INSTITUTIONAL REVIEW BOARD PROTOCOL REVIEW REQUEST

1) **Date:** 12/18/23

2) **Study Title:** Supplementation with a Next Generation Synbiotic in Individuals with Overweight or Obesity: A Triple-Blinded Randomized Controlled Trial

3) **Principal Investigator (must be a TCU faculty or staff):** Elisa Marroquin, PhD

4) **College/School:** Texas Christian University

5) **Other Investigators: List all faculty, staff, and students conducting the study. For those not affiliated with TCU, please provide their names and institution.**
Ryan Porter, Sarah McKinley-Barnard, Tim Ritter, Melissa Fernandez, Jessica Mrosla, Katelyn Harnen, Genevieve Aiwonegbe, Olivia Landis, Jade Nesbitt, Malia Shipsey, and Meggan Duncan

6) **If you *have internal or* external funding for this project –**
Funding Agency: The supplement company Pendulum will provide the product (probiotic and placebo with a market value of $50,400). The Dean's Opportunity Fund will provide $49,512 for blood analyses, blood and stool sample tubes, and stool sample whole genome sequencing. Dr. Marroquin's will provide part of her start up funds ($6,000) for participants compensation. **Project #: IRB#2023-345** **Date for Funding:** November 2023

7) **If you *intend to seek/are seeking* internal/external funding for this project –**

Funding Agency: TCU Graduate SERC Grant

Amount Requested From Funding Agency: $2,000

Due Date for Funding Proposal: October 23rd, 2023

8) **Purpose: Describe the objectives and hypotheses of the study and what you expect to learn or demonstrate:** Our proposed triple-blinded, placebo controlled, randomized clinical trial is divided into two stages.

The first stage will consist of exploring the effect of next generation synbiotic supplementation for 12 weeks on body composition, insulin sensitivity, gut microbiota composition and diversity, depression, anxiety, and food cravings in 60 individuals with overweight or obesity who have no diagnosis of diabetes mellitus (DM). For this first stage, we hypothesize that this next generation synbiotic will decrease adiposity, overall body weight, anxiety, depression, and food cravings and increase insulin sensitivity, and gut microbiota diversity. We also hypothesize that the levels of bacterial species in the symbiotic will increase in the gut microbiome.

The second stage will only be conducted if enough external funds are ensured by using the results from the first stage as preliminary data. The second stage would consist of performing 16S rRNA on blood samples to analyze microbial composition in blood, and metabolomic analysis to evaluate the effect of the probiotic on gut-derived metabolites in blood and stools. The hypothesis of this second stage of the project is that the next generation symbiotic will change blood microbial composition as well as the presence and concentration of gut-derived metabolites in blood and stool samples. If relationships exists between changes in body composition, insulin sensitivity, depression, anxiety, or cravings (first stage variables) with blood microbial composition and derived-

metabolites (second stage variables), we could show not only a cause-effect relationship but also the mechanism of action through which the next generation symbiotic acts.

9) <u>Background:</u> Describe the theory or data supporting the objectives of the study and include a bibliography of key references as applicable.

Obesity is a strong risk factor for the development of the majority of the leading causes of death.[1] Recent research has pointed to the gut microbiota as a potential mechanism of action through which body weight can be regulated.[2] The gut microbiota of people with obesity has a lower alpha diversity[3] and lower abundance of *Akkermansia muciniphila*.[4-7]

Next Generation Probiotics have been created as a result of recent scientific advances.[8] *A. muciniphilia* is one of the next generation species that has independently shown anti-obesity potential.[9] *A. muciniphila* is inversely correlated with body weight, fat mass, fasting glucose, and hedonic eating.[10-12] *A. muciniphila* is a gram-negative anaerobic bacteria and therefore it is difficult to grow in the lab. Pendulum® (the company that will donate the product to TCU), which possesses a patented strain of *A. muciniphila*, has been able to create an oxygen-free manufacturing process to cultivate and grow this bacterial specie. In mice, its administration has shown to be sufficient to protect from diet induced obesity and insulin resistance.[13] In humans, *A. muciniphila* supplementation at a concentration of 10^{10} bacteria per day for 12 weeks is safe and effective and can improve metabolic parameters such as body weight, fat mass, and insulin resistance in volunteers with overweight or obesity who are insulin-resistant.[14] In addition to physical benefits, preliminary research indicates *A. muciniphila* to have potential psychological benefits.[15] In mice and humans, depression and anxiety are significantly associated with decreased *A. muciniphila* abundance.[16-22] Furthermore, *A. muciniphila* supplementation in mice with chronic restraint stress-induced depression resulted in significantly diminished depression-like behavior.[19] Additionally, FMT from healthy patients decreased anxiety and depression in patients with refractory irritable bowel syndrome or ulcerative colitis, changes that were associated with higher abundance of *A. muciniphila*.[21,22]

Synbiotics combine probiotics (alive bacteria) and prebiotics (bacteria food) in order to improve microorganism survival and activity within the host.[23] The synbiotic that will be used in this trial contains the oligosaccharide-degrading probiotics, *A. muciniphila* and *Bifidobacterium infantis*; butyrate producers such as *Clostridium beijerinckii, Anaerobutyricum hallii,* and *Clostridium butyricum*; and chicory-derived inulin, a prebiotic dietary fiber. Butyrate is a gut-derived short chain fatty acid suspected to play a role in weight regulation and insulin resistance.[24] Butyrate administration in mice models of obesity improves weight and fat mass regulation, increases lipid oxidation in brown adipose and liver tissue, and stimulates the production of gut-derived neuropeptides involved in energy homeostasis and food intake, such as GIP, GLP-1, PYY, and serotonin, and decreases fasting insulin.[25-27] A previous pilot clinical trial indicated the synbiotic that will be used in the current study to be safe and tolerable as well as effective in reducing HbA1c and improving post-prandial glucose control in individuals with type 2 diabetes mellitus (T2DM) and overweight or obesity,[28] however, no studies have analyzed the effect of the synbiotic on body composition, anxiety, and depression in individuals with overweight who do not have T2DM.

