1) Protocol Title

Title: *The influence of honey-flavored yogurt on low-grade inflammation and gut health in middle to older aged women.*

Protocol Version Date: *September 25, 2019*

2) Objectives

To assess the influence of four weeks of honey flavored yogurt intake on pro-inflammatory CD4+ T Helper (TH)17 cytokines, via Luminex Bead Assay, with a focus on Interleukin (IL)-23 (primary outcome). The secondary outcomes will be to assess the influence of microbial derived metabolites on pro-inflammatory cytokines and oxidative stress. In addition, intestinal microbial populations will be determined in order to assess the impact of the intervention on this population and metabolism.

3) Background

The goal of the proposed research is to assess the influence of short-term honey intake on low-grade inflammation, oxidative stress, and gut microbial-derived metabolites in healthy adult women. Honey is a phenolic-rich food that has long been used for beneficial digestive health, yet only limited scientific evidence exists that documents this potential health benefit, including its effects on measures of immune function and microbial-derived metabolites.

Obesity is a risk factor for type 2 diabetes and cardiovascular disease (CVD) with both characterized by a low-grade systemic inflammatory phenotype. Inflammation in CVD is initiated by endothelial damage, and promoted by a number of cell types, including white blood cells and platelets that in themselves produce substantial amounts of lipid mediators, cytokines and reactive oxygen species (ROS) that can amplify the immune response. Research has focused on the role of adipose tissue dysfunction and insulin resistance in the promotion of inflammation, however, recent studies suggest a role for a disturbance in gut homeostasis. The gut epithelial barrier and immune system tolerates non-pathogenic bacteria, protects against pathogenic subtypes, and absorbs needed nutrients and other dietary and intestinal bacterial-derived factors. Diet can significantly influence gut bacterial populations within a short period of time. TH17 cytokines, including IL-23, play a role in a number of autoimmune diseases including inflammatory bowel disease. In conjunction with diet, the gut microbiota produces short chain fatty acids (SCFAs), secondary bile acids (BAs), and phytochemical metabolites that influence both local and systemic physiologic and immune responses. Therefore, the proposed study will focus on the assessment of the influence of short-term daily honey intake on pro-inflammatory Th17 cytokines and the influence of known microbial-derived metabolites.

Polyphenols and phenolics from a number of foods have been observed to produce positive physiologic effects from circulating metabolites produced after primary small intestinal absorption or after bacterial metabolism. A limited number of studies have shown a positive influence of an increased intake of certain polyphenolics on the gut microbiome, as well as microbial derived metabolites. As genetics, health status, and diet can lend to increased variability in the selected biomarkers among individuals, we have included in our research plan a metabolomics analysis as a secondary objective. This approach can potentially

capture phenotypic differences associated with habitual diet that can be related to physiological response for which traditional methods of dietary intake and associated databases are not sufficiently sensitive to discriminate individual differences in diet and metabolism. In addition, individual differences in urolithin metabotype have been observed. As urolithins are the microbial metabolites of the tannin ellagitanin, which is found in walnuts, strawberries, and some honeys, urolithin metabotype may also allow for further discrimination of biomarker response in our study population.

Finally, we have observed that the intake of phenolic-rich honey can increase plasma antioxidant activity within hours of intake. While intriguing, regulatory agencies now prefer outcomes of oxidative stress of known physiological relevance and mechanism. Therefore, in order to capture the impact of honey intake on ROS production, we will measure NADPH oxidase (NOX2), uric acid (UA) and total nitrate/nitrite and related nitric oxide (NO) metabolites (RSNO). These measures were selected based on their contribution to oxidative stress, physiologic function, and disease pathology, and they are influenced by the diet and microbiome.

