-fat diet (HFD) supplemented with SPI alone, mice fed an isocaloric HFD supplemented with GP-SPI exhibited greater resistance to weight gain, adiposity, and glucose intolerance (6). Compared to HFD control group, mice fed the HFD supplemented with GP-SPI had lower levels of bacteria-derived lipopolysaccharide (LPS; endotoxin) in circulation. LPS triggers well-characterized systemic inflammatory responses in humans and mice. Coincidentally, analyses of intestinal tissue of mice fed the GP-SPI supplemented HFD revealed: i) a decrease in inflammatory mediators (TNFα, IL-6, iNOS), ii) increased expression of the tight junction protein occludin, indicating improved intestinal barrier integrity, iii) increased expression of Fiaf, indicating reduced peripheral deposition of fatty acids, iv) lower Glut2 expression, suggesting decreased glucose absorption, and v) higher expression of proglucagon (6). Collectively, these data suggested that GP-SPI supplementation improves metabolic resilience in a gut stressed by HFD. Fecal and cecal samples collected from mice fed HFD supplemented with GP-SPI showed higher levels of the gut bacterium *Akkermansia muciniphila* and a lower Firmicutes/Bacteroidetes ratio (6). In other words, polyphenols extracted from Concord grape or cranberry fruits and used as supplements triggered dramatic shifts in the type of bacteria that live in the gut, referred to as the “gut microbiota”.

We recently showed that GP-SPI can induce the *A. muciniphila* bloom within 14 days, independent of dietary fat, and that PACs, the major constituent of GP, was sufficient to induce the bloom (14). We also found that in addition to promoting intestinal abundance of *A. muciniphila* in mice, GP-SPI induced changes in several genera (i.e. *Oscillibacter, Clostridium IV*, *Intestinimonas, Acetatifactor, Blautia, Gemella, Romboutsia, Weissella*) in a direction that may also contribute to intestinal health (14). While there are differences at the strain/species level, mouse and humans share 89% of bacterial genera (40). Compared to controls, mice fed HFD supplemented with GP-SPI, but not LFD supplemented with GP-SPI, showed improved oral glucose tolerance after 2 weeks (14).

A. muciniphila is a Gram-negative obligate anaerobe that colonizes the mucus layer covering the intestinal epithelium. *A. muciniphila* can metabolize mucin as its only source of carbon and nitrogen (41) and was shown to modulate mucosal gene expression pathways involved in basal metabolism and immune tolerance toward the gut microbiota (42). For these reasons, it is thought that the bacteria species *A. muciniphila* has beneficial effects on carbohydrate metabolism. Consistent with this, treatment of Type 2 diabetic patients with the commonly used drug, metformin, triggered an increase in *A. muciniphila* population (43; 44). Increases in *A. muciniphila* were similarly observed in pregnant women with normal weight gain as compared to those with excessive weight gain (44; 45), and in normal weight and post-gastric-bypass subjects (46; 47). Prebiotic (oligofructose) treatment of *ob/ob* mice resulted in an improved metabolic profile (increased glucose tolerance, L-cell number, intestinal proglucagon gene expression and plasma GLP-1 levels alongside reduced adiposity, oxidative stress, and low-grade inflammation) concomitant with a ~100 fold increase in *A. muciniphila* compared to control *ob/o*b mice (48). Probiotic administration of *A. muciniphila* to HFD-fed mice reduced adiposity, metabolic endotoxemia, adipose inflammation and insulin resistance in association with increased intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion (49). Significantly, although it represents only 1 - 4 % of the gut microbiota in healthy subjects, the presence of *A. muciniphila* in the gut is inversely related to intestinal bowel disease (IBD), Crohn's disease, ulcerative colitis and appendicitis, suggesting its utility as a biomarker of intestinal health (41). A recent intervention study of sweetened dried cranberries, which are rich in PACs, observed a trend towards increased *Akkermansia* species after 2 weeks compared to baseline (50).

1.5 Sample Size Justification

Using conservative estimates for mean relative abundance (RA) of *A. muciniphila* in human subjects (i.e. baseline mean RA of 0.1% increasing to 0.4% on day 10 with standard deviation of 0.5%), a sample size of 16 subjects would be required to detect significant difference from baseline levels (target power = 95%, actual power = 96.2%; $\alpha = 0.05$, Hotelling-Lawley Trace test for repeated measures, GLIMMPSE).

