Methods Supplement

Protocol supplement

Protocol Amendments

Two modifications to the original protocol were made during the course of the study, both in response to slower than expected enrollment rates. The original protocol excluded participants using sulfonylureas. In response to input from study-site investigators that this was a significant recruitment impediment, this exclusion was removed. Contemporaneous to this change in exclusion criteria, two additional study sites were added. Both the original and final protocols are provided as Supplementary Methods.

An error in the notation in the adjusted statistical model was discovered after unblinding. When modeling the 12-week change from baseline of glucose $AUC_{0\text{-}180\ min}$, we intended to account for both main effects and administration:term interactions, as metformin, sulfonylurea, sex and age have all been reported to effect the composition of the host microbiome. Complete details of the statistical analysis plan and the aforementioned correction are available in Supplementary Materials, Statistical Analysis Plan.

Missing Data and Imputation

To assess the possible impacts of non-random missingness, efficacy endpoints were assessed in the intent-to-treat population using last observation carried forward (LOCF) for missing values. Intent-to-treat analysis was consistent with per-protocol analysis, however, as expected, LOCF biases toward the null of no probiotic formulation effect, as subjects assigned to placebo tended to deteriorate, and subjects assigned to probiotic formulation tended to improve. As our aim was to gain insight into the effects of the intervention, we focus on the per-protocol population containing 16, 21 and 21 subjects assigned to receive placebo, WBF-010 and WBF-011 respectively.

Preparation of Synbiotic Formulations

Manufacturing of strains

Study synbiotic strains AMUC, EHAL, CBEI, and CBUT were produced at Pendulum Therapeutics according to current Good Manufacturing Practices (cGMP) for food products in an FDA registered food manufacturing facility. Strains were individually grown in 2000-litre anaerobic batch cultures using media free of animal products and then harvested mid-log phase by centrifugation. The cell paste was combined with a cryoprotectant and lyophilized for downstream characterization and processing into a free-flowing powder. The individual strains and prebiotic were subsequently combined to create the final formulations which were then encapsulated and bottled.

Characterization of the strains and manufacturing process includes: (1) quantitation of live and dead cell counts using a Becton Dickinson Accuri C6 flow cytometer and Becton Dickinson Cell Viability Kit (Becton Dickinson Biosciences, San Jose, CA), (2) 16S sequencing of strain DNA to ensure identity and purity, (3) whole-genome sequencing to search for any known toxin or antibiotic resistance gene signatures, (4) third-party heavy metal testing, and (5) third-party pathogen test of final bottled product. The complete characterization of these strains along with data from preclinical rat toxicity and human-safety pilot studies were combined into a Generally Recognized as Safe (GRAS) dossier which was reviewed and accepted by an independent third party (Ramboll Environ, Arlington, VA).

Study Product Preparation and Dosing

In addition to the 4 strains described above, the commercially available strain *B. infantis* was purchased from Nutraceutix (Redmond, WA) as lyophilized powder and then combined according to supplementary table S1. Combined study product was encapsulated in acid-resistant opaque capsules made of hydroxypropyl methylcellulose, hydroxypropylmethylcellulose phthalate, and titanium dioxide from Capsuline, and stored at 4° C until use.

The first study product, WBF-010, contained 3 bacterial strains, and the second study product, WBF-011, contained two additional strains. Each formulation contained between $9.0x10^8$ and $1.6x10^{10}$ Active Fluorescent Units (AFU), a quantitative measure of viable-cell count, of each of the specified bacteria [1]. The placebo study product, WBF-009, was composed of colloidal silicon dioxide (CSD).

All participants were instructed to consume 3 capsules of their assigned study product twice daily prior to consuming their morning and evening meals. Upon completion of the 12-week intervention period, subjects were followed for an additional 4-week wash-out to assess strain persistence.

