Blueberries, Gut Microbiota, and Metabolites in Depressed Older Adults - A Pilot Study aka Berries, Bugs, and the Blues

Study Protocol
IRB# Pro00068528
NTC# 05817383

Version 5
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CHAPTER 1. INTRODUCTION

Emerging evidence indicates the trillions of microbes residing in the gut play an integral role a mediating physiological pathways of depression (e.g., neuro-inflammation and neurotransmitters). Given that diet is a potent modulator of the gut microbiota, the long-term goal of this project is to identify nutrients that interact with the gut microbiota processes/pathways relevant to depression. Our preliminary data suggests that intake of dietary fiber and anthocyanins (class of flavonoids found in berries) are associated with reductions in depressive symptoms. Coincidentally, fiber and anthocyanins are metabolized by microbes, and influence the gut microbial communities and metabolites relevant to depression (i.e., short chain fatty acids, SCFA). However, it is unknown if the nutrient-gut microbiota-interaction is the mechanism underlying the beneficial association between fiber and anthocyanin intake with depression. Our objective is to determine if consuming a whole-food source of dietary fiber and anthocyanins (via freeze-dried blueberry powder) modulates the gut microbiota, SCFA, and improves depressive symptoms. This study is an ancillary project to a currently funded randomized, placebo-controlled 12-week intervention in older, sedentary adults with depressive symptoms (**IRB# Pro00064749**). This specific project proposes distinct aims to the trial by gathering preliminary data on the synergistic impact of dietary fiber and anthocyanins on the gut-microbiota, gut-derived metabolites, and depressive symptoms in older adults. This study will provide evidence on the utility of fiber- and anthocyanin-rich agricultural foods as a new therapeutic opportunity to improve human health by modulating the gut microbiota and relevant metabolites.

CHAPTER 2. BACKGROUND

2.1 Urgency to Address Age-related Depression: Between 8-16% of community-dwelling older adults have clinically relevant depressive symptoms,[1] which is marked by severe feelings that significantly impair daily function.[2] As a result, individuals with depressive symptoms/depression have increased risk of disability, chronic depression, and suicidal ideation.[3-7] Older adults prefer non-pharmacological approaches,[8] yet underutilize psychotherapy (an effective treatment regimen for depression)[9] due to its high cost and inaccessibility, which prevents many older adults from obtaining adequate care[8] and underscores the need for novel therapeutic strategies.

2.2 Diet, the Gut, and Depression: The gut microbiota directly impact several pathophysiological pathways of depression (e.g., neuro-inflammation).[10-31] Dietary modification of the gut microbiota[30-34] offers an accessible, low-cost approach to help improve depression in the millions of older adults with depressive symptoms. Epidemiological studies have linked intake of dietary fiber (i.e., fiber found naturally in foods) or anthocyanins (a class of flavonoids with antioxidant properties) with the prevention of depression.[35-39] Our preliminary data from a prospective cohort study also suggests that increasing dietary fiber and anthocyanin intake were associated with reduced depressive symptom severity in older adults (**Table 1**). Human studies increasing intake of dietary fiber and anthocyanins are needed to confirm their potential to improve depression and elucidate the role of the gut microbiota as an underlying mechanism.

Table 1. Increased nutrient intake and reduced CES-D scores in 399 older adults with depressive symptoms

Dietary Component	Beta*(SE)	P-value
Anthocyanins (per 60 mg)	-1.9(0.6)	0.01
Fiber (per 60 mg)	-1.2(0.4)	0.01

Adjusted for age, sex, and calorie intake. CES-D, center for epidemiological studies depression scale; SE, standard error;

2.3 Fiber and Anthocyanins to Modulate the Gut: Dietary fiber[40-43] and anthocyanins[32,44-51] interact with the gut microbiota, which may contribute to their protective associations with depression.[52] Both nutrients are metabolized by gut microbes, which helps shape the number and types of microbes present in the gut, as well as the gut-derived metabolites.[52] Certain types of dietary fiber are metabolized by some microbes to directly produce short chain fatty acids (SCFA). Increased production of SCFA may improve depressive symptoms given their ability to reduce neuro-inflammation and increase proteins essential for neuron formation.[30] Research suggests that anthocyanins promote the enrichment of SCFA-producing microbes to indirectly increase production of SCFA.[53-55] *We hypothesize* intake of dietary fiber and anthocyanins synergistically increase gut-derived SCFA and exert a protective effect on depression (**Figure 1**).