16S rRNA gene sequencing data will be compared longitudinally within each subject and in aggregate and consists of surveying the relative composition of gut microbiota populations in fecal samples and mine data for differences in phyla (e.g. *Bacteriodetes* vs. *Firmicutes*), genera, and specific microbes (*Akkermansia* spp.) as far as resolution allows. Power calculations will be performed using data obtained from this pilot time course study to assist in finalizing design of a future randomized, placebo-controlled intervention study.

1.6 Study Variables

A. Independent Variables, Interventions, or Predictor Variables
The independent variables will be supplementation with SPI or GP-SPI.

B. Dependent Variables or Outcome Measures
Outcome measures are gut microbial community analyses, CMP results, urine and serum metabolites, and bacterial genes, species/strains.

1.7 Drugs/Devices/Biologics
N/A

1.8 Specimen Collection

A. <u>Primary</u> Specimen Collection

- **<u>Types of Specimens</u>**:

Blood samples: A blood samples will be collected by study nurse at screening visit for CMP test. If CMP is within normal ranges, subject will be enrolled in study. Blood will be collected again at baseline (day 0) and at end of study (day 11). Each blood draw will be no more than 30 mL (2 tablespoons).

Stool samples: Subjects will be provided with Omnigene®Gut OMR-200 kits or collection tubes containing 95% ethanol and/or tubes containing 50% glycerol (50% water) along with paper toilet accessories (DNA genotek, Ottawa, Canada) for easy self-collection of a pea-sized amount of fecal sample. Each participant will be instructed on use of stool collection materials.

Urine samples: Subjects will be provided with sterile collection containers (100 mL) for self-collection of urine samples on same days fecal samples are collected and asked to keep samples in 4 °C fridge until transport to the IFNH laboratory to maintain metabolite stability (20).

- **Annotation**:

  Blood samples: CMP tests will be performed on blood samples collected at screening visit, day 0, and day 11. Targeted and untargeted metabolomics analyses will be performed on de-identified blood samples collected on days 0 and 11 from participants enrolled in study.

  Stool samples: Microbial community structure will be analyzed from de-identified fecal samples as previously described (51) and used for shot gun metagenomics studies.

  Urine samples: De-identified urine samples will subject for targeted and untargeted metabolomics analyses.

- **Transport**:

  Blood samples: Blood samples collected and processed by study nurse at Clinical Academic Building will be picked up by a study staff member of Roopchand lab and transported to IFNH on dry ice.

  Stool samples: The Omnigene®Gut OMR-200 kit allows transport and storage of stabilized DNA at ambient temperature for 60 days and 95% ethanol stabilizes samples for 8 weeks. Subjects will be asked to bring their stool samples to the IFNH laboratory as soon as possible, within 1-3 days of collection, for processing. Subjects will be asked to freeze fecal samples collected in 50% glycerol as soon as possible to minimize shifts in the microbiota, which can use glycerol as a carbon source.

  Urine samples: Subjects will be instructed to keep urine samples in their 4°C fridge until transport to the IFNH laboratory to maintain metabolite stability (20).

- **Processing**:

  Blood samples: Initial processing will be performed by study nurse. Further processing for metabolomics analysis will be performed by Roopchand lab research personnel working on this study.

  Stool and urine samples: Processing will be performed by Roopchand lab research personnel working on this study.

- **Storage**:

Blood, stool and urine samples: Deidentified samples will be aliquoted and stored at -80 °C

De-identified specimens will be stored in locked -80°C freezers belonging to the Roopchand laboratory in IFNH (61 Dudley Rd, New Brunswick, NJ 08901) until analysis. Specimens may be used for future experiments which may include: 1) new sample analyses that are necessary for this study but are not listed in the current protocol and sample analyses for future studies. Consent to use stored samples for experiments is required for participation in this study. Specimens will be stored in IFNH laboratory freezer until they are no longer in a fit state for scientific analyses and may only be accessed by authorized research personnel associated with this study.

- **Disposition**:
  Blood, stool, and urine samples: Specimens will be stored in IFNH laboratory freezer until they are no longer in a fit state for scientific analyses, after which they will be disposed of by incineration by REHS. Only de-identified study data will be associated with the specimens banked for future use by this PI, removing need for a repository.