References:

1. Censin JC, Peters SAE, Bovijn J, et al. Causal relationships between obesity and the leading causes of death in women and men. *PLoS Genet*. Oct 2019;15(10):e1008405. doi:10.1371/journal.pgen.1008405

2. Muscogiuri G, Cantone E, Cassarano S, et al. Gut microbiota: a new path to treat obesity. *Int J Obes Suppl*. Apr 2019;9(1):10-19. doi:10.1038/s41367-019-0011-7
3. Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature*. Jan 22 2009;457(7228):480-4. doi:10.1038/nature07540
4. Million M, Maraninchi M, Henry M, et al. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. *Int J Obes (Lond)*. Jun 2012;36(6):817-25. doi:10.1038/ijo.2011.153
5. Da Silva CC, Monteil MA, Davis EM. Overweight and Obesity in Children Are Associated with an Abundance of Firmicutes and Reduction of Bifidobacterium in Their Gastrointestinal Microbiota. *Child Obes*. Apr 2020;16(3):204-210. doi:10.1089/chi.2019.0280
6. de la Cuesta-Zuluaga J, Corrales-Agudelo V, Carmona JA, Abad JM, Escobar JS. Body size phenotypes comprehensively assess cardiometabolic risk and refine the association between obesity and gut microbiota. *Int J Obes (Lond)*. Mar 2018;42(3):424-432. doi:10.1038/ijo.2017.281
7. Crovesy L, Masterson D, Rosado EL. Profile of the gut microbiota of adults with obesity: a systematic review. *Eur J Clin Nutr*. Sep 2020;74(9):1251-1262. doi:10.1038/s41430-020-0607-6
8. O'Toole PW, Marchesi JR, Hill C. Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat Microbiol*. Apr 25 2017;2:17057. doi:10.1038/nmicrobiol.2017.57
9. Chang CJ, Lin TL, Tsai YL, et al. Next generation probiotics in disease amelioration. *J Food Drug Anal*. Jul 2019;27(3):615-622. doi:10.1016/j.jfda.2018.12.011
10. Santacruz A, Collado MC, Garcia-Valdes L, et al. Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. *The British journal of nutrition*. Jul 2010;104(1):83-92. doi:10.1017/S0007114510000176
11. Albaugh VL, Banan B, Antoun J, et al. Role of Bile Acids and GLP-1 in Mediating the Metabolic Improvements of Bariatric Surgery. *Gastroenterology*. Mar 2019;156(4):1041-1051 e4. doi:10.1053/j.gastro.2018.11.017
12. Dao MC, Everard A, Aron-Wisnewsky J, et al. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut*. Mar 2016;65(3):426-36. doi:10.1136/gutjnl-2014-308778
13. Everard A, Belzer C, Geurts L, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America*. May 28 2013;110(22):9066-71. doi:10.1073/pnas.1219451110
14. Depommier C, Everard A, Druart C, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med*. Jul 2019;25(7):1096-1103. doi:10.1038/s41591-019-0495-2
15. Xu R, Zhang Y, Chen S, et al. The role of the probiotic Akkermansia muciniphila in brain functions: insights underpinning therapeutic potential. *Crit Rev Microbiol*. Mar 2023;49(2):151-176. doi:10.1080/1040841X.2022.2044286
16. Song J, Ma W, Gu X, et al. Metabolomic signatures and microbial community profiling of depressive rat model induced by adrenocorticotrophic hormone. *J Transl Med*. Jul 15 2019;17(1):224. doi:10.1186/s12967-019-1970-8
17. Park YS, Kim SH, Park JW, et al. Melatonin in the colon modulates intestinal microbiota in response to stress and sleep deprivation. *Intest Res*. Jul 2020;18(3):325-336. doi:10.5217/ir.2019.00093
18. McGaughey KD, Yilmaz-Swenson T, Elsayed NM, et al. Relative abundance of Akkermansia spp. and other bacterial phylotypes correlates with anxiety- and depressive-like behavior following social defeat in mice. *Sci Rep*. Mar 1 2019;9(1):3281. doi:10.1038/s41598-019-40140-5
19. Ding Y, Bu F, Chen T, et al. A next-generation probiotic: Akkermansia muciniphila ameliorates chronic stress-induced depressive-like behavior in mice by regulating gut microbiota and

metabolites. *Appl Microbiol Biotechnol*. Nov 2021;105(21-22):8411-8426. doi:10.1007/s00253-021-11622-2
20. Aatsinki AK, Keskitalo A, Laitinen V, et al. Maternal prenatal psychological distress and hair cortisol levels associate with infant fecal microbiota composition at 2.5 months of age. *Psychoneuroendocrinology*. Sep 2020;119:104754. doi:10.1016/j.psyneuen.2020.104754
21. Huang HL, Chen HT, Luo QL, et al. Relief of irritable bowel syndrome by fecal microbiota transplantation is associated with changes in diversity and composition of the gut microbiota. *J Dig Dis*. Aug 2019;20(8):401-408. doi:10.1111/1751-2980.12756
22. Kump P, Wurm P, Grochenig HP, et al. The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis. *Aliment Pharmacol Ther*. Jan 2018;47(1):67-77. doi:10.1111/apt.14387
23. Rabin Gywali NN, Rita Fiagbor, Tahl Zimmerman, Robert H. Newman, Salam A. Ibrahim. *The Role of Prebiotics in Disease Prevention and Health Promotion*. Dietary Interventions in Gastrointestinal Diseases Foods, Nutrients, and Dietary Supplements; 2019:151-167.
24. Mayorga-Ramos A, Barba-Ostria C, Simancas-Racines D, Guaman LP. Protective role of butyrate in obesity and diabetes: New insights. *Front Nutr*. 2022;9:1067647. doi:10.3389/fnut.2022.1067647
25. Lu Y, Fan C, Li P, Lu Y, Chang X, Qi K. Short Chain Fatty Acids Prevent High-fat-diet-induced Obesity in Mice by Regulating G Protein-coupled Receptors and Gut Microbiota. *Sci Rep*. Nov 28 2016;6:37589. doi:10.1038/srep37589
26. Yadav H, Lee JH, Lloyd J, Walter P, Rane SG. Beneficial metabolic effects of a probiotic via butyrate-induced GLP-1 hormone secretion. *J Biol Chem*. Aug 30 2013;288(35):25088-25097. doi:10.1074/jbc.M113.452516
27. Lin HV, Frassetto A, Kowalik EJ, Jr., et al. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS One*. 2012;7(4):e35240. doi:10.1371/journal.pone.0035240
28. Perraudeau F, McMurdie P, Bullard J, et al. Improvements to postprandial glucose control in subjects with type 2 diabetes: a multicenter, double blind, randomized placebo-controlled trial of a novel probiotic formulation. *BMJ Open Diabetes Res Care*. Jul 2020;8(1)doi:10.1136/bmjdrc-2020-001319

10) <u>Location</u>: Specifically describe where the research will take place. If on TCU campus please list the exact location. If off campus please describe the exact location(s) where you plan to conduct your research and be prepared to provide a <u>letter of support</u> with your submission.
The informed consent and all research procedures will take place at the Kinesiology Department (Rickel building, rooms 256 and 259).

11) <u>Subject Population:</u> Describe the characteristics of the participant population.
<u>Please list out and state the</u> age range (for adults and/or children), inclusion and exclusion criteria and the total number of participants you plan to recruit:
60 participants ages 18-50 that have a BMI between 25.0-40.0 kg/m^2, who do not also have type 1 or type 2 diabetes mellitus (T1DM or T2DM) will be recruited. Exclusion criteria include: being vegetarian, vegan, carnivore, or keto; pregnancy or planning pregnancy during the study period; lactating women; having a history of inflammatory bowel disease, colon cancer; having been diagnosed with T1DM or T2DM; active cancer; currently participating in a weight loss intervention (dietetic or medication); use of antibiotics, antifungals, or antivirals in the last 3 months; currently taking metformin, GLP-1 agonists, insulin, or fiber supplement; having taken PPI, stimulant laxatives, probiotics, or immunosuppressants in the last month; history of recent (within 30 days) diarrhea illness (including bacterial or viral enteritis or enteric parasitic infection); having a known

hypersensitivity to any component of the study product; having an acute infection or inflammatory condition over the past 4 weeks; having >10% weight variation in the last 6 months; history of bariatric surgery in the past with exemption of laparoscopic band that has been removed.