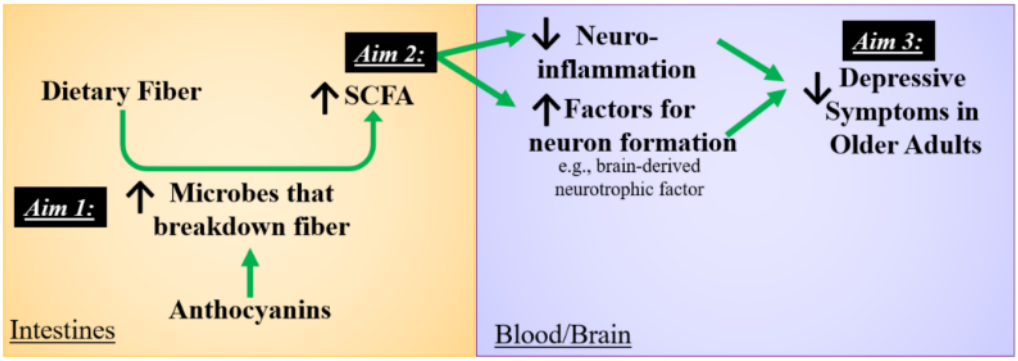


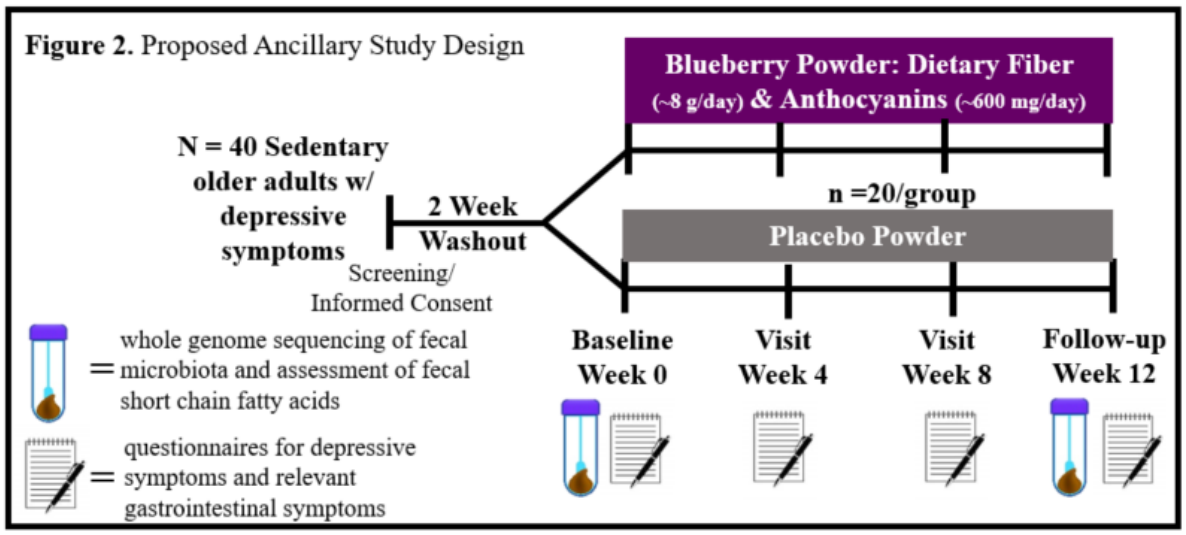
Figure 1. Conceptual Model. We hypothesize the dietary fiber in freeze-dried blueberry powder will be metabolized by gut microbes to directly increase SCFA, and the anthocyanins in freeze-dried blueberry powder will increase the prevalence of gut microbes that metabolize fiber into SCFA to indirectly increase SCFA. The SCFA, which have been associated with reduced neuro-inflammation and increase production of factors needed for neuron formation, will reduce depressive symptoms. SCFA, short chain fatty acids;

CHAPTER 3: RESEARCH DESIGN

3.1 Study Objectives and Aims

Our objective is to gather preliminary evidence on the synergistic impact of dietary fiber and anthocyanin intake on the gut microbiota-related pathways/processes relevant to depression utilizing a whole-food approach. We propose an ancillary study to a currently funded (**IRB Approval Pro00064749; Co-PI Dr. Millar**) randomized, double-blind, placebo-controlled intervention in older adults (≥ 65 y) with depressive symptoms (see **§3.2** for details on the ancillary study design; **Figure 2**). The aims of this ancillary project are:

Aim 1: Determine the impact of daily dietary fiber and anthocyanin (via freeze-dried blueberry powder) intake on microbes that produce gut-derived SCFA in older adults with depressive symptoms by performing a randomized, placebo-controlled, double-blind trial.
Aim 2: Determine the effect of daily dietary fiber and anthocyanin intake on fecal SCFA in older adults with depressive symptoms, participating in the clinical trial.
Aim 3: Determine the effect of daily dietary fiber and anthocyanin intake on depressive symptom severity in older adults with depressive symptoms, participating in the clinical trial.



3.2 Overview

This proposal leverages a currently funded individual-level, randomized, double-blind, placebo-controlled, parallel pilot study funded by a National Institute of Aging Roybal Pilot Grant, in 40 sedentary, older adults with depressive symptoms (**IRB Approval Pro00064749; Co-PI Dr. Millar**). The primary goal of the parent study is to evaluate the impact of daily dietary fiber and anthocyanin intake on serum inflammatory markers and the engagement of physical activity (via a wrist-worn accelerometer). Recruited participants of the parent trial will be randomized to consume 48 g/day of freeze-dried blueberry powder (a source anthocyanins and dietary fiber) or 48 g/day of a nutritionally matched placebo powder (devoid of anthocyanins and dietary fiber) mixed in water for 12 weeks. *As an expansion to the parent study, this ancillary study (**Figure 2**) aims to determine the impact of both dietary fiber and anthocyanins on the gut microbiota, gut-derived metabolites, and depressive symptom severity.* Thus, individuals recruited for the parent trial will also be asked to provide a fecal sample for the measures related to the gut microbiota and to answer additional questionnaire-based assessments.

3.3 Inclusion and Exclusion Criteria

The target population of the parent study is sedentary older adults, with minor depressive symptoms. Individuals expressing an interest in participating after recruitment out-reach will be screened in-person (e.g., at HRC or at participant's home). As a part of the parent study, participants will complete several assessments to confirm that they meet the following inclusion criteria.

Inclusion Criteria

- Men and women aged ≥65 years
- Self-reporting ≥ 8 hours of sitting per/day (e.g., sedentary behavior)
- Depressive symptoms (defined as ≥4 and <16 points on the center for epidemiological studies depression-scale)

Exclusion criteria have been selected to ensure safety and optimize compliance, while minimizing confounds due to overt disease or conditions that may significantly influence study outcomes. Exclusions may be during the in-person screening as described below:

Exclusion Criteria

- Unwilling to follow the study protocol
- A median daily step count >7,500 steps per day (as measured by the ActiGraph), or per discretion of the PI
- Cognitive impairment (defined as Montreal Cognitive Assessment,[37] MoCA <22 points)
- Self-reporting a history of inflammatory bowel disease/syndrome, major depression, bipolar, schizophrenia, or other psychotic or neurological disorders (e.g., Parkinson's disease), or per discretion of the PI
- Self-reporting type one or type two diabetes
- Allergic to intervention or control products
- Recent use (within the last 3 months) of antibiotics, or per discretion of the PI
- Recent use (within the last 3 months) of probiotics, or per discretion of the PI
- Current substance use disorder (Drug Abuse Screening Test,[40,41] DAST-10>2 points)
- Current alcohol use disorder (Alcohol Use Disorders Identification Test,[42,43] AUDIT-C≥4 points)
- Unstable anti-depressant use (e.g., change in medication within last 3-6 months), or per discretion of the PI
- Current homicidal or suicidal ideation (assessed via the P4 Suicidality Screener[44])
- Current psychosis (via the Psychosis and Hallucinations Questionnaire,[45] PsycHQ>12 points)
- Manic symptoms (assessed by the Mood Disorder Questionnaire,[46] MDQ >5 points)

The majority of subjects that will enroll in this ancillary study will undergo screening as a part of the parent study as outlined in **IRB Pro00064749.** However, if a participant wants to enroll only in this ancillary study, the participant will need to undergo the telephone screening and in-person screening visits as outlined in the parent study (**IRB Pro00064749**).

3.4 Number of Subjects and Study Duration

The parents study aims to recruit a total of 40 individuals (both men and women), in proportion to the gender and racial distribution of the greater Boston population, through local newspaper and internet advertisements, physician referrals, our registry of research volunteers, Hebrew SeniorLife (HSL) senior housing sites, and patient registries (e.g., HRC or Beth Israel Deaconess Medical Center). Participants will remain in the study for a total of 14 weeks.