B. Secondary Specimen Collection
N/A

1.9 Data Collection (Interviews, Focus Groups, or Surveys)
N/A

1.10 Timetable/Schedule of Events

	Dec 2018 - Feb 2019	Mar - Aug 2019	Sept 2019 - Mar 2020	April 2020 - ongoing
Staff/student training, research material production				
Eligibility screening, recruitment, and sample collection				
Sample processing, data generation and analyses				
Publications and results dissemination				

2.0 Project Management

2.1 Research Staff and Qualifications

Diana Roopchand, PI, Rutgers IFNH Dr. Roopchand is an Assistant Professor in the Food Science Department with a laboratory located at IFNH. Her research focuses on dietary components and

interventions that promote metabolic health by altering the gut microbial community. She pioneered development of dietary polyphenol-protein complexes and has experience in phytochemical analysis, molecular biology, and gut microbiota community analysis. She will oversee research material production and will direct day-to-day research and training activities including participant recruitment, scheduling, sample organization, data entry, and analyses.

Susette Coyle, Co-investigator, RWJMS Nurse Ms. Coyle has several years of experience in clinical protocol development and performing clinical studies. She will perform blood draws for CMP tests and collection of blood samples for metabolomics analyses.

Marie Macor, Co-investigator, RWJMS Nurse Ms. Macor has several years of experience in clinical protocol development and performing clinical studies. She will perform blood draws for CMP tests and collection of blood samples for metabolomics analyses.

Rocio M. Duran, Study Staff, Research Technician, Rutgers IFNH Ms. Duran is the lead research technician in the laboratory of Dr. Roopchand. She brings extensive experience in molecular biology, microbial genetics, and biochemical analysis of microbial metabolites. She will assist with participant recruitment, scheduling, sample organization, storage and processing, data entry, and analyses.

Esther Mezhibovsky, Study Staff, Graduate Student, Rutgers IFNH
Ms. Mezhibovsky has experience with prior clinical studies, specifically as a study coordinator at the University of Wisconsin for the CAREDS2 Study. She has experience in participant screening, data collection, entry, and quality assurance and is trained in handling confidential information as defined by HIPAA. She organized research material production and will assist with participant recruitment, scheduling, sample organization, data entry, and analyses.

David M. Krol, MD, Study Physician, Medical Director, Rutgers IFNH and RWJMS
Dr. Krol is the Medical Director at IFNH and Associate Professor and Chair of Pediatrics at RWJMS. He serves as Medical Director for the NJ Healthy Kids Initiative, a partnership between the IFNH and Child Health Institute of NJ. Dr. Krol will order CMP tests and review CMP data. As onsite study physician, the clinical study team and/or study participants will consult with Dr. Krol in case of adverse events or safety concerns.

2.2 Research Staff Training

RESEARCH TRAINING. All investigators and research personnel will complete the Collaborative Institutional Training Initiative (CITI) training and the Lab Safety/Biological Safety/Blood Borne Pathogens Training from Rutgers Environmental Health & Safety.

The PI and RWJMS collaborators have had several meetings to develop the study. Prior to starting the study, PI will meet with study personnel to review the protocol, procedures, study forms, and each one's responsibilities going forward. Meetings will be held periodically throughout the study to review progress and make changes if necessary.

2.3 Resources Available

IFNH and CAB are equipped with conference/interview rooms for eligibility screening visits. The GP-SPI and SPI materials will be stored in freezers of Food Science pilot plant, which has space dedicated for production of food-grade study materials.

2.4 Research Sites

Rutgers Robert Wood Johnson Medical School (RWJMS)
Rutgers SEBS, Institute for Food, Nutrition, and Health

3.0 Multi-Center Research

N/A

4.0 Subject Considerations

4.1 Subject Selection and Enrollment Considerations

A. Method to Identify Potential Subjects

Flyers will be posted locally to recruit volunteers and emails will be sent to Rutgers university email lists.

B. Recruitment Details

Recruitment of the 30 participants will start in March 2019. Prospective participants will be recruited through flyers on Rutgers University campuses and email lists. Interested individuals will be asked to contact the research team by email or phone to obtain an overview of the study. We expect to recruit the majority of our participants from the New Brunswick campus of Rutgers University. We conservatively expect a recruitment rate of 3-6 participants per month over 6 months.