12) **Recruitment Procedure: Describe your recruitment strategies in great detail including how and where the potential participants will be approached and by whom. Will recruitment involve a point of contact on behalf of the study team? Who and how will recruitment material(s) be distributed? Describe precautions that will be taken to minimize the possibility of undue influence or coercion. Include copies of the recruitment emails, flyers, social media post, etc. in your submission as attachments or appendices following the recruitment guidelines.**
TCU staff and students will be approached for recruitment via IRB-approved email (colleagues will be asked the favor to send the recruiting email to their current students), posters, flyers, and social media ads in English and Spanish. Posters and flyers will be posted in strategic locations such as dinning halls, TCU cafeterias, dorms, bathrooms, gym, etc. as well as electronically on the Nutrition Department's webpage and Pendulum's webpage (supplement company). Ads will also be posted in strategic locations outside TCU such as gyms, cafeterias, health care centers, recreation centers, etc. Social media adds will be published in the professional Instagram page of the PI (Elisa_Marroquin_PhD) and the professional Instagram and Facebook pages of the Nutrition Department (TCUNutrition and TCU Department of Nutritional Sciences, respectively), as well as through paid adds both in Facebook and Instagram. These recruitment methods will contain a QR code that links to a screening survey to evaluate participant eligibility. Precautions that will be taken to minimize undue influence or coercion: reiterate that participation is voluntary when dispersing recruitment materials, include all required elements aligned with TCU's recruitment guidelines for advertisement materials, and refrain from singling out students and staff that are familiar with or have personal ties to individuals on the research team.

13) **Consenting Procedure: Describe the consenting procedure, whether participation is completely voluntary, whether the participants can withdraw at any time without penalty, the procedures for withdrawing, and whether an incentive (describe it) will be offered for participation. If students are used as participants, indicate an alternative in lieu of participation if course credit is provided for participation. If a vulnerable population is recruited, describe the measures that will be taken to obtain surrogate consent (e.g., cognitively impaired participants) or assent from minors and permission from parents of minors. If you need to request consent or HIPAA waivers you can do so in this section. If including Non-English-speaking participants, please include a plan to have consent documents translated after initial approval, by either student or faculty that is fluent in the desired language (give names and document translation and back translation) or a translation service/vendor. If the study involves deception explain the process of debriefing statement that will be given to participants and attach the script that will be used by the investigators to orally explain the study.**
Advertisements, fliers, and emails contain a QR code that leads participants into a screening questionnaire which provides us with information regarding their eligibility to participate in this study. Email and phone number, but not other personal information, are collected in this screening form with the intention of contacting back the participants who are eligible. Eligible participants will be contacted through phone or email to schedule their first visit to the Kinesiology Department, Rickel Building 259. A trained, IRB-approved member of the research team will perform the informed consent. The consent document is attached to this protocol. Non-English-speaking participants may be included in this study. An IRB-Spanish version of the informed consent will be provided to the participants. The IRB Spanish version was written by Dr. Elisa Marroquin who is

fluent in Spanish and obtained her B.S. in México. Dr. Marroquin will conduct all Spanish-speaking informed consents. Students can take part in this project but will not receive extra credits. No vulnerable populations will be recruited.

The triple blinding nature of this study means that participants, researchers (including the PI), and statisticians cannot be made aware of the randomization allocation. Therefore, debriefing to participants can only take part until after data has been analyzed. Once all participants have finalized their interventions and all data has been analyzed, researchers will contact back participants to verbally give them the debriefing statement and inform them which supplement/placebo they received.

14) <u>Study Procedures:</u> Provide a chronological description of the procedures, tests, and interventions that will be implemented during the course of the study. Indicate the number of visits, length of each visit, and the time it would take to undergo the various tests, procedures, and interventions. If blood or tissue is to be collected, indicate exactly how much in volume and all procedures that will be done with collected samples. Describe when, where and how collection will take place. Flow diagrams may be used to clarify complex projects. For Non-TCU study team members please explain their study team role in detail. If the study involved deception justify the use of deception and explain why deception is necessary to achieve the goals of the study. Explain if alternative methods not involving use of deception were considered and why these methods are not being used.

STAGE 1

For stage 1, participants will come to TCU on three days total:

<u>In the first visit,</u> participants will come for 45 minutes to the Rickel Department where they will receive a complete explanation of the protocol and will process to sign the "Informed Consent to Participate in Research and Authorization to Collect, Use, and Share their Health Information" if agreeing to participate. Participants' height and weight will be measured to ensure they meet BMI inclusion criteria. Height will be measured using a standard stadiometer and weight will be measured using a standard calibrated digital scale. Their race will be asked to help in data interpretation. Height, weight, race and other information relevant to antibiotic prescription such as date of birth, allergies to medications, and preferred pharmacy will be collected at this time point using our document titled "checklist 2". All symbiotic and placebo bottles will be entirely similar with exemption of individual codes that will vary every 3 bottles. This is because each participant will be provided at the beginning of the study with 3 bottles (with similar code) to ensure enough supply for the 12-week supplement intervention. The company Pendulum will perform an *a priori* randomization of the entire study by using blocked randomization with randomly selected blocked sizes with the intention to reduce bias and ensure balance between groups. This randomization will be added into an Excel document in which they will specify the order of the participant in column 1 (example: Participant 001, Participant 002), the code of the bottles in column 2, and the interpretation of the code (placebo vs probiotic) in column 3. A researcher, independent from this study (Meggan Duncan), will save in different protected locations (Box, secured laptop, protected USB) the randomization document. Meggan will share with Dr. Marroquin an Excel document containing column 1 and column 2 of the provided document (the order of participant and the code of the bottles, without the interpretation of each code). Dr Marroquín will use the supplement code as the participants' IDs and study data will be added to this Excel document. Both documents, from, Dr. Marroquín and from Meggan, will be merged at the end of the study, once data has been analyzed to figure out the intervention group to which each participant belonged. During the first