3.5 Study Endpoints

The primary outcomes of this study are:

1) **Abundance of microbes that produce SCFA** (e.g., *Bifidobacteria, Eubacterium, and Clostridium*[56,57]) will be evaluated by whole genome sequencing (WGS). WGS will be performed in fecal samples before and after the 12 week intervention to obtain a holistic representation of the microbes in the gut and to confirm that the blueberry powder interacts with the gut microbiota WGS was chosen because it enables identification of microbes down to the specific species or strain of bacteria whereas other methods can only identify the genus level. Also, WGS tends to be less biased compared to 16S rRNA sequencing. A reputable lab (e.g., Microbial Omics Core at the Broad Institute) will perform the DNA extraction from fecal samples and whole genome sequence library construction and sequencing. Then the Fastq sequences will be processed and analyzed by a reputable lab (e.g., the Harvard Chan Microbiome Analysis Core).

2) **Fecal SCFA** will be measured by a reputable lab (e.g., West Coast Metabolomics Center at University of California Davis). Following sample preparation outlined in **§5.2,** a biphasic extraction will be performed to extract the SCFA from the fecal samples. SCFA will be separated and identified using gas chromatography mass spectrometry. Internal standards and an external curve will be used to calculate the concentration of SCFA) in each sample (acetate, propionate, and butyrate).

3) **Depressive symptom severity** will be assessed by the original CES-D[58] and the CESD-R,[59] which are validated questionnaires of questions regarding feelings of depression, worthlessness, loneliness, energy level, and fear. See §5.2 for more information.

3.6 Study Intervention Products

In the parent study, participants will be randomized to consume either 48 g of freeze-dried blueberry powder (***equivalent to ~2 cups of fresh, whole blueberries***) or 48 g of placebo powder each day for this 12 week intervention. A single batch of freeze-dried blueberry powder and a single batch of placebo powder will be provided by the US Blueberry Council. A 48 g dose of the freeze-dried blueberry powder provides ~8 g of dietary fiber and ~600 mg of anthocyanins. The powder will be packaged into individualized packets (24 g/packet), and individuals will be instructed to consume 1 packet in the morning and 1 in the afternoon (i.e., 48 g/day). The placebo powder is a nutritionally matched powder (primarily consisting of maltodextrin, fructose, and dextrose) that is devoid of dietary fiber and anthocyanins. *Given that microbes are ubiquitous (even on blueberries[60]), we will evaluate the microbial content of the freeze-dried blueberry and placebo powder. We will preliminarily explore if microbes on foods are similar to the microbes that reside and proliferate in the gut.* Prior to the intervention, similar methods will be used as described for the fecal microbiota techniques in **§3.5**

To allow for blinding, the placebo powder will be labeled with either a letter or number that is different from the intervention powder. This is a double-blind intervention, so neither the participants nor the study staff will know which powder is the placebo and/or which is the blueberry powder. See **§**3.6 for further information on dietary intervention.

CHAPTER 4 RECRUITMENT AND DATA COLLECTION

4.1 Recruitment Overview

Participants will be recruited from the Boston area community, including senior housing facilities in urban/suburban areas and research recruitment repositories. We will utilize a multi-pronged approach to meet our recruitment goals:

- We will recruit from the research repository that resides at HRC
- We will connect with social workers in and outside the HRC
- We will perform medical record reviews to identify potentially eligible individuals at the Hebrew SeniorLife (HSL) geriatric medicine practices.
- We will advertise through direct mailings to all residents of HRC's seven supportive housing facilities (over 3,000 residents).
- We will give presentations at each Hebrew SeniorLife (HSL) facility.
- We will use the Harvard Catalyst (CTSA) Shared Health Research Information Network (SHRINE) to identify volunteers from Harvard-affiliated hospitals and clinics.
- We will advertise our study within numerous local media outlets, on HRC's Hinda and Arthur Marcus Institute for Aging Research and other websites (*e.g.*, Craig's List), and at www.clinicaltrials.gov.

4.2 Informed Consent

All interested individuals will be asked to provide verbal consent to complete an initial eligibility screen during a phone conversation with study personnel. Potentially eligible participants will then schedule an in-person screening visit. Potential participants may be sent by email or conventional post (per request, and according to their preference) a copy of the informed consent form for them to review at their own pace prior to the in-person screening. Written informed consent will be obtained by study personnel at the beginning of the in-person screening visit.

4.3 Participant Withdrawal

Any participant who expresses a desire to discontinue participation in the study will be withdrawn at their request immediately. All data collected prior to withdrawal will be maintained in the study data set.

Additionally, a subject may be withdrawn from the study prior to completing all of the study related procedures due to the following conditions:

- Subject safety issues
- Failure of subject to adhere to protocol requirements
- Disease progression
- Subject decision to withdraw from the study (withdrawal of consent)

Withdrawn subjects may not reenter the study unless there are extenuating circumstances (e.g. family emergency or required travel out of town) that interfere with the start of the study before any medications are administered. In this case, they may be scheduled to start over again. If new medical conditions arise or are exacerbated during the study intervention, the withdrawal of a participant will be evaluated by the PI, SO, and/or study psychiatrist.

4.4 Methods to Protect Participant Privacy

The following are the planned procedures for effectively protecting against and minimizing loss of participant privacy:

1. Phone screening will be conducted in a private office space.
2. Study visits will be conducted in private rooms.
3. Each participant will be given a unique study identification number and data will not include any of the participant's PHI.
4. All participant-identifying information will be stored and managed on a secured database server. The information will be password protected.
5. Participant confidentiality will be maintained in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations.
6. Only the PI, study personnel, and laboratory personnel approved by the IRB and authorized to view PHI will have access to the information.

7. PHI will not be used during discussion, presentation or publication of any research data.
8. Files containing PHI data collected for recruitment and screening purposes will be kept in locked, secured filing cabinets accessible only to designated study personnel (research assistants and investigators)

4.5 Minimization of Bias

This study is designed as a double-blind intervention so neither staff nor participants are aware of their assigned intervention arm.

4.6 Maximizing Compliance and Minimizing Attrition

At the start of an individual's study participation, he/she will be given a schedule of their study visits. Visits will be scheduled at a time of day that the participant determines is most convenient for them, and will repeated at the same time for each visit. Transportation will be provided for each visit as needed, snacks will be available, and stipends will be provided for each study milestone. If necessary, reminder calls will be made to participants on approximately 2 days prior to study visits.

Participants will be tracked throughout their enrollment. Each study visit will be documented. Some study visits will be followed with a brief telephone check-in to ask the participant questions about medication compliance, adverse effects, and their experience during the most recent visit. All calls to the participant and their feedback will be carefully tracked. Notes that may facilitate compliance, such as "call before 10 am," etc., will be kept in participant files.