C. Subject Screening

Interested subjects will call in to receive further information about the study. A co-investigator/coordinator will give a brief summary of the study. If the subject requests a screening appointment, the investigator will ask for verbal consent to proceed with a list of questions. Collecting this information at this time assists in expediting the paperwork during the visit and spares ineligible subjects the inconvenience of a futile appointment.

At the screening visit, a co-investigator/coordinator will explain the study in detail to the subject. Full explanation of procedures and potential risks will be provided. The subject will have ample time to read the consent and ask questions before signing and proceeding with screening procedures. A signed copy will be given to the subject.

Inclusion Criteria:

A subject will be deemed as "healthy" and eligible for inclusion in this study if all of the following criteria are met:

1. Healthy as assessed based on a medical evaluation including a comprehensive metabolic panel (CMP) test with values in normal range, medical history, and not presently taking any medication
2. Adults between 18 and 35 years
3. BMI 18.5 - 29.9
4. Have at least one bowel movement per day
5. Capable of giving written informed consent, which includes compliance with the requirements and restrictions listed in the consent form.

Exclusion criteria:

1. History/current cancer, rheumatoid arthritis immunologic, renal, hepatic, endocrine, neurologic or heart disease, hypertension, diabetes, GI dysfunction, or CMP test results showing values outside of normal range.
2. Cannot provide written informed consent.
3. Exposure to any experimental agent or procedure within 30 days of study.
4. Pregnancy or breast-feeding
5. Taking dietary supplements
6. Current smoker or have smoked within previous 6 months
7. Taking medications regularly (prescription, over the counter, supplements etc.)

8. Treated with antibiotics during the past 6 months
9. Have an allergy to soy or grapes

4.2 Secondary Subjects

N/A

4.3 Number of Subjects

A. Total Number of Subjects

30 subjects will be enrolled after successfully passing screening. The anticipated lost to follow-up rate for this study will be 20% (i.e. 6 non-completers)

B. Total Number of Subjects If Multicenter Study

N/A

C. Feasibility

We expect to recruit the majority of our participants from the New Brunswick campus of Rutgers University. With a relatively flexible study schedule and no particularly stringent eligibility criteria, we do not anticipate difficulties in engaging interest and accommodating schedules of participants. We conservatively expect a recruitment rate of 3-6 participants per month over 6 months.

4.4 Consent Procedures

A. Consent Process

- **Location of Consent Process**
  The Consent Process will take place at the New Jersey Institute for Food, Nutrition & Health

- **Ongoing Consent**
  N/A

- **Individual Roles for Researchers Involved in Consent**
  Principal Investigator Dr. Diana Roopchand, Co-Investigators Susette Coyle or Marie Macor, Rocio Duran, or graduate student Esther Mezhibovsky will conduct the consent process to explain all aspects of the study and answer any questions the prospective participants may have. Dr. Roopchand, Susette Coyle, Marie Macor, Rocio Duran, or Esther Mezhibovsky will sign the consent form.

- **Consent Discussion Duration**
  It is expected that the consent discussion will last approximately 45 min.

- **Coercion or Undue Influence**
  In order to minimize the possibility of coercion or undue influence, the consent process will not be conducted by research personnel who have any known relationship or conflict of interest with the prospective participants.

- **Subject Understanding**
  Throughout the consent process the prospective participants will be encouraged to ask any questions should they feel the need to do so. The consent process will be divided into a series of small sections. Members of the research team will summarize each section, use probe questions to check for sufficient understanding before proceeding to the next section. An illustrated Study Summary will be provided to assist subject understanding of study procedures.

B. Waiver or Alteration of Consent Process
N/A

C. Documentation of Consent

- **Documenting Consent**
  Please see attached Consent form.

- **Waiver of Documentation Of Consent (i.e., will not obtain subject's signature)**
  N/A

4.5 Special Consent/Populations

A. Minors-Subjects Who Are Not Yet Adults
N/A
B. Wards of the State
N/A
C. Non-English-Speaking Subjects
N/A
D. Adults Unable to Consent / Cognitively Impaired Adults (for interventional studies)
N/A

4.6 Economic Burden and/or Compensation for Subjects

A. Expenses
Participants may incur expenses when they travel to our research facilities.

B. Compensation/Incentives
Subjects will receive parking for each blood draw study visit and compensated $5 for each of 9 stool, 7 urine, and 2 blood samples delivered on days -5 through 10 and an additional $110 on Day 11 visit for completing all requested study samples, completion of all study visits and delivery of complete digital and written food diary for a total of $200 paid in cash. One missed or incomplete visit will result in only $5 for each blood, urine and stool sample delivered on Days -5 through 10.