4) Inclusion and Exclusion Criteria

Prior to enrollment, all volunteers that meet the initial inclusion and exclusion criteria based on a telephone interview will be asked to participate in a clinical screening visit. This screening visit will assess the individual's overall health, and whether the individual meets the inclusion and exclusion criteria for the study. Participants will arrive in the morning to the Department of Nutrition's Ragle Human Nutrition Research Center. The participants will provide their written consent, after which time their weight and height are measured. The subjects will undergo an office blood pressure measurement. After completion of the blood pressure measurement, a fasting blood sample (30mL ~ 2 Tbsp.) will be collected. This will conclude the screening visit. The blood sample will be sent to the UC Davis Medical Center Pathology Lab used for the measurement of a complete blood cell count, plasma lipids and a comprehensive metabolic panel.

We seek to enroll individuals that are overweight, not obese, with no self-reported disease. As hormonal changes throughout the menstrual cycle can potentially influence the outcome measures we will only enroll women who are postmenopausal with an absence of a period for at least 2 years. To further limit potential variability, we will also exclude those individuals who are on prescription medications, supplement use, and/or consume a high plant based diet.

Inclusion and Exclusion Criteria

Inclusion:

- Postmenopausal female: 45-65 years
- Women: lack of menses for at least two years.
- Subject is willing and able to comply with the study protocols.
- Subject is willing to participate in all study procedures

- BMI 25.0 – 30.0 kg/m^2

Exclusion:

- BMI ≥ 31 kg/m^2
- Food allergies
- Self-reported use of daily anticoagulation agents including aspirin, NSAIDs
- Vegan, Vegetarians, food faddists or those consuming a non-traditional diet
- Use of concentrated food supplements/powders and extracts
- Fruit consumption > 2 cups/day
- Vegetable consumption >3 cups/day
- Self-reported restriction of physical activity due to a chronic health condition
- Self-reported chronic/routine high intensity exercise
- Self-reported diabetes
- Blood pressure ≥ 140/90 mm Hg
- Self-reported renal or liver disease
- Self-reported heart disease, which includes cardiovascular events and stroke
- Abnormal Metabolic or CBC panels (laboratory values outside the reference range) if determined to be clinically significant by the study physician.
- Self-reported cancer within past 5 years
- Self-reported malabsorption
- Currently taking prescription drugs or supplements.
- Supplement use other than a general formula of vitamins and minerals that meet the RDA
- Not willing to stop any supplement use, including herbal, plant or botanical, fish oil, oil supplements a month prior to study enrollment.
- Indications of substance or alcohol abuse within the last 3 years
- Cannabis use
- Screening LDL ≥ 190 mg/dl for those who have 0-1 major risk factors apart from LDL cholesterol (i.e. family history of premature coronary artery disease (male first degree relative < 55 years; CHD in female first degree relative < 65 years), cigarette smoker, HDL-C ≤ 40 mg/dL]. (using NCEP calculator http://cvdrisk.nhlbi.nih.gov/calculator.asp)
- Screening LDL ≥ 160 mg/dl for those who have 2 major risk factors apart from LDL cholesterol [i.e. family history of premature coronary artery disease (male first degree relative < 55 years; CHD in female first degree relative < 65 years), cigarette smoker, HDL-C ≤ 40 mg/dL]. (using NCEP calculator http://cvdrisk.nhlbi.nih.gov/calculator.asp);
- Screening LDL ≥ 130 mg/dl for those who have 2 major risk factors apart from LDL cholesterol [i.e. family history of premature coronary artery

disease (male first degree relative < 55 years; CHD in female first degree relative < 65 years), cigarette smoker, HDL-C ≤ 40 mg/dL], and a Framingham 10-year Risk Score 10-20% (using NCEP calculator http://cvdrisk.nhlbi.nih.gov/calculator.asp).

- Current enrollee in a clinical research study.