We will employ specific strategies to maximize participation and compliance:

- ***Positive Framing about Benefits:*** Information will be presented in terms of the possible gains rather than the avoidance of losses as this is a more effective motivational approach.
- ***Feedback and Recognition of Progress:*** Participants will be acknowledged throughout their participation with thank you notes, and will be recognized for their contributions to the study through regular brief flyers/newsletters such as "Partners in Progress – Mobility and Falls updates". We will remain in close contact with individuals throughout their participation with follow-up calls each month.
- ***Incentives and Rewards:*** Participants will receive snacks at each visit, cards for achieving milestones, such as birthdays, holidays, etc.; and certificates of completion.

CHAPTER 5. RESEARCH METHODS

5.1 Participant Visit Schedule

All study visits will take place at the Clinical Research Laboratory at HRC, Roslindale, MA, an HRC-affiliated housing site, or at the participants home (if possible).

Participant eligibility will be determined during an in-person screening that is a part of the parent study. If eligible and interested, the participant will be asked to continue with 5 additional visits. The assessments/activities for the study visits pertaining to this specific ancillary study are outlined below. Some measures (e.g., safety measures) will overlap with the parent study and are indicated with a *.

The majority of subjects that will enroll in this ancillary study will undergo screening as a part of the parent study as outlined in **IRB Pro00064749.** However, if a participant wants to enroll only in this ancillary study, the participant will need to undergo the telephone screening and in-person screening visits as outlined in the parent study (**IRB Pro00064749**).

Visits	Assessments and Medication Administration at Each Visit

Baseline Visit, Week 0:	Assessments/Activities: 1. Turn in Diet Records* 2. Provide Fecal Sample 3. Depressive Symptoms 4. Positive/Negative Affect 5. Suicidal Ideation* 6. Symptoms*
Week 4 Visit:	Assessments/Activities 1. Turn in Diet Records* 2. Depressive Symptoms 3. Positive/Negative Affect 4. Suicidal Ideation* 5. Symptoms* 6. Compliance*
Week 8 Visit:	Assessments/Activities 1. Turn in Diet Records* 2. Depressive Symptoms 3. Positive/Negative Affect 4. Suicidal Ideation* 5. Symptoms* 6. Compliance*
Week 12 Visit:	Assessments/Activities: 1. Turn in Diet Records* 2. Provide Fecal Sample 3. Depressive Symptoms 4. Positive/Negative Affect 5. Suicidal Ideation* 6. Symptoms* 7. Compliance*

5.2 Study Visits and Assessments

A summary of this ancillary study's assessments is provided in the table below. Given that this is a pilot study, we will allow for flexibility for the administration of some assessments at visits, as long as these changes do not impede the scientific interpretation.

Assessment	Baseline	4 Weeks	8 Weeks	12 Weeks
Fecal Sample	X			X
Suicidal Ideation (via P4SS)*	X	X	X	X
Depression Severity (via CES-D)	X	X	X	X
3-Day Diet Record*	X	X	X	X
Relevant Symptoms*	X	X	X	X
Positive and Negative Affect	X	X	X	X
Compliance*		X	X	X

* Reflects that this measure is also a part of the parent study

The parent study consists of up to 6 study visits: a screening visit, a baseline visit, 3 mid-point visits, and a final follow-up visit. The ancillary study adds additional assessments to the baseline visit, 2 of the 3 mid-point visits, and the follow-up visit in week 12. Information on the study procedures at the study visits related to the ancillary study is outlined below.

Informed Consent: In order to participate in this study, all interested and eligible participants will be required to provide informed consent. They will be given ample time to ask any questions about the study. A trained research staff will answer any questions and if the individual is interested in participating in the study, they will be offered to sign the informed consent form. When the staff is confident that the participant is completely

familiar with the document and understands all the aspects of the informed consent form, it should be signed by the participant in the presence of the staff member, and should then be signed by the staff member. All consent forms will be double checked to make sure they are properly signed and dated. Copies of completed consent forms will be given to the participant and the original signed document will be kept on file at the Hinda and Arthur Marcus Institute for Aging Research. As a part of the informed consent process, potential participants will be clearly informed that this intervention is not a treatment option for depressive symptoms or depression, but rather studying feasibility of a dietary strategy for health. If seeking a treatment, they will be directed to their primary care.

2-Week Washout (Pre-intervention Activities): To ensure participants have a stable level of dietary influence on the gut microbiota, participants will be asked to avoid consumption of fiber-rich and anthocyanin-rich foods (i.e., washout period) for the two weeks prior to their baseline visit. Additionally, the week before baseline participants will be asked to fill a 3-day diet record to estimate nutrient intake. As a part of the parent study, the participants will be wearing an accelerometer to measure physical activity, which will be done for the remainder of the study.

Baseline Visit (~30 minutes): At the baseline visit, participants will be asked to turn in their 3-day diet records. Participants will also be asked to collect a fecal sample as is, in a sterile collection tube without any preservation medium that will be provided to participants. Participants will temporarily store the self-collected fecal sample in their own freezer (-17°C), and then transported to the lab at HSL for sample processing and long-term storage until analysis. Other questionnaire based assessments will be administered (e.g., depressive symptom severity, positive and negative affect, suicidal ideation, relevant symptom assessment).

3-Day Diet Records: Diet records (consisting of 2 weekdays and 1 weekend) will be reviewed by research staff for accuracy and completeness. Records will be entered into a dietary analysis program (e.g., Nutrition Data System for Research) to estimate dietary intake of nutrients.

Center for Epidemiological Studies Depression Scale (CES-D) and CESD-revised (CESD-R): Depressive symptoms will be assessed by the original CES-D[58] and the CESD-R,[59] which are validated questionnaires of questions regarding feelings of depression, worthlessness, loneliness, energy level, and fear. The original CES-D questionnaire is a validated 20-item assessment. It was more recently revised to the CESD-R, which changes some of the items to ask about symptoms that are more consistent with the updated Diagnostic and Statistical Manual of Mental Disorder-5 criteria for major depression. We will implement both questionnaires since the original CES-D is most widely used in the literature and due to the high internal consistency (r=0.90) and a test-retest reliability of 0.51 of the CESD-R.[61]

Relevant Symptoms: Information on relevant symptoms including gastrointestinal distress, appetite, pain etc, will be collected by self-report. Symptoms of anxiety and sleep will also be evaluated by self-report and/or validated measures.

Positive and Negative Affect: The Positive Affect Negative Affect Scale (PANAS) is a validated questionnaire that assesses two of the most prominent dimensions of emotional experience: positive and negative affect. The questionnaire is a 20 item assessment that consists of 10 items that evaluate positive affect and an additional 10 items that evaluate negative affect.[62]

Week 4 and Week 8 Mid-point Visit (~30 minutes): The Week 4 and Week 8 Mid-point Visit questionnaire based assessments will be administered (e.g., depressive symptom severity, suicidal ideation, relevant symptom assessment, positive/negative affect). We will also measure compliance.