C. Compensation Documentation
Each participant will be asked to sign a form confirming receipt of stated amount of compensation at end of their participation in the study. See page 3 of "**Log of Study Visits & Receivables**" form; once signed this page will be moved to separate binder and kept in a separate locked cabinet.

4.7 Risks of Harm/Potential for Benefits to Subjects

A. Description of Risks of Harm to Subjects

- **Reasonably Foreseeable Risks of Harm**
  Study Supplement – there are no known risks to ingesting the study supplement. To minimize the risk of an allergic reaction the study excludes persons allergic to soy or grapes.

  Blood Sampling – may cause a bruise or bleeding at the site. Infection at the site is rare.
  A total of 65 mL (~ 4 Tablespoons) will be drawn during the course of the study. Healthy subjects can tolerate this blood loss without any side effects.

  Urine and Stool collection – There is risk of discomfort with sample collection and storage.

These risks are considered minimal and are addressed in the protocol and consent form. To minimize expected risk, study staff will communicate with subjects during intervention period and do a final follow up by phone or email 2 - 3 weeks after end of intervention.

- **Risk of Harm from an Intervention on a Subject with an Existing Condition**
  N/A

- **Other Foreseeable Risks of Harm**
  All efforts will be made to keep personal information in subject research record confidential, but total confidentiality cannot be guaranteed.

- **Observation and Sensitive Information**
  N/A

B. Procedures which Risk Harm to Embryo, Fetus, and/or Pregnant Subjects
Soy protein and dietary polyphenols are often consumed as part of a normal diet; however, in case of pregnancy within the 15-day study period, the risk of GP-SPI to a newly formed embryo/fetus is uncertain. This study will not include pregnant subjects and a urine-based pregnancy test will be performed to confirm inclusion of only non-pregnant participants.

C. Risks of Harm to Non-Subjects
N/A

D. Assessment of Social Behavior Considerations
N/A

E. Minimizing Risks of Harm
All procedures will be explained to subjects at the time of obtaining consent and subjects will be encouraged to ask questions about any concerns throughout the study. Every effort will be made to maintain subject confidentiality.

- **Certificate of Confidentiality**
  This study is partially funded by NIH-NCCIH therefore a Certificate of Confidentiality is automatically issued to protect data collected.

- **Provisions to Protect the Privacy Interests of Subjects**
  Should any participants desire not to interact with or provide personal information to specific members of the research team, they may report such a need to the Principal Investigator and the arrangement of the research personnel will be adjusted accordingly. Participants will not be required to provide a reason if they desire not to do so. Should changing of the research personnel is not possible, the Principal Investigator will discuss with the participants about alternative solutions.

  In order to ensure the protection of personal health information of the participants, there will be no identifiable information on any of the data collection instruments. Each participant will be assigned a unique identification code. A master code identifier which links the identification code and personal information (name, date of birth and contact information) will be maintained separately from the study data. Only the Principal Investigator and approved research personnel who have a specific need for identifiable information will have access (e.g. their primary physician, research student/staff to schedule appointments). All data and materials

collected during this study are for research purposes only, and the data will be kept in strict confidence. No information will be given to anyone without permission from the participants.

F. Potential Benefits to Subjects

There are no direct benefits for the subjects. Subjects will receive a copy of results from health assessment blood tests, if abnormal they will be encouraged to see their doctor.

5.0 Special Considerations

5.1 Health Insurance Portability and Accountability Act (HIPAA)

HIPPA will be collected with consent.

5.2 Family Educational Rights and Privacy Act (FERPA)

N/A

5.3 NJ Access to Medical Research Act (Surrogate Consent)

N/A

5.4 General Data Protection Regulation (GDPR)

N/A

5.5 Code of Federal Regulations Title 45 Part 46 (Vulnerable Populations)

N/A

6.0 Data Management Plan

6.1 Data Analysis

Statistical comparisons will focus on individual and aggregate comparisons with respect to baseline to follow-up change in gut microbiota populations and biochemistry profile in blood and urine samples.