visit, depending on the order they enrolled, participants will receive the 3 bottles containing the assigned code. Participants will also be provided with two stool sample collection kits, a 3-day diet record form, a 3-day prescription of antibiotics (500 mg of generic Vancomycin every 8h), date for second appointment, and a start date for the antibiotic regimen. The 3-day antibiotic treatment provided prior to beginning the 12-week synbiotic intervention is given with the intention to increase the number of available bacterial niches (bacterial homes/space) and thus increase the engraftment of the newly ingested bacteria. Although participants will be asked to pay the cost of the generic antibiotic treatment initially (which should have an estimated cost of $20-25), we will offset the cost of the antibiotic by providing the participants with a $50 Amazon gift card in their second appointment, so they will not be responsible for any costs to participate in this study. This initial gift card is in addition to their $100 Amazon gift card provided at the end of the study in appreciation for having taken part in this project. Their insurance will not be charged for any study-related testing; however, they are welcome to (but don't have to) use their insurance to buy the antibiotic. During this first visit, participants will have their 1) first 12h fasted blood collection and will be instructed to 2) fill out a 3 day food log before each stool sample collection, 3) collect a stool sample before the start of the antibiotic regimen, 4) collect a stool sample within 12-48 hours after the last dose of the antibiotic regimen, 5) bring both stool samples and diet records with them to the second appointment and, 6) fast 12 hours prior to the second appointment (refrain from the consumption of all food and liquids, except water). The first appointment is expected to take no greater than 45 minutes.

For the second visit, participants will meet researchers at the Rickel building. This appointment is expected to take approximately 45 minutes and includes the completion of a DEXA scan, an air displacement plethysmography test (via BOD POD), body weight measurement, the Beck Anxiety and Depression Inventories, the RED-9 questionnaire, and a blood draw. Participants will turn in their first (before antibiotic prescription initiation) and second (after antibiotic prescription completion) stool samples and food logs to researchers. The blood draw will consist on 2 serum separation tubes (10 mL each), 1 EDTA tube (3 mL), and 1 Zymo tube (3 mL). The time to complete each test is approximately as follows: 10 minutes for the blood draw, 10 minutes for the anxiety and depression survey, 20 minutes for the DEXA scan, and 5 minutes for an air displacement plethysmography test. Air displacement plethysmography tests via the BOD POD will be used to measure body fat percentage. Participants will be asked to wear minimal clothes and no shoes for the air displacement plethysmography test. The DEXA scans will be used to measure fat mass, visceral fat, lean mass, and bone mineral density. The DEXA scan uses a two-sided low emission X-ray to quantify lean body mass, fat mass, and bone density. To conduct the test, participants will be asked to void their bladder, remove all metal objects (piercings, rings, etc.) and shoes, and will lay supine on a padded table for approximately 6 minutes while the scanner moves down the length of the participant's body. Measurements will be conducted by a General Electric Certified DEXA Operator (Dr. Ryan Porter or Dr. Sarah McKinley-Barnard). Blood samples will be collected using sterile techniques and only by qualified investigators (Dr. Ryan Porter or Dr. Sarah McKinley-Barnard). Blood samples will be used to measure fasting glucose, fasting insulin, and HbA1C. Blood will be sampled using venipuncture. The venipuncture samples will be collected using a traditional blood draw needle to extract blood from a vein in the antecubital region of the arm. Two 10 mL vials of blood will be collected, centrifuged to separate the serum. One of those tubes will be stored in a -80°C freezer in the Exercise Physiology Laboratory (Rickel 259) whereas the other will be transported to BioReference for fasting glucose and insulin analysis. The 3 mL for EDTA analysis will also be transported to BioReference for HbA1C analysis. The Zymo collection tube (3 mL) will be stored at the Rickel 259 building. All blood collection vials and plasma/serum containers will be

labeled using participant numbers (deidentified) for participant privacy. The leftover blood samples will be stored in the laboratory for 10 years from date of collection at which point they will be discarded as biohazard waste. Participants agree to allow their deidentified samples to be used for future research by signing the informed consent. These procedures have been approved by the TCU Institutional Biosafety Committee (IBC Protocol). At the end of the second appointment, participants will be provided with the 12 weeks of the probiotic+prebiotic supplement. Each person will receive 3 bottles (one per month) with the three of them having the same ID, this same ID will be used to identify all questionnaires and samples obtained from that person. This ID will also be different between each participant. Participants will also be instructed to 1) record everything they eat for 72 hours before stool sample collection, 2) collect a stool sample within 24 hours of finishing the last dose of the intervention, 3) bring the stool sample, diet record, and remnant of their supplement or placebo with them to the third appointment, and 4) fast 12 hours prior to the third appointment (refrain from the consumption of all food and liquids, except water).

The third and final appointment is expected to also take approximately 45 minutes and will include a replication of all tests conducted in the second appointment (completion of a DEXA scan, an air displacement plethysmography test [via BOD POD], body weight measurement, the Beck Anxiety and Depression Inventories, the RED-9 questionnaire, and a blood draw [consisting on 2 serum separation tubes of 10 mL each, 1 EDTA tube of 3.5 mL, and 1 Zymo tube of 3mL]). Participants will turn in their third and final stool sample, as well as the remnant of their supplements or placebo for analysis of adherence and compliance. Participants will receive a $100 Amazon gift card at the end of the study in compensation for their time. After all stool samples have been collected, they will be sent in the same batch to the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine in Houston for posterior whole genome sequencing analysis.

Whole Genome Shotgun Metagenomic Sequencing will reveal not only the characterization of all the species present in the sample down to the strain level but also their functionality according to their genetic capacity. Unlike 16S rRNA which sequences only a specific region of the ribosome, whole metagenome sequencing sequences all the genomic material present in the sample and thus allows for higher resolution down to the strain level. Whole metagenome sequencing also allows for the identification of viruses and other important microorganisms whose importance we are just now starting to understand. That being said, the main goal of incorporating whole metagenome sequencing in this study is to explore the effect of the probiotic on the gut microbiota composition and functionality when it is preceded by short antibiotic exposure. The following represents the whole metagenome sequencing methods employed by the CMMR at Baylor College of Medicine.

> *CMMR Bioinformatics WGS Pipeline*. Raw data files in binary base call (BCL) format are converted into FASTQs and demultiplexed based on the dual-index barcodes using the Illumina 'bcl2fastq' software. Demultiplexed raw fastq sequences are processed using BBDuk (sourceforge.net/projects/bbmap/; BBMap version 38.82) to quality trim, remove Illumina adapters and filter PhiX reads. Trimming parameters are set to a k-mer length of 19 and a minimum Phred quality score of 25. Reads with a minimum average Phred quality score below 23 and length shorter than 50 bp after trimming are discarded. The trimmed fastqs are mapped to a combined PhiX (standard Illumina spike in) and host reference genome database using a two-step BBTools approach (sourceforge.net/projects/bbmap/; BBMap version 38.82). Briefly, the trimmed reads are first processed through the bloomfilter script, with a strict k=31 to remove reads identified as human. The remaining reads are mapped to the reference genome

with BBMap using a k-mer length of 15, the bloom filter enabled, and fast search settings in order to determine and remove host/PhiX reads.