Compliance: Compliance with our dietary intervention will be evaluated throughout the study. Participants will be asked to keep all of their used powder packets. At the study visit, participants will return all unused and used powder packets to estimate number of intended doses that were consumed.

Week 12 Final Follow-up Visit (~30 minutes): At the follow-up visit, participants will be asked to turn in their 3-day diet records and repeat the questionnaires/assessments that were assessed at baseline visit. Participants will also be asked to collect another fecal sample as is, in a sterile collection tube without any preservation medium that will be provided to participants. Participants will temporarily store the self-collected fecal sample in their own freezer (-17°C), and then transported to the lab at HSL for sample processing and long-term storage until analysis. Dietary compliance will also be estimated as previously described.

5.3 Dietary Intervention

The duration of this study will be a total of 14 weeks. The first two weeks will require participants to avoid eating fiber- and anthocyanin-rich foods.

Individuals will then be randomized to either the intervention or the control group. This is a double-blinded study, so the study staff not the participant will know what powder the participants are assigned to. Those randomized to the intervention group will be asked to consume approximately 48 g of freeze-dried blueberry powder as a source of ~8 g of fiber and 600 mg of anthocyanins. The proposed dose of 48 g of freeze-dried blueberry powder is equivalent to approximately 2 cups of whole blueberries. Individuals randomized to the control group will be asked to consume approximately 48 g of a nutritionally matched placebo powder that is devoid of anthocyanins and fiber.

The powders will be individually packaged in 24 g amounts. Participants will be instructed to consume their respective powder, mixed in an 8 fl oz glass of water twice per day for 12 weeks. They will be asked to consume 24 g in the morning and 24 g in the afternoon. Given that intervention and control products are regularly consumed, we do not anticipate any toxicities.

5.4 Outcome Measures

Our primary endpoint is depressive symptom severity after 12 weeks of the dietary intervention. Secondary outcomes include aspects of the gut microbiota; however, we will also explore alternative outcomes.

Name	Type	Timeframe	Brief description
Depressive Symptom Severity	Primary	Baseline, Week 4, Week 8, Week 6, and Final Follow-up (Week 12)	Severity of depressive symptoms will be measured each week with the CES-D
Abundance of SCFA producing microbes	Secondary	Baseline and Final Follow-up (Week 12)	The abundance of microbes that produce SCFA (e.g., *Bifidobacteria, Eubacterium, and Clostridium*[56,57]) will be evaluated by whole genome sequencing
Fecal SCFA	Secondary	Baseline and Final Follow-up (Week 12)	The concentration of SCFA in feces will be measured using gas chromatography mass spectrometry.

CHAPTER 6. STATISTICAL DESIGN

6.1 Statistical Analysis

All analyses will be performed by intent to treat, and further evaluated per protocol. As a first step, we will assess distribution characteristics of the primary and secondary outcomes. Where appropriate, transformation of variables to combat skew or other irregularities will be employed. Participant characteristics will be summarized using means, medians, standard deviations, interquartile regions and ranges for continuous variables and sample counts and proportions generated for discrete characteristics. Comparability of treatment arms will be assessed on potentially confounding characteristics using tabular and graphical methods.

6.2 Aim Statistical Methods and Power

Aim 1 Hypothesis: The abundance of microbes that produce SCFA increase after 12 weeks of dietary fiber and anthocyanin supplementation compared to placebo. **Aim 1 Analysis:** To determine taxa and pathways that differ significantly by treatment (blueberry vs. placebo powder), multivariate analyses that accounts for covariates will be carried out using MaAsLin2[63] (Multivariate Analysis by Linear Models). MaAsLin2 performs a mixed-effects linear regression of individual log-transformed microbial features (response) on categorical or continuous factors (explanatory variables). A random effects model will be used for repeated measures (i.e., longitudinal data). Linear models will be constructed to assess each feature independently, and adjusted for false discovery using Benjamini-Hochberg FDR. The output of MaAsLin will reveal whether cohort participants with different dietary exposures also differ in relative abundance of a particular bacterial species or pathway in the gut**. Aim 1 Expected Results/Power:** With our proposed sample size of 20/group, we anticipate power 0.95 to detect a 0.314-unit difference in common taxa (~14310 sequences and 0.0954 relative abundance at 5% read usage rate), and a 0.122-unit difference in rare taxa (~2222 sequences and 0.0148 relative abundance at 5% read usage rate) collected at the 2 time points. Approximating as above, the corresponding detection limit for 250 pathways is power 0.95 for a 0.0013-unit difference (1282 sequences at a 33% read usage rate). We assumed we will observe 250 pathways.

Aim 2 Hypothesis: The fecal SCFA's increase after 12 weeks of dietary fiber and anthocyanin supplementation compared to placebo. **Aim 2 Analysis**: Since butyrate is implicated as the gut-derived metabolite important for depression,[30] we will focus on the change in fecal butyrate, but others SCFA and combinations will be explored. Change in fecal butyrate will be calculated between baseline and the final 12 week follow-up. Distribution of change will be summarized using sample quantities and kernel density estimates. We will develop 80% confidence interval estimates of inter- and intra- individual variation (i.e., standard deviation) of change in fecal butyrate, the upper limit will be used to estimate sample size calculations for a subsequent clinical trial. Formal inference and estimation of treatment effects will use a Student's t-test to compare mean change in fecal butyrate in the intervention group compared to placebo. **Aim 2 Expected Results/Power**: A 12 week intervention in non-depressed older adults supplementing usual diet with prebiotic fiber, report mean percent of fecal butyrate concentration increased by 0.4%.[64] Assuming standard deviation of mean percent change of fecal butyrate = 0.32% and an alpha=0.05, a sample size of 20/group is required to detect a 0.3% change in fecal butyrate.

Aim 3 Hypothesis: Depressive symptom severity (evaluated by CES-D) declines after 12 weeks of dietary fiber and anthocyanin supplementation compared to placebo. **Aim 3 Analysis:** Change in depressive symptom severity (CES-D) will be calculated between baseline and the final 12 week follow-up (we will consider change between baseline and visits at week 4 and 8). The same analyses described for Aim 2, will be implemented for Aim 3. **Aim 3 Expected Results/Power**: Other lifestyle interventions, including dietary strategies, that were designed to reduce depressive symptoms report between a 2-4 point reduction in CES-D scores.[65-67] Assuming standard deviation of change of depressive symptoms = 3.5 estimated from other lifestyle interventions and an alpha=0.05, a sample size of 20/group is needed to detect a 2 point reduction in CES-D scores.