For each outcome, the equality of the outcome means for GP-SPI versus SPI will be tested against a two-directional alternative, controlling the type 1 error at $\alpha = 0.05$. The analyses will be conducted using t-tests for between group analysis and with repeated measures to conduct analysis of variance. Post-hoc tests will be conducted for pairwise comparisons of GP-SPI and SPI employing the Tukey method for controlling the global comparison-wise significance at 0.05 level. Logarithms or other transformations will be applied for data seriously deviating from normality; alternatively, non-parametric analyses may be conducted.

Handling of missing data: The data will be analyzed using the procedures PROC MIXED and PROC GLIMMIX SAS (v 9.3 or later). Thus, mixed effects models for repeated measures will be employed to analyze all available data irrespective of missing or incomplete data attributable to dropouts or other phenomena. In anticipation of the possibility of missing data, we plan to conduct sensitivity analyses that compare our findings against protocol completer analyses and last observation carried forward analyses in addition to investigating characteristics of participants who fail to protocol.

6.2 Data Security

A code will be assigned to every participant and the collected data will be recorded using the participant's unique code for identification purposes. The Principal Investigator, study nurses, and research staff/students who schedule appointments will be the only ones with access to the master code identifier. Participants' hard copy files will be kept in locked cabinets only accessible by authorized research personnel in IFNH, a secured building. Electronic files will be password-protected and stored on the university's secure server and will only be accessible to authorized research personnel. The master code identifier and the data files will be kept in two separate locations. All personnel who will

have access to data of any form will be required to complete CITI training and will only have access to the data during a specific duration deemed necessary by the Principal Investigator.

6.3 Data and Safety Monitoring

A. Data/Safety Monitoring Plan

An Independent Monitoring Committee (IMC) was formed, composed of three Rutgers University faculty and staff members who are not directly involved with the study. The IMC will monitor recruitment, retention, adherence, and review participant safety including adverse events, proposed major protocol modifications, and reports of related studies as appropriate.

Study progress and safety will be reviewed monthly (and more frequently if needed) by study team and IMC. The IMC will receive monthly email updates (i.e. IMC Report, attached) on study progress and meet once every two months or as needed.

Data will be collected on prepared forms for Adverse Event, Serious Adverse Event, Unanticipated Problems, and Protocol Deviation Tracking Log.

An Annual Report (i.e. IMC Report attached) will be compiled and will include a list and summary of AEs. In addition, the Annual Report will address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The Annual Report will be sent to the Independent Monitors and will be forwarded to the IRB and NCCIH, and FDA. The PI will send copies of signed recommendations and comments from the Independent Monitors to the NCCIH Program Officer within 1 month of each monitoring review.

B. Data/Safety Monitoring Board Details

The Independent Monitoring Committee for this study is comprised of Dr. Amy Davidow (Biostatistician), Dr. Thomas Gianfagna (expert in plant-derived natural products), and Dr. Vinod Rustgi (Clinician, gastroenterologist). Dr. Rustgi has previously served on a monitoring committee. Drs. Davidow, Gianfagna, and Rustgi are not associated with this research project and work independently of the PI, Dr. Roopchand. They are not part of the key personnel involved in this grant. No member of the Committee has collaborated or co-published with the PI within the past three years. They are qualified to review the patient safety data generated by this study because of their unique expertise.

Any serious adverse event that is life-threatening or results in death, that is possibly, probably, or definitely related to the research protocol, will trigger an immediate suspension of the research.

C. Source and Preparation of Study Supplement

Concord grapes are a rich source of dietary polyphenols. Concord grapes are pressed to produce polyphenol-rich Concord grape juice, which also large amounts of fruit sugars, mainly glucose and fructose (10). Concord grape pomace is comprised of the seeds and skins of the grapes that are left over after the juicing process. The pomace is also rich in dietary polyphenols, mainly proanthocyanidins (PAC), but in contrast with the juice, contains low amounts of sugars (9).

We have previously demonstrated that, due to a natural affinity of polyphenols and proteins, dietary polyphenols can be stabilized for at least 24 weeks (and up to one year, unpublished data) at 37 ºC when sorbed to a protein-rich food matrix, such as soy protein isolate (SPI), without compromising subsequent release from the matrix or efficacy (7; 9; 11). Studies performed in models of the human intestine have also demonstrated that protein-polyphenol particles provide an efficient vehicle for delivering intact polyphenols, e.g. anthocyanins, to the lower part of the intestine (52). We will therefore use this protein sorption process to maintain stability of extracted Concord grape polyphenols.