Taxonomic profiling – MetaPhlAn2. Taxonomic profiling of the sequenced samples is done using MetaPhlAn2 (4). Processed fastq reads are first mapped against the MetaPhlAn2 marker gene database (mpa_v20_m200) using bbmap (3) with the bloom filter enabled and fast search settings. Each sample is run through the metaphlan.py script to generate the kingdom-specific taxonomic profile per sample, using the flag to generate relative abundances and estimated read counts. The MetaPhlAn2 utility scripts are employed to merge the output for all samples into a single sample per taxon table for each kingdom and relative abundance and estimated read count output. Finally, the tables are converted into biom-format for further statistical analysis.

Taxonomic profiling – MetaPhlAn3. Taxonomic profiling of the sequenced samples is done using MetaPhlAn3 (7). Processed fastq reads are first mapped against the MetaPhlAn3 marker gene database (mpa_v30_CHOCOPhlAn_201901) using bbmap (3) with the bloom filter enabled and fast search settings. Each sample is run through the metaphlan.py script to generate the kingdom-specific taxonomic profile per sample, using the flag to generate relative abundances and estimated read counts. The MetaPhlAn3 utility scripts are employed to merge the output for all samples into a single sample per taxon table for each kingdom and relative abundance and estimated read count output. Finally, the tables are converted into biom-format for further statistical analysis.

Functional profiling – HUMAnN2. Functional profiling of the microbial community will be done using HUMAnN2 (5). The standard recommended workflow will be followed with modifications to the nucleotide and translated alignment steps. Briefly, nucleotide alignment is performed using bbmap (3) with the bloom filter enabled (bloomk=22) and fast search settings generating a HUMAnN2 compatible SAM file output. The translated alignment step is performed using diamond (6; version 0.9.26). This creates the default pathway abundance and coverage tables, as well as gene family abundance output files per sample. Post-processing of the per-sample tables is done using a combination of HUMAnN2 utility scripts and in-house code designed to clean up the tables for better readability. The three default outputs are each merged across samples using 'humann2_join_table' script. Merged pathway abundance and genefamilies tables are also normalized to relative abundances using 'humann2_renorm_table' script. All tables are split into stratified (by Taxa) tables and unstratified (metagenome) tables. Additional tables are generated by regrouping the UniRef90 genefamilies into other functional categories using the 'humann2_regroup_table' script with 'uniref90 to ko' and 'uniref90 to ec' utility mapping databases. The output tables for KEGG Orthogroups (KOs), molecular functions represented in terms of functional orthologs, and Level-4 enzyme commission (EC), categories of numerical nomenclature that classifies enzymes based on the overall reaction catalyzed, are merged across samples as described above. Using the legacy KEGG databases included with HUMAnN 1.0, genefamilies outputs are converted to KEGG Pathways (a collection of manually drawn pathway maps representing our knowledge of the molecular interaction, reaction and relation networks) and KEGG Modules (manually defined functional units of gene sets and reaction sets). This is done by processing the genefamilies default output tables through the 'humann2' script specifying the 'pathways-database' for the KEGG Pathways and KEGG Modules. The abundance and coverage output tables are merged as described above.

Functional profiling – HUMAnN3. Functional profiling of the microbial community will be done using HUMAnN3 (7). The standard recommended workflow will be followed with modifications to the nucleotide and translated alignment steps. Briefly, nucleotide alignment is performed using bbmap (3) with the bloom filter enabled (bloomk=22) and fast search settings generating a HUMAnN3 compatible SAM file output. The translated alignment step is performed using diamond (6; version 0.9.26). This creates the default pathway abundance and coverage tables, as well as gene family abundance output files per sample. Post-processing of the per-sample tables is done using a combination of HUMAnN3 utility scripts and in-house code designed to clean up the tables for better readability. The three default outputs are each merged across samples using 'humann_join_table' script. Merged pathway abundance and genefamilies tables are also normalized to relative abundances using 'humann_renorm_table' script. All tables are split into stratified (by Taxa) tables and unstratified (metagenome) tables. Additional tables are generated by regrouping the UniRef90 genefamilies into other functional categories using the 'humann_regroup_table' script with 'uniref90 to ko' and 'uniref90 to ec' utility mapping databases. The output tables for KEGG Orthogroups (KOs), molecular functions represented in terms of functional orthologs, and Level-4 enzyme commission (EC), categories of numerical nomenclature that classifies enzymes based on the overall reaction catalyzed, are merged across samples as described above. Using the legacy KEGG databases included with HUMAnN 1.0, genefamilies outputs are converted to KEGG Pathways (a collection of manually drawn pathway maps representing our knowledge of the molecular interaction, reaction and relation networks) and KEGG Modules (manually defined functional units of gene sets and reaction sets). This is done by processing the genefamilies default output tables through the 'humann' script specifying the 'pathways-database' for the KEGG Pathways and KEGG Modules. The abundance and coverage output tables are merged as described above.

References

1) Human Microbiome Project, C. (2012). "Structure, function and diversity of the healthy human microbiome." Nature 486(7402): 207-214.
2) Human Microbiome Project, C. (2012). "A framework for human microbiome research." Nature 486(7402): 215-221.
3) BBMap – Bushnell B. – sourceforge.net/projects/bbmap/
4) Nicola Segata et al. Metagenomic microbial community profiling using unique clade-specific marker genes. 2012, Nature Methods, 8, 811–814.
5) Franzosa EA et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods 15: 962-968 (2018).
6) Buchfink B, Xie C, Huson DH, "Fast and sensitive protein alignment using DIAMOND", Nature Methods 12, 59-60 (2015).
7) Francesco Beghini et al. Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3.
   bioRxiv 2020.11.19.388223; doi: https://doi.org/10.1101/2020.11.19.388223

After stool samples have been analyzed by Baylor College of Medicine and after all statistical analyses have taken place, researchers will verbally communicate to individual participants the debriefing statement to inform them which supplement/placebo they received. If enough external funds are ensured by using the results from the first stage as preliminary data, stage 2 will proceed.

STAGE 2

The second stage would use the stored blood samples from stage 1 to perform 16S rRNA on blood samples to analyze microbial composition in blood and metabolomic analysis to evaluate the effect of the next generation synbiotic on gut-derived metabolites in blood and stools. Blood samples (which will be deidentified by fabricated ID) will be sent to the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine for 16SrRNA analysis and blood and stool samples will be sent to Metabolon for mass spectrometry analyses. 16S rRNA will be used in blood as this is the technique preferred when there is low bacterial biomass.