The following special populations will be excluded:

- Individuals who are not yet adults (infants, children, teenagers)
- Pregnant women
- Prisoners

5) Study Timelines

We expect that it will take approximately 3 years to recruit, enroll and complete the study, as well as, to complete the data analysis. The screening day visit will take approximately 1 hour to complete the consenting process, anthropometric measures, complete the health and dietary questionnaire and the blood draw. Participants eligible for enrollment will be asked to participate in a 12 week study that includes a pre-visit, plus 4 study day visits, with each visit lasting about 2 hours. They will also be asked to arrive at the facility after 2 weeks of their assigned yogurt intake in order to pick up yogurt for an additional 2 weeks of intake. This visit should take no longer than 30 minutes Participants will be asked to complete daily compliance logs that will document product intake, and 3 day food records during the last two weeks of each study phase

6) Study Endpoints

The objective will be to assess the influence of four weeks of honey flavored yogurt intake on pro-inflammatory CD4+ T Helper (TH)17 cytokines, via Luminex Bead Assay, with a focus on Interleukin (IL)-23. Cytokines included in the panel are: Il-1b, IL-4, IL-6, IL-10, IL-17a, IL-21, IL-22, IL-23, IL-31, IL-33, IFN□, TNF□, IL-17f.

Secondary outcomes will be to assess the influence of microbial derived metabolites on pro-inflammatory cytokines and oxidative stress. In addition, intestinal microbial

populations will be determined in order to assess the impact of the intervention on this population and metabolism.
The outcomes will be as follows:
Gut Microbial Derived Metabolites:

- Plasma secondary bile acids (BAs)
- Fecal short chain fatty acids (SCFAs)
- Conjugated and unconjugated urolithins and other ellagitannin-derived plasma metabolites

Oxidative Stress:

- Plasma soluble NADPH oxidase (NOX2)
- Plasma Uric Acid (UA)
- Plasma total Nitrate/Nitrite and Nitric Oxide related metabolites (RSNO)

Microbiome/Metabolism:

- Serum and Urine Metabolomics
- Fecal microbiome

7) Procedures Involved

Experimental Approach:

A randomized, double-blind, crossover dietary intervention trial will test the effects of 4 weeks of daily honey-flavored yogurt intake on markers of inflammation (Th17 cytokines) and oxidative stress (NOX2, UA, RSNO) and associative changes with microbial derived metabolites (SCFAs, BAs, ellgaitanins), metabolism and the fecal microbiome.

Study Design:

Twenty overweight (25-30 kg/m2) women 45-65 years of age will be enrolled into a 12 week dietary intervention trial. The study design will be a 2-arm crossover, with each arm 4 weeks in length. A 4 week washout period will be in between the two ars. blinded and controlled crossover study design. Prior to study enrollment, potential volunteers will be initially screened for eligibility using a telephone scree, then prescreened with a fasting blood sample (30mL ~ 2 Tbsp.), and a health interview. The blood samples will be sent to the UC Davis Medical Center Pathology Lab for a comprehensive metabolic and lipid panels and complete blood counts.

Those qualified for enrollment will be randomized to consume 2 morning servings of a 0.6 cup (150g) of plain yogurt with : A) cane sugar added in an isocaloric level as the honey or, B) 1 tbsp of phenolic-rich honey for 4 weeks. A one-month washout between periods will be used. The participants will be instructed to consume one yogurt in replacement of or as part of breakfast, and as a late morning snack. They will also be instructed to not add any additional items to the yogurt. Therefore, each study participant will consume 2 tbsp. of honey a day for four weeks, which is a realistic amount typically consumed by honey users. They will be provided two weeks worth of yogurt, which they will be instructed to keep frozen until 1-2 days prior to intake, which at that time they can

store in the refrigerator. After the first two weeks of intake they will be asked to arrive to the Ragle center in order to pick up an additional two weeks of yogurt.

One week prior to each study visit, the participants will be asked to refrain from the intake of non-steroidal anti-inflammatory medications, and from using mouth wash as these can affect the microbiome and inflammatory markers.

Study volunteers will be asked to participate in 4 study visits each scheduled before yogurt intake and after 4 weeks of daily yogurt intake.

For each study visit:

The study participants will be asked to collect a stool sample with the kit provided at home 24-48 hours prior to each study visit. They will also be asked to collect a "first" morning urine prior to their arrival to the facility.