The biochemical composition of the grape pomace extract used in this study and the pilot scale production of grape polyphenols (GP) sorbed and stabilized to the SPI matrix (i.e. GP-SPI) have been previously described in detail (9). Aliquots of extracted GP will be biochemically characterized using LC-MS and quantified for total polyphenols and PAC using previously described colorimetric methods (9; 14).

GP-SPI will be manufactured in the Rutgers Food Science Pilot plant. Briefly, Concord grape pomace (supplied by Welch's) will be extracted with 50% ethanol (1:5 solids:solvent; wt./vol. ratio) with pH adjusted to 2 (using sulphuric acid) at 80°C for 2 h with agitation and ambient pressure. The cooled polyphenol extract is then separated by filtration and the concentration of total polyphenols is quantified using the Folin-Ciocalteu assay. A calculated amount of SPI is mixed with the grape polyphenol extract and dried until moisture is < 10% to produce the GP-SPI complex containing 5% total grape polyphenols. Since the basic process involves the mixing of two foods and removal of water and ethanol, it is equivalent to cooking. The resulting product remains a food, and we feel it can be Generally Regarded as Safe (GRAS) http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/. GP-SPI is not intended to cure, prevent or mitigate a disease. Its suggested use is to maintain normal functions of the human body.

Safety Monitoring: A representative sample of the GP-SPI product will be provided to the laboratory of Prof. Donald Schaffner (Distinguished Professor and Extension Specialist, Food Science Department) to perform microbiological testing using same procedures they use for weekly testing of Rutgers University dining hall foods (including total aerobic plate count, coliforms, generic *E. coli*, *Salmonella*, *S. aureus*, *B. cereus*, *C. perfringens*, *L. monocytogenes*, etc.).

Based on our quantification of total polyphenols in commercial Concord grape juice (e.g., Welch's), the daily dose of GP-SPI we propose to test in this clinical study (40 g per day) is equivalent to the concentration of polyphenols that can be found in four-to-six 250 mL glasses of Concord grape juice.

Drinking 4-6 glasses of Concord grape juice would normally deliver 150 g – 225 g sugar along with 1860 – 2160 mg of total polyphenols while 40 g of GP-SPI product will deliver this level of polyphenols without the excess sugar (4g total sugars/100g GP-SPI). Grape pomace polyphenols are mainly catechins and PACs (catechin polymers), which are compounds commonly found in pigmented fruits and spices. Given that subjects will be instructed to consume a diet low in PAC-rich foods, we don't expect that consuming 2000 mg/day of grape polyphenols delivered in GP-SPI will promote side effects. Average daily polyphenol intake has been reported to be 1000 mg/day (53). Total polyphenol intake has been reported to be 3000 mg/day in individuals consuming a Spanish diet, typically higher in polyphenols (54). Subjects consuming GP-SPI would be consuming the level of total polyphenols within the normal range of a total diet.

6.4 Reporting Results

A. Individual Subjects' Results

Subjects will receive a copy of their CMP blood test results. Subjects will receive a copy of their individual gut microbiota community analysis data after all study data is analyzed.

B. Aggregate Results

Aggregate results will be accessible to participants after study is published in the peer-reviewed literature.

C. Professional Reporting

Scientific manuscripts reporting study findings will be prepared and submitted for publication in peer-reviewed academic journals. The study findings will also be prepared for presentation at regional, national, and international research conferences. Key finding will be disseminated to the public by press release.

D. Clinical Trials Registration, Results Reporting and Consent Posting
This study will be registered on ClinicalTrials.gov.

6.5 Secondary Use of the Data
N/A

7.0 Research Repositories – Specimens and/or Data

Data: De-identified electronic data will be stored on the university's secure server indefinitely. Data banked for future use will only be accessible by the Principal Investigator. Release of these data, irrespective of whether the Principal Investigator has ownership of the research project(s) that require the banked data, will need to obtain separate IRB approvals. When the banked data are transferred or shared, they will not be associated with any identifiable data, personal information of the research participants, or the master code identifier for deciphering the codes.

Specimens: The stool, urine, and blood samples collected during the study will be coded and stored in a locked -80°C laboratory freezers until samples can be processed. With consent of participants, de-identified samples from the clinical study will be stored for use by PI only in future studies.

8.0 Approvals/Authorizations

NIH Approval letter
Roopchand IBC, Foty IBC

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