CHAPTER 7 DATA MANAGEMNT AND QUALITY

7.1 Data Management Plan

All data collected for analysis will be de-identified and assigned a unique study number. Any data collected on paper forms will be kept in a locked file cabinet at HRC. Data collected on paper forms will be entered and stored on a password-protected secure server at HRC. When possible, data will be collected directly via our electronic data capture system (e.g, REDCap).

Expected data type: this project includes both digital and non-digital data. Non-digital data may include hand-written questionnaires and dietary records that will be collected on/by each participant, as well as intervention compliance (via physical return of used/un-used packets). All non-digital data, will be documented and transferred to a digital database (e.g., REDCap) that is used for secure data acquisition and storage. Digital

data will include the genomic sequences of the gut microbiota acquired from the fecal and powder samples, as well as quantification of fecal short chain fatty acids. All data from this study will be primary data.

Data format: Non-digital data acquired from this study will be kept as papers forms, but also will be transferred to our data capture/storage software, REDCap. The Marcus Institute for Aging Research (Marcus Institute) primarily employs the REDCap system to facilitate digital data management operations. REDCap is a full-featured clinical trials data management system (DMS) accessible to data entry and data analysis workstations using secure Web technologies. The REDCap product is developed and maintained by Vanderbilt University in cooperation with REDCap Consortium members, including our institute. Hebrew SeniorLife hosts and maintains a dedicated instance of REDCap for use across our research enterprise. REDCap has the added benefit of offering data exportation the datasets into several various spreadsheet-type formats (e.g., .csv, .xlm, .r, .pdf, .sps, etc.). To transpose non-digital data to digital formats, questionnaires will transferred to our study-specific, secure REDCap database. Some questionnaires may be captured digitally in real-time, via direct data capture with an iPad that uploads the data to our secure REDCap database. For the data that is not directly captured or entered into REDCap, the data will have to be merged into a master data file. Dietary records will first be entered into dietary analytic software (Nutrition Data System for Research, NDSR), utilizing required formats. Once all data is entered into NDSR, it will be exported into an spreadsheet-type format, (e.g., excel to produce a .csv file) to facilitate data merging and long-term storage in the REDCap database. The other digital data gathered from fecal samples (e.g., quantification of short chain fatty acid and abundances of specific microbes) will be stored in a spread-sheet like format (e.g., .csv) to facilitate data merging with the data stored on REDCap. All data related to this project will be merged into a final master dataset that will be used for analyses and will contain enough information to allow independent use of the data. The various types of formats provided by REDCap (e.g., .csv, .xlm, .r, .pdf, .sps, etc.) will help ensure that the data will be readily accessible and usable datasets in community-recognized standard and machine readable formats.

Data storage and preservation: During the intervention, any data collected on paper forms will be transposed to the digital data capture software (e.g., REDCap) as soon as possible. The de-identified, non-digital data (e.g., paper forms) collected as a result of this study will be stored in a locked cabinet at the Marcus Institute, and the PI will be responsible for the key to the cabinet. Only approved individuals (e.g., study staff) will be able to access the paper forms in the locked cabinet. For long-term preservation, digital data will be stored indefinitely on the secure server employed by the Marcus Institute, that will be accessible by qualified individuals (e.g., study staff and monitors of the USDA). Paper forms will be stored for at least 5 years following the completion of this study. The final digital dataset will be uploaded to a secure, password protected digital cloud (e.g., ShareFile) to avoid loss of data.

Data sharing, protection, and public access: Data resulting from this study will be shared upon request. Individuals that would like to use de-identified data may do so providing the appropriate approvals are acquired (e.g., IRB). To protect the confidentiality of the participants of this study, we will make the identifiable data and associated documentation available to users under a data-sharing agreement that provides for: 1) a commitment to using data only for research purposes and not to identify any particular participant; 2) a commitment to securing the data using appropriate computer technology; and 3) a commitment to destroying or returning the data after analyses are completed. The availability of data will be advertised over the Internet through websites maintained by Hebrew SeniorLife and Harvard Medical School. All investigators wishing to access the data will submit a brief proposal to Dr. Millar describing their research project, data needs, regulatory approvals (e.g., IRB and USDA approval), and mechanisms to assure patient confidentiality. Upon affirmative review by the PI and co-investigators of this study, a data-sharing agreement will be signed and the requesting investigators will be given a working electronic data file and appropriate documentation. Although identifiable information will be collected, the master data file will be de-identified to avoid breach of confidentiality. All recruited individuals will be given a unique study ID number that will be used in place of identifiable information when possible. To minimize the risk of breach in confidentiality, all computer datasets that contain personal identifiers (e.g., name, address, and phone numbers) will be stored in a separate encrypted dataset. The pass phrase to gain access to the encrypted files will be known only to qualified individuals (e.g., the PI, data analyst, and research staff). Any form or printout that contains personal identifiers will be stored under lock and key. All digital data will be entered and stored on a password-protected secure

server at Marcus Institute. Each research study is provided separate project workspace on REDCap, in which all of the study data are stored in a MySQL relational database on the private corporate network behind several firewalls and located physically within the Hebrew SeniorLife data center.

7.2 Participant Tracking

Each recruited participant will be tracked closely throughout study enrollment. If desired, a study events calendar will be created for each participant. Any outstanding or incomplete visits will be accessible in real time to the project director and study team. The study team will maintain regular communications with each study participant throughout enrollment, through regularly scheduled follow up calls, and established retention strategies will be used as discussed in **§**4.6.

CHAPTER 8 DATA SAFETY MONITORING PLAN

8.1 Participant Risks

Participation in this ancillary study may be associated with minor risks or safety concerns. The potential risks of this study fall into 5 categories: 1) those related to research participation; 2) those related to testing procedures; 3) those related to the intervention or control products; and 4) those related to depressive symptoms. The risks are outlined for each category below:

Minor Risk of Participation in Research: With any study, risk of breach of confidentiality is possible since we are collecting personal health information.

Minor Risk of Testing Procedures: The potential risks of the testing procedures are minor since the majority will be questionnaire based assessments. It is possible that participants may find the questionnaires tedious or may be uncomfortable being asked about sensitive topics like suicidality. It may also be unpleasant to collect a fecal sample.

Minor Risk Related to Intervention or Control Products: We do not anticipate any major risks for the participants with consumption of either the intervention (freeze dried blueberry powder) or control (placebo powder) products. Participants might grow weary and uninterested in consuming the products each day over the 12-week period. Additionally, the proposed intervention will provide an older adult with 30% of the recommended daily intake of dietary fiber. One may experience side effects that commonly occur with increased dietary fiber consumption if the individual typically consumes relatively low amounts of dietary fiber.

Risk Related to Depressive Symptoms: It is possible that individuals with depressive symptoms may progress in symptom severity during the study period. Although we do not anticipate a change to a more severe category of depression, we recognize that our proposed population of older adults with prevalent depressive symptoms are already predisposed to development of more severe depressive disorders. Importantly, our population of interest is also more likely to experience suicidal ideation.