Collection procedures will be performed at the same time of the day to minimize circadian effects. Anthropometric measures will be collected, and blood pressure will be measured after a 15 minute resting period (3 measurements taken 5 minutes apart). A fasted (12 hour no food or beverage, except water) blood sample (50 mL, ~3.5 tbsp) will be collected for the biochemical measurements, and a spot urine will be collected for metabolomics

Plasma and blood will be sent to the UC Davis Medical Center Pathology Lab for a comprehensive metabolic and lipid panels and complete blood counts.

Dietary intakes will be assessed during each dietary phase with three-day food records. Compliance will be assessed through the use of a daily intake log, and return of used and return of unused packaging.

Potential gastrointestinal symptoms and bowel changes will be assessed via a daily log and a weekly questionnaire.

Test Yogurts:

The honey will be provided from commercial producers from the National Honey Board, and will be one of the following: alfalfa, clover, orange blossom or buckwhet. The final selected honey will have the highest total polyphenolic content. Plain yogurt and sugar will be purchased through a grocery or restaurant supply. Plain Greek non-fat yogurt (for example Chobani) that provides S. Thermophilus, L. Bulgarious, L. Acidophilus, Bifidus and L. Casei as a culture will be selected. The test yogurt will include 1 tablespoon per serving of the selected honey and will provide 149 total calories and 13g of protein. The control yogurt will be isocalorically matched, and sweetened with 17g of cane sugar (such as "C&H" brand), a commonly used commercial sweetener in yogurts. The yogurts will be portioned out into individual serving sizes and stored frozen. The participants will be instructed to thaw their daily portions 2-3 days prior to intake in the refrigerator. The yogurts will be mixed and packaged at the Ragle Human Nutrition Center's commercial kitchen facility. The formulated yogurts will be kept frozen until they are distributed

8) Data and/or Specimen Management and Confidentiality

☒I understand that the UC Davis Health Electronic Health Record (EMR/EPIC) also contains the clinical data for Marshall Medical Center (MMC). I understand that MMC patient data cannot be accessed for research purposes and that I must take the necessary steps to ensure that MMC data is not accessed, used, or disclosed for UC Davis Health research purposes.

Statistical Analysis: IL-23 is considered a key regulator of the inflammatory TH17 response 7, therefore, the response of IL-23 to 4 weeks of honey intake will be the primary outcome measure. The study is powered using data from our previous study in overweight or obese children and 4-week dietary changes in IL-23, using a mean difference of 28.9 pg/mL with a SD of 30 pg/mL, an n =18 will be needed for an α =0.05 at 80% power. We will enroll 20 participants in order to account for potential attrition, which typically has been less than 10% in our recent clinical studies among postmenopausal women. Statistical testing will be conducted by Repeated Measures Two-way ANOVA with Tukey's post hoc testing, using treatment and time as factors. Confirmation of potential period effects will be analyzed, as well as, comparing the baseline after washout to the "pre-testing" period sampling that will occur on an individual's baseline diet prior to treatment assignment. Multivariate analysis will be conducted to assess relationships between the changes in cytokine response to circulating markers, and bacterial taxa with honey intake. Multiple comparison adjustments will follow the False Discovery Rate correction.

All data derived from this study will be identified through an alpha numerical code. The code will be associated with the individual's name and contact information in an enrollment log kept as separate file stored on a password protected departmental servers. Only the clinical coordinator and research staff that are responsible for the daily conduct of the research study will access this information.

The enrollment log will contain the subject's name, contact information, enrollment details and study identifier that will be kept in a file and folder that is separate from study data. The enrollment log satisfies IRB requirements for subject enrollment data, and only enables the study coordinator with study flow, by linking study folders for the current study day with the correct subject identifier. This enrollment log will be saved and maintained on a server in the Department of Nutrition. All departmental servers are housed in a secure server room with an alarm system and restricted access (physical access to the drives is restricted and only designed study coordinators can access the server drive in question). No identifiable human subject data will be accessed on any mobile devices (USB flash drives, laptops, cell phones, etc). If any de-identified data is stored on a mobile device, such as a laptop, it would be encrypted, as all departmental laptop hard drives are encrypted with bitlocker.