Method of 16S rRNA Analysis at Baylor College of Medicine: The hypervariable region V3 and V4 of the bacterial 16S rRNA gene will be captured using the Illumina Nextera protocol (Part # 15044223 Rev. B; Illumina, San Diego, CA). A single amplicon of 460bp will be amplified using the 16S Forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, as described in the Illumina protocol. The polymerase chain reaction (PCR) product will be cleaned using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA). The Illumina adapter and barcode sequences will be ligated to the PCR amplicons in order to attach them to a MiSeqDx flow cell for multiplexing. The quality and quantity of each sequencing library will be assessed using a bioanalyzer and PicoGreen (Molecular Probes, Inc., Eugene, OR) measurements, respectively. About 6pM of pooled library will be loaded onto a MiSeqDX flow cell and sequenced using a PE300 (Paired end 300 bp) v3 kit. Raw FASTQ files will be demultiplexed based on unique barcodes and assessed for quality. Samples with more than 50K QC pass sequencing reads will be used for downstream 16S OTU analysis. Taxonomic classification and Operational Taxonomic Unit (OTU) abundance analysis will be done using a CLC Bio Microbial Genomics Module (QIAGEN, Redwood City, CA). Individual sample reads will be annotated with the Greengenes database, and taxonomic features of the sample will be determined. Blood microbiome compositions will be compared pre- and postintervention. Alpha and beta diversity analyses will be computed to understand the within-sample and betweens amples diversity, respectively. Raw FASTQ files will be submitted to the Sequence Read Archive (SRA).

Metabolomic Analysis at Metabolon: Aliquots of 1mL will be divided in four aliquots of 250 µL to be sent to Metabolon company (Morrisville, US) for further process. Samples will be prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards will be added prior to the first step in the extraction process for QC purposes. For the metabolomic analysis, a total of 100 µL of sample will be extracted under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) with methanol 80% containing the following recovery standards: DL-2-fluorophenylglycine, tridecanoic acid, d6-cholesterol, and DL-4-chlorophenylalanine. The resulting extract will be divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI and one for analysis by HILIC/ UPLC-MS/MS with negative ion mode ESI. The remaining aliquot will be reserved for backup. Samples will be placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts will be stored overnight under nitrogen before preparation for analysis. All methods utilized a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at R = 35,000 mass resolution. The sample

extract will be dried then reconstituted in solvents compatible to each of the four methods. For each sample, two aliquots of each sample will be reconstituted in 50 µL of 6.5mM ammonium bicarbonate in water (pH 8) for the negative ion analysis and another two aliquots of each will be reconstituted using 50 µL 0.1% formic acid in water (pH ~3.5) for the positive ion method. Each reconstitution solvent will contain a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The internal standards consist of a variety of deuterium labeled or halogenated biochemicals specifically designed both to cover the entire chromatographic run and to not interfere with the detection of any endogenous biochemicals. One aliquot will be analyzed using acidic positive ion conditions (LC pos), chromatographically optimized for more hydrophilic compounds. In this method, the extract will be gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 µm) using water and methanol, containing 0.05% per fluoropentanoic acid (PFPA) and 0.1% formic acid (FA) at pH=2.5. Elution will be performed at 0.35 mL min−1 in a linear gradient from 5% to 80% of methanol containing 0.1% FA and 0.05% PFPA over 3.35 min. A second aliquot will be also analyzed using acidic positive ion conditions; however, it will be chromatographically optimized for more hydrophobic compounds. In this method, the extract will be gradient eluted from the same afore mentioned C18 column using methanol 50%, acetonitrile 50%, water, 0.05 % PFPA, and 0.01 % FA at pH=2.5 and will be operated at an overall higher organic content. Elution will be performed at 0.60mL/min in a linear gradient from 40% to 99.5% over 1min, hold 2.4 min at 99.5% of methanol 50%, acetonitrile 50%, 0.05% PFPA, and 0.01% FA. A third aliquot will be analyzed using basic negative ion-optimized conditions with a separate dedicated C18 column (LC neg). The basic extracts will be gradient eluted from the column using methanol 95% and water 5%, with 6.5mM ammonium bicarbonate at pH 8. Elution will be performed at 0.35mL min−1 with a linear gradient from 0.5% to 70% of methanol 95%, water 5% with 6.5mM ammonium bicarbonate over 4min, followed by a rapid gradient to 99% in 0.5min. The sample injection volume will be 5 µL and a 2× needle loop overfill will be used. Separations utilized separate acid and base-dedicated 2.1mm× 100mm Waters BEH C18 1.7 µm columns held at 40 °C. The fourth aliquot will be analyzed via negative ionization following elution from an HILIC column (LC HILIC) (Waters UPLC BEH Amide 2.1 × 150mm, 1.7 µm, held at 40 °C) using a gradient consisting of water (15%), methanol (5%), and acetonitrile (80%) with 10mM ammonium formate, pH 10.16. Elution flow rate will be 0.5mL/min with a linear gradient from 5% to 50% in 3.5 min, followed by a linear gradient from 50% to 95% in 2min, of water (50%), acetonitrile (50%) with 10mM ammonium formate, pH 10.6. The MS analysis will alternate between MS and data-dependent MSn scans using dynamic exclusion.

Due to the length of the Metabolomic Methods followed by Metabolon, the description of quality assurance and quality, compounds identification and quantification, and data analyses are described in the following article: https://doi.org/10.1038/s41467-019-13498-3

Our hypothesis is that the short antibiotic intervention will help to improve probiotic colonization and metabolic effects. A material transfer agreement will be completed through the Office of Sponsored Programs and an IRB amendment will be submitted and approved prior to the shipping of any biological materials. Shipments of all materials will be done following all International Air Transport Association (IATA) and U.S. Department of Transportation (DOT) regulations. See figure below for study design.