8.2 Risk Minimization

We will attempt to minimize the identified risks as specified below:

Risk Minimization of Participation in Research: To minimize the risk of breach in confidentiality, all primary study data will be recorded with computer tablets on electronic case report forms (CRF) or as digital files generated from laboratory equipment. All data recording will be in accordance with procedures and guidelines outlined in the study's Manual of Procedures (MOP) authored by the study team. Participant confidentiality will be maintained by recording subject data using a unique subject identifier. Identifiable data, such as contact information and medical record numbers, will be recorded and stored separately from the clinical study data. Any paper-based study material and any identifiable data will be kept separate in a locked file cabinet accessible by authorized study staff only. Only the

study staff directly responsible for the data collection and the safety of the participant will have access to identifiable information. All electronic CRF data will be stored securely in an electronic data capture and management system. Raw electronic instrumentation data will be organized and saved on a private network file dedicated to the research project. Only those listed on the approved IRB protocol will have access to subject data. Subject data will be coded and locked in a file cabinet in a locked office. Identifying information will not be used during discussion, presentation or research publication. All documents and electronic data will be archived for a minimum of three years, or as required by the IRB and federal regulations, after the completion of the clinical trial. The study will be registered at clinicaltrials.gov.

The Hinda and Arthur Marcus Institute for Aging Research employs the Research Electronic Data Capture (REDCap) system for data capture and data management operations. REDCap is a full-featured clinical trials data management system (DMS) accessible to data entry and data analysis workstations using secure Web technologies. While REDCap can be used to collect virtually any type of data (including 21 CFR Part 11, FISMA, and HIPAA-compliant environments), it is specifically geared to support online or offline data capture for research studies and operations. REDCap is developed and maintained by Vanderbilt University in cooperation with REDCap Consortium members, including HRC. HSL hosts and maintains a dedicated instance of REDCap for use across our research enterprise. Each research project is provided separate workspace in which all of the study data are stored in a MySQL relational database on the private corporate network behind several firewalls and located physically within the HSL data center.

Risk Minimization of Testing Procedures: The majority of the testing procedures will be questionnaire based. Participants will be advised that they can refuse to answer any of the questions. Participants will be permitted to rest between studies to prevent fatigue. To minimize the risk of being uncomfortable during questionnaires on sensitive topics like suicidality, only trained research staff will administer the questionnaires. Research staff will be trained to administer them in a calm, welcoming demeanor and will reassure the participants that the assessment(s) can stop at any time.

To avoid any unpleasant experience collected a fecal sample, study staff will provide detailed instruction on how to collect the sample. Furthermore, to avoid the burden of storing it in the participants freezer, study staff will pick up the collected sample as soon as possible from the study participant.

Risk Minimization of Intervention Product: Individuals will allergies to intervention products will not be included in this study to avoid any adverse effects/allergic reactions. We anticipate that the blueberry and placebo powder will be well-tolerated by participants since they consist of dietary nutrients that are regularly consumed. Regardless, every 4 weeks we will ask participants about any complaints or adverse events that are directly related to the study intervention products. We plan to track diarrhea, gas, bloating, abdominal pain, constipation etc. Since the intervention arm will be increasing fiber consumption and sudden changes in fiber intake may cause gastrointestinal distress, participants will be counseled to consume their dietary intervention products alone (i.e. without other foods) to avoid consumption of large quantities of fiber at one time. Since our intervention provides only ~30% of the recommended intake of daily fiber, we do not anticipate this amount will result gastrointestinal distress, if consumed alone. Nevertheless, symptoms of gastrointestinal distress will be captured at each visit and appropriately addressed by the study team. Dr. Millar, who has a PhD in Nutrition, will consult with participants on their experience with the fiber and reconcile any gastrointestinal stress. If a participant develops a health problem or a potential health problem (in addition to the ones outlined below), the PI will be notified ASAP. If necessary, the SO will be contacted to help decide whether the participant should continue in the study, and/or what further steps regarding medical evaluation should be performed.

Risk Minimization of Depression Severity: Although we do not anticipate a change to a more severe form of depression, we recognize that our proposed population of older adults with prevalent

depressive symptoms are already predisposed to development of more severe depressive disorders. The level of depression will be evaluated and monitored at all visits (except for the telephone screen) to proactively monitor participant safety. If at any time during the study an individual's assessments indicate they have progressed to a more severe forms of depression, then specific safety protocols will be followed (e.g., notifying the study psychiatrist and/or PI). Our population of interested is also more likely to experience suicidal ideation, which will also be monitored at every visit to identify any individuals, who may need psychiatric care outside this study. If the participant is deemed to be dangerous or at imminent risk of harm, study staff will contact emergency services (i.e., #911) for immediate medical assistance.

General Risk Minimization: The proposed protocol requires 5 visits over a total 14 weeks and therefore imposes a moderate amount of participant burden with respect to time and effort. Our institute has a strong track record of successful clinical research requiring similar participation, and retention has been high in these projects. The Clinical Research Laboratory at the Marcus Institute is located near a cafeteria and rest room, and is equipped with comfortable seating, a TV, movies, books, and magazines to keep individuals occupied during rest periods. Several additional strategies will be employed to minimize participant burden and maximize adherence to the protocol. We will:

- Develop a personal relationship between participants and members of the staff.
- Schedule appointments at convenient times with familiar staff.
- Explain to participants all aspects of their participation and follow up. We will demonstrate and practice study procedures before beginning data collection.
- Provide reminders of all appointments and follow-up phone calls.
- Include personal notes in the participant's data file to remember events in the life of the participant; these can be commented on at the next visit (e.g., birthday, birth of a grandchild).
- Provide snacks and lunch during all visits.
- Provide transportation for all visits, if required.
- Provide valet or dedicated on-site parking spaces.
- Compensate participants for visits.

8.3 Quality Assurance and Safety Monitoring

The Principal Investigator (PI) will assume primary responsible for ensuring participants' safety on a daily basis. Since this is a single-site, phase 1 pilot study, without high risk, our study will not require an official Data and Safety Monitoring Board (DSMB). However, to ensure and monitor participant safety, any adverse event will be documented. The PI will have the support of her co-investigators, Drs. Lewis Lipsitz and Douglas Kiel, who are both geriatricians and will assist the PI assessing severity and relatedness of any safety events.

8.4 Adverse Event Collection and Reporting

Any adverse or serious adverse events will be logged using forms either provided by or modeled after the forms that are provided by the NIA Clinical Research Toolbox. Since there is no separate screening visit for this specific study, the baseline visit will mark the beginning of when any potential adverse or serious adverse events could occur/be collected. All other AE's will be collected as a part of the parent study.