All study folders are identified by the study participant code, and all contents within the folders will only contain this code. This will include all source documents. A source document will be maintained for each study visit, to include the screening. The source document serves as a check for the adherence of study procedures including, and checks for the proper conduct of the consenting process during the screening visit.

All questionnaires and consent forms will be kept in a locked cabinet in a departmental office that is accessible only to the study researchers. All samples, including those sent out for outside analysis (for example, lipid analysis at UCDMC) will have only a numerical identifier, and date/time.

All data resulting from the sample analysis will also be kept in a spreadsheet associated with the numerical identifier that will be saved and maintained on the Department of Nutrition server, in a drop-box that is accessible only to study researchers; it will be ID and password protected. Department servers are maintained in a highly secured facility due to the proprietary nature of some UC Davis, Department of Nutrition research.

9) Data and/or Specimen Banking

Plasma/serum samples will be stored coded for up to 5 years at -80oC freezer in a secured laboratory at the Department of Nutrition. After the 5 year period the samples will be destroyed. Data will be continued to be maintained indefinitely in the Department of Nutrition as described above. Only those individuals involved in the analyses will have direct access to the samples. Upon appropriate IRB approval, and subjects' permission, de-identified samples and data may be shared with outside researchers or analytical core facilities. These outside researchers will not have access to any identifiable subject information. De-identified samples will be provided to investigators that specialize in the measurement of the Th17 cytokines, microbiome and metabolomics, and the Department of Pathology.

10) Provisions to Monitor the Data to Ensure the Safety of Subjects

Participants will be monitored for intolerance/adverse reaction to yogurt or honey intake throughout the study period by direct questioning by study staff. They will also be monitored for adverse effects of phlebotomy during the blood draws.

11) Withdrawal of Subjects

In the event that volunteers withdraw from the research, they will receive prorated compensation for whatever portion of the study they have completed. If the investigators determine that a volunteer no longer meets the eligibility criteria, they will be withdrawn

from the research study. Blood samples from time points that occurred prior to withdrawal will still be analyzed, and will not be destroyed.

In case of the incidence of any serious adverse effects. We will consult with the volunteer as to their symptoms, and if necessary, withdraw them from the study. Any serious adverse event as well as other unanticipated problems will be reported to the IRB within five days of the investigator becoming aware of such an event.

12) Risks to Subjects

Risks and side effects related to consumption of the yogurts may include a change in bowel sensation or function. This might include abdominal fullness/bloating, abdominal gurgling, increased gas leading to increased pressure, or gas expulsion. More extreme changes like cramping, diarrhea or constipation may occur.

Other risks include those associated with venipuncture, which may include potential discomfort, bruising, dizziness, and rarely, infection. We have rarely encountered instances in which subjects reported serious discomfort, and therefore, we assess the likelihood and seriousness of this risk to be small.

13) Potential Benefits to Subjects

No direct benefits to the individual participants are anticipated.

14) Sharing of Results with Subjects

The participants will receive copies of their clinical laboratory reports, which will give them general information regarding their overall health status.

15) Prior Approvals

No prior approvals needed.

16) Provisions to Protect the Privacy Interests of Subjects

During the telephone screening process the individuals will be informed that they do not need to answer any questions that they feel uncomfortable answering. Additional health or dietary habit information will be obtained during the clinical screening visit, via written questionnaire that will not include the person's name in order for them to answer in private. Situations where private health information will be obtained, such as weight

or blood pressure measurement will be conducted in a private area, away from the view or hearing of others.

17) Economic Burden to Subjects

Participation in this study will not be an economic burden.

18) Review Requirements

Are there any contractual obligations or other considerations that require IRB review of this research, or review at intervals other than those required by the Common Rule or FDA? If yes, check box:

☐ Yes

X No