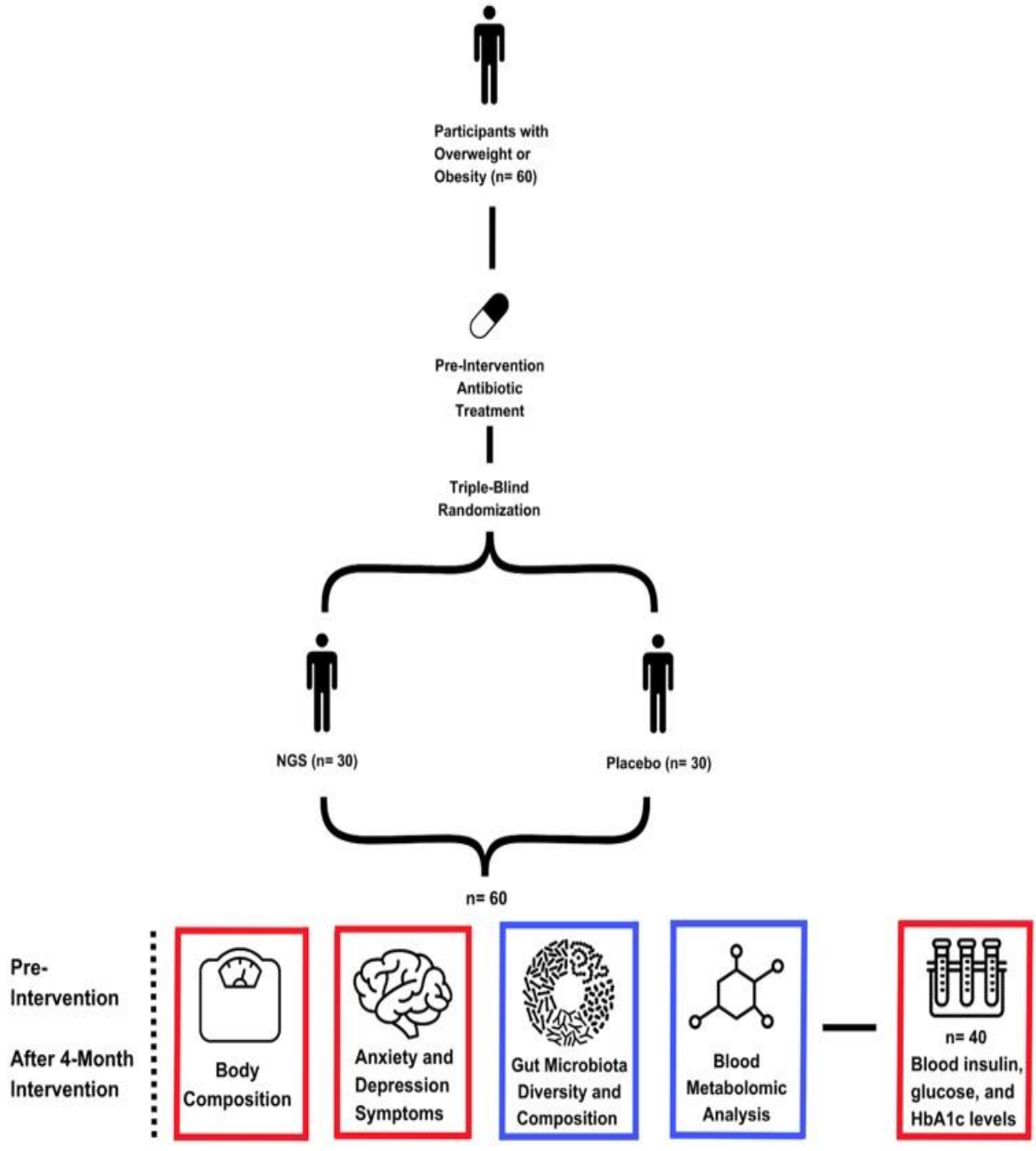

Figure 1. Conceptual framework. By using a randomized triple blind, placebo-controlled clinical trial, we will test the effects of a 4-month next generation synbiotic intervention on glucose control, body composition, and anxiety and depression symptoms. NGS: Next generation synbiotic.

Project Stages are denoted as follows: Red- Stage 1 and Blue- Stage 2 (dependent on futher external funding)

15) <u>Potential Risks and Precautions to Reduce Risk:</u> Include, as may be useful for the IRB's consideration, a description of the probability, magnitude, duration, and reversibility of the risks. Consider physical, psychological, social, legal, and economic risks as well as risks to privacy and/or confidentiality. Also describe what measures have been or will be taken to prevent and minimize each of the risks identified. If any deception is to be used, explain if use of deception is likely to cause the participant psychological discomfort (i.e., stress, loss of self-esteem, embarrassment) while the deception is taking place. Explain how this risk will be minimized during the experiment and after the experiment is complete (i.e. full debriefing).

There are some potential moderate risks in this study.

The moderate risks include radiation exposure from the DXA scans (which is equivalent to less than 10% of the radiation produced by an X-Ray) and gastrointestinal discomfort due to the 3-day antibiotic exposure and/or the symbiotic intervention.

1) There are a total of 2 DEXA scans in this study. This will expose participants to low-dose radiation, the amount of radiation exposure is small and is about the same as one day of normal background radiation per scan. Exposure to ionizing radiation at low levels has the potential of causing cancer later in life. The risk of this occurring increases as the amount of exposure increases. The amount of radiation exposure in this study is very small and so the additional risk of causing radiation related effects does not change significantly by participating in this study. Prior to any body composition measurements, participants who are female and of childbearing age will be required to complete a pregnancy questionnaire. To minimize the radiation-related risks, females who become pregnant will be unenrolled from the study and will not undergo DEXA scanning. While radiation exposure is low, the consent form includes a statement that instructs participants to let the researchers know if they are in any other studies involving DXA scans in order to consider cumulative radiation exposure.

2) Although the antibiotic intervention is short (3 days) and might not be enough to cause gastrointestinal discomfort is possible that susceptible people may still experience gastrointestinal symptoms such as bloating and diarrhea. Secondary infections to antibiotic treatment, such as *Clostridiodies difficile*, although unlikely are also possible. Short antibiotic interventions, however, cause minimal microbiota changes that are temporary and reversible (DOI: 10.1016/j.cell.2018.08.047). The antibiotic and dose chosen in this trial were the most highly used according to a systematic review our team published in 2022 in which we compared RCTs evaluating the effect of "antibiotic" versus "antibiotic + probiotics" (DOI: 10.1099/jmm.0.001625). Gastrointestinal discomfort at such short antibiotic interventions is unlikely, however, if present, symptoms tend to subside within a short period of time.

3) Likewise, supplementation with live bacteria strains (probiotics) and/or prebiotics may induce gastrointestinal discomfort secondary to microbiota composition changes.

4) Individual verbal treatment debriefing (placebo vs symbiotic) will take place after participant recruitment and data analysis have finalized as this is the time point in which we will reveal the randomization process.

5) Risk of being asked questions that might be sensitive for you and that pertain to your anxiety and depression levels. If you feel that answering the questionnaires caused psychological distress you have the following resources available:

   i. If you are a TCU student you can access to psychological health through several ways: psychiatric evaluations, 24/7 telephone counseling helpline, peer support communities, personalized interventions for student athletes, etc. (https://counseling.tcu.edu/student-services/#StudentAthletes)
   ii. If you are not a TCU student, you can find free government support through Texas Health and Human Services by accessing the following link and filling out the form (https://resources.hhs.texas.gov/pages/find-services). This form will be used to provide you with the best phone number to contact based on your needs and based on your location. If you prefer to use a phone number you can call 855-937-2372 and they will help you to get in touch with a health professional.
   iii. If you live in Tarrant County you can directly call MHMR of Tarrant County (My Health My Resources) at 800-866-2465.

6) Lastly, there is always a potential risk of data breach, however, only personnel that has been certified and approved by the IRB will have access to the participants and their related data. Samples, questionnaires, and other forms pertaining to the participants will be labeled with an ID that do not include any personal health information of the participant. The document containing the link between the names of the participants and their IDs will be saved in a password protected computer. Informed consents will be saved in the office of the PI which is locked at all times the PI is not present and which is located in a card locked department.