Adverse Event Definition and Categorization

An adverse event is any untoward medical occurrence in a participant, whether or not it is causally related to the study. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom or disease temporally associated with the study. Adverse events will be recorded on the appropriate case report forms and source documents. The PI and/or trained staff member will evaluate all adverse events as to their severity and relation to the study. The severity of adverse events will be graded as follows:

Mild: Awareness of a sign or symptom but easily tolerated.
Moderate: Discomfort sufficient to cause interference with usual activity or to affect clinical status.
Severe: Incapacitating with inability to do usual activity or to significantly affect clinical status.
Life Threatening: The participant was at immediate risk of death from the adverse event as it occurred.

The Investigator will also assess the relationship of any adverse event to the study, based upon available information, using the following guidelines:

0 = Unlikely: No temporal association, or the cause of the event has been identified
1 = Possible: Temporal association, but other etiologies are likely to be the cause; however, involvement of the study procedures cannot be excluded.
2 = Probable: Temporal association, other etiologies are possible, but not likely.

To determine the attribution and temporal association of an adverse event we will consider the following:

1) Whether the participant reports they have experienced the same symptom prior to the study intervention.
2) Whether the symptom occurred and resolved within 24 hours of taking the study intervention.

The PI and study staff will consider the symptom according to the conditions stated above and determine temporality as per clinical judgment.

Definition of a Serious Adverse Event

A serious adverse event is any experience that results in any of the following outcomes:

- Death
- Is life-threatening
- Inpatient hospitalization or prolongation of hospitalization

A persistent or significant disability/incapacity. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. We do not anticipant any Serious Adverse Events with our intervention.

Adverse and Serious Adverse Event Reporting

There is a potential for adverse events and incidental findings during this study. A structured questionnaire asking about adverse events will be assessed during each visit of the intervention period. However, when any adverse has been identified, the study team will take appropriate action necessary to protect the study subject and then complete the Adverse Event form that will be modeled after the form provided by the NIA Clinical Research Toolbox. This form requires Principal Investigator review and signature. After review by the Principal Investigator any adverse event will be reported to the IRB as appropriate.

AE's that 1) are unexpected in nature, severity, or frequency, 2) are possibly, probably, or definitely related, and 3) suggests that the research places participants at a greater risk of harm than previously known or recognized, will be reported to the IRB as appropriate (e.g., within 2 weeks of the event).

If a serious event occurs, it will be brought immediately to the attention of the Principal Investigator, who will discuss the event with the co-investigators and together decide if immediate treatment is necessary. If such treatment is necessary, the PI, co-investigators, or the study staff will coordinate such treatment at an appropriate hospital or urgent care setting, contact the primary care physician, and notify the IRB. A Serious Adverse Event form that is modeled after the one provided by the NIA Clinical Research Toolbox will be completed, which requires Principal Investigator review and signature. If an AE is defined as a SAE, the Principal Investigator will be notified as soon as the event is known about. Routine reporting of any SAEs will be reported to the IRB within as appropriate.

Unanticipated problems or adverse events will be reported according to Advarra's IRB written guidelines for interventional studies. Unanticipated problems and serious adverse events that are probably, possibly, or definitely related to the study will be reported as soon as possible from the time of learning of the event, but reported within 10 days to Advarra's IRB per Advarra IRB guidelines. Advarra will be provided a written report submitted and a submission of the incident via the eIRB system. This form will record any adverse symptoms and/or study protocol deviations. Study staff will reference a Subject Safety Event Reporting Decision Chart

provided and updated regularly by Advarra to determine whether an event needs to be reported to the Advarra IRB.

All other adverse events/study incidents will be logged on an Adverse Event log and reported to the IRB following the appropriate reporting times as defined by the Advarra IRB.

For less serious or incidental findings the Principal Investigator will speak with the participant about the finding. If necessary, the PI may suggest appropriate follow-up with their clinical provider and/or provide a letter describing the findings and need for follow-up.

Any adverse events that take place during testing will be reported to the PI and recorded in the database. The PI will have ultimate responsibility for monitoring participant safety in the trial. The investigators will be responsible for reviewing each adverse event in a timely fashion, and reporting all incidents to the appropriate regulatory agencies according to written guidelines.

8.5 Participant and Study Stopping Rules

Participant Stopping Rules: If a participant experiences any adverse event that is deemed "severe" as outlined in **§**8.4 (Adverse Events Collection and Reporting) their continuation in the study will be determined by the PI. If necessary, the PI will consult the co-investigators to gain insight on participant continuation. Additionally, if a serious adverse event (SAE) occurs, it will be carefully reviewed by the study team. Any report of a serious adverse event (SAE) that is thought to be directly related to the study products or study procedures, will result in the participant's discontinuation from the study.

Study Stopping Rules: Similar to the participant stopping rules, all serious adverse events (SAE) will be carefully reviewed by the study team to determine if study termination is warranted.

8.6 Potential Benefits

Participants may not receive any significant health benefit from participation, although some may benefit from knowledge of their health status, as well as potential therapeutic effects freeze-dried blueberry powder. This will be a first of its kind trial that evaluates a dietary intervention in sedentary, older adults with depressive symptoms. Confirmation that a dietary intervention modulates the gut microbiota and/or depressive symptoms may provide older adults with a safe, well-tolerated, cost effective alternative strategy to ameliorate depression. If our findings confirm our hypotheses, subsequent trials will be appropriately designed using the variation in change of our outcomes gathered from this study. Such a study will be adequately powered to determine definitive efficacy of a dietary intervention to target the gut microbiota and depressive symptoms in sedentary, older adults with depressive symptoms.

8.7 Participant Compensation

Participants will be provided up to $50 stipend to compensate them for their time spent completing study procedures.

CHAPTER 9. TRAINING

A manual of operations will be created with standard participant instructions for each question and assessment. All research staff will review and sign the Site Signature Log – Delegation of Authority Log that is modeled after the log provided by the NIA Clinical Research Toolbox to confirm their responsibilities related to the study. During startup, staff will undergo intensive training, and all training sessions will be logged and signed accordingly. They will conduct all study procedures on a few volunteers (more if necessary) with oversight from the PI to ensure consistency of raters and equipment setup. Quality checks will be done every six months throughout the data collection period.

Training will be based on standardized materials developed for the study, and coordinated by the Project Director/Study Coordinator. Every six months, the staff will undergo training review and quality checks on all

assessments and drug distribution protocols. Additionally, any time there is an amendment to the study protocol, the change will be logged on a Change in Protocol Log. All study staff will be provided a summary of the protocol modifications and under-go re-training for the new protocol. The date, duration, and certification of all training will be documented and signed by the Principal Investigator on the appropriate training logs.

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