16) Potential Benefits: Describe the potential benefits of the research to the participants, to others with similar problems, and to society (Compensation is not a benefit to participation). If using deception describe how the potential benefits of the research justify the deception.
Participants may or may not directly benefit from their participation in this project. If in the synbiotic group, participants may have the potential to see beneficial changes in body composition, insulin sensitivity, anxiety, depression, and cravings, however, these are potential benefits and cannot be guaranteed as group designation is random and as research in this synbiotic is still underway. The research outcomes may reveal this synbiotic to be a new therapeutic option for others with overweight or obesity to induce weight loss, insulin sensitivity, and/or diminish anxiety, depression, and/or cravings. Society may benefit from this research because the outcomes from this study will further scientific understanding of the microbiome's effect on overweight and obesity treatment. Furthermore, this clinical trial will provide the scientific foundation and the preliminary data to apply for NIH funding to examine this next generation synbiotic's effect on the gut microbiota through whole metagenome sequencing in stools and on the gut-derived metabolome in blood.

17) Compensation: Describe in detail if participants will be compensated for their time and effort to complete the study procedures. Compensation can take on many forms and can include monetary (cash, gift cards, etc.) and/or non-monetary (gifts, course credit, extra credit, SONA credit etc.)

payments to subjects. Compensation cannot be random, if utilizing a raffle, you must describe how you will choose which participants (every 5th or 10th or 15th for example). Your consent document should clearly specify what form(s) and how compensation would be provided to participants in your study and the amount of payment. You must also include and describe if participants can still receive full or partial compensation should they either withdraw or are not able to complete the study procedures for any reason. For non-monetary items, please provide an approximate value.

Participants will receive two cards, a $50 Amazon gift card after the 2nd appointment and a $100 Amazon gift card upon completion of the study. Participants will not receive compensation if they withdraw or are unable to complete the study. Additionally, participants will receive free body composition results and interpretation of the BOD POD and DEXA scans at the end of the study. Two BOD PODs and two DEXA scan amount to ~$400 in monetary market value. Participants will also receive at the end of the study their blood work results (glucose, insulin, HbA1c) and their psychological results (anxiety, depression, and cravings). Results will be disclosed to participants earlier if researchers consider that not disclosing could jeopardize the health and well-being of the individual. Participants with high anxiety and/or depression scores can use one of the following options to obtain psychological support:

- If you are a TCU student you can access to psychological health through several ways: psychiatric evaluations, 24/7 telephone counseling helpline, peer support communities, personalized interventions for student athletes, etc. (https://counseling.tcu.edu/student-services/#StudentAthletes)
- If you are not a TCU student, you can find free government support through Texas Health and Human Services by accessing the following link and filling out the form (https://resources.hhs.texas.gov/pages/find-services). This form will be used to provide you with the best phone number to contact based on your needs and based on your location. If you prefer to use a phone number you can call 855-937-2372 and they will help you to get in touch with a health professional.
- If you live in Tarrant County you can directly call MHMR of Tarrant County (My Health My Resources) at 800-866-2465.

18) <u>Procedures to Maintain Confidentiality:</u> **Describe how the data will be collected, de-identified, stored, used, and disposed to protect confidentiality. If protected health information is to be re-identified at a later date, describe the procedure for doing so. All signed consents and hard data must be stored for a minimum of 3 years in a locked filing cabinet (and locked room) in the principal investigator's office, lab, or storage closet at TCU. Your professional society may recommend keeping the materials for a longer period of time.**

Only personnel that has been certified and approved by the IRB will have access to the participants and their related data. <u>Samples, questionnaires, and other forms pertaining to the participants will be labeled with an ID that do not include any personal health information of the participant. The document containing the link between the names of the participants and their IDs will be saved in password protected computers. Informed consents will be saved in the office of the PI which is locked at all times the PI is not present and which is located in a card locked department.</u>

19) <u>Data Analyses:</u> **Describe how you will analyze your data to answer the study question. Describe any procedures that will be used to ensure the accuracy and quality of collected data. If participants withdraw from the study, describe what options they will have in terms of their data being used or removed from the study. If collecting blood or other biospecimens please indicate what and where analysis (on campus or off campus) will take place. If transcribing data please**

indicate and the provide the name of the person or service/vendor that will conduct the transcription.

If participants withdraw from their study the data collected up to that point will remain available for research purposes with the intention to use intent to treat analysis. Blood and stool samples will be stored in Dr. Ryan Porter's lab located within the Rickel building room 259. For the first stage of the project, blood samples will be analyzed at Dr. Ryan Porter's lab room 259. Once the Nutritional Sciences Department laboratory has been finalized, samples will be transferred to the Nutritional Sciences Department and further storage and analyses could take place here (BASS, room 1203). If enough funds become available to perform the second stage of the study, blood and stool samples (which will be identified by fabricated ID) will be sent to Baylor College of Medicine and to Metabolon for posterior 16S rRNA/WMS and mass spectrometry analyses, respectively. For statistical analysis of the first stage, two sample T-tests will be used to compare within (pre- versus post- values on each group) and between-group (placebo versus probiotic) differences in body composition, adherence, metabolic, dietary, and psychological variables. SPSS will be used for statistical analysis. A p value <0.05 will be considered as statistically significant. Dietetic variables collected via the NCI Automated Self-Administered 24-hour Dietary Assessment Tool including protein, carbohydrate, fat, and fiber will be controlled statistically in the case that significant intra-individual or between-group differences are observed.

20) <u>Check List for the Items That Need to be Submitted:</u> Please submit protocol and consent documents separately in MS word, supplemental documents (interview guides, surveys, recruitment materials etc. can be submitted as separate pdfs) before submitting the materials electronically to the IRB. To prevent any delay in the approval of your protocol, use the most recent template for the protocol, consent document, and HIPAA form by downloading them from https://research.tcu.edu/research-compliance/irb/irb-forms-templates/ **each time you prepare your materials.**

a.	Protocol	X
b.	Consent document	X
c.	Protecting Human Research Participants Training complete for each investigator	X
d.	Recruitment flyers, letters, ads, etc.	X
e.	Questionnaires or other documents utilized in screening and data collection	X

Principal Investigator Assurance

21) <u>By signing below, I certify to the following</u>:

- The project described herein will be conducted in accordance with applicable TCU policies and procedures, as determined by the IRB of record. All Human Subject Research projects occurring at TCU must be conducted in compliance with the Office of Human Protection ("OHRP") regulations at 45 CFR 46 and all other applicable federal and state laws and regulations (collectively "Applicable Law")

- I have a working knowledge of Applicable Law

- All personnel who work with human participants under this protocol have received, or will receive, appropriate training in protocol procedures and protection of human subjects prior to working with humans.

- All experiments involving human participants will be performed only by the qualified individuals listed in this protocol and individuals not listed in this protocol will not participate in the protocol experiments.

- Procedures on experimental subjects described in this IRB protocol accurately reflect those described in the funding applications and awards, if externally supported.

- I and all personnel have read and will comply with any pertinent safety information, IRB requirements, and security procedures.

- I will maintain records of all human participants and the procedures carried out throughout the entire term of my project.

- As Principal Investigator, I am aware that I have the ultimate responsibility, on a day-to-day basis, for the proper care, treatment, and protection of the human participants.

_____ September 29th, 2023

Signature of Principal Investigator Date