Fecal Microbiome Analyses

Stool Collection and Homogenization

Whole stool was collected by participants using a Commode Specimen Collection System (Biomedical Polymers, Sterling, MA) at baseline, 4 weeks, 12 weeks, and 16 weeks (4 weeks after discontinuing use of study product). Upon collection, subjects were instructed to immediately freeze (home freezer, approximately -20 °C) their stool samples within a sponsor-provided thermally-insulated container. The insulated container was returned to the clinic at the next scheduled visit and then shipped on dry ice to Pendulum Therapeutics for long-term storage at -80 °C. Stool samples were first thawed for homogenization, which consisted of: (1) suspension in a 2:1 ratio (m/m) of 50 mM Tris HCl buffer (pH 8.0) with 5 mM EDTA, (2) homogenization for five minutes in a paddle stomacher (Stomacher 3500, Seward, UK), (3) filtration through a coarse 280 µm mesh, and (4) preparation of 1 mL aliquots subsequently refrozen and maintained at -80 °C. Most samples provided an adequate volume for the storage of 24 separate aliquots, yielding a total of 5,760 barcoded and catalogued aliquots of stool homogenate.

Strain-specific qPCR

Primer-pairs were developed by first identifying unique regions in the genomes of each of the 5 strains, designing primer-pairs specific to those regions, and then selecting specific and sensitive pairs for synthesis and testing. Briefly, unique 1,000 - 40,000 bp regions of a target's genome were identified by whole genome alignment using mummer3 [2] to the taxonomically closest available genome fetched from NCBI's Taxonomy Database [3]. Primer-pair search on these unique genomic regions was conducted via Primer3 [4]; and top-scoring primer-pairs with predicted amplicons of 100 to 500 bp in length were selected for synthesis and testing. Primers were synthesized by Integrated DNA Technologies (Skokie, IL). Candidate primer-pairs performance was tested in a serial titration of target organism gDNA in an aqueous background of DNA extracted from human stool naive to the target organism. Selected primer-pairs exhibited the best sensitivity and specificity and minimal or no unspecific amplification in no-template controls. The primer-pair sequences used in the study are shown in supplementary table S3.

For qPCR, genomic DNA (gDNA) was extracted and purified using the DNeasy PowerSoil HTP 96 Kit (Qiagen, Venlo, Netherlands) as per manufacturer's instructions. gDNA concentration was measured using the Quant-iT dsDNA high sensitivity kit (ThermoFisher, Waltham, MA). qPCR reactions were performed on a CFX384 Real-Time PCR Detection System (Biorad, Hercules, CA) using SYBR green detection. Reactions were set up in a 20 **µ**L volume by combining six **µ**L of gDNA template with 10 **µ**L of PowerUp mastermix (ThermoFisher, Waltham, MA), and 4 **µ**L of 2.5 mM primer mix (500 nM final reaction concentration of each primer). Reaction conditions were as follows: 50 °C for 2 minute, then 95 °C for 2 minute, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute with plate reads at the end of each cycle. Next, a melt curve was taken from 65 °C to 95 °C ramping 0.2 °C/s. Duplicate eight point standard curves were run each with three technical replicates by diluting known quantities of purified genomic DNA from target organisms titrated into a background of stool gDNA. These standard curves were used to relate threshold cycle (Ct) values to the mass of DNA of target organism for each sample. Only reactions exhibiting a Ct < 40 and a single melt curve

peak at the same temperature as corresponding standard (+/- 0.6 °C) were considered successful. All qPCR primer pairs were evaluated on at least four DNA-extraction replicates.

Short-read Amplicon Sequencing of 16S rRNA gene V4 Region

For 16S rRNA gene V4 region amplicon sequencing (heretofore “16SV4”), DNA extraction was accomplished in three separate protocols; specifically (1) using DNeasy PowerSoil HTP 96 Kit with 650 uL each homogenate, (2) using DNeasy PowerSoil HTP 96 Kit as above but with a 4 μL of a defined whole cell standard (ZymoBIOMICS Community Standard, Zymo Research, Irvine, CA) spiked into the homogenate prior to extraction, and (3) using Zymo Research Quick-DNA Fecal/Soil Microbe 96 Kit (Zymo Research, Irvine, CA) with 200 uL each homogenate and 16.6 μL ZymoBIOMICS Community Standard spiked into the homogenate prior to extraction.

Extracted DNA template was standardized to 9 ng in each 25 μL PCR reaction. Primers and reaction conditions used in the study were described previously (Kozich et al.). Briefly, 3 μL of 5 uM primers were added to 19 μL Accuprime Pfx Supermix (ThermoFisher, Waltham, MA). Samples were denatured for 2 min at 95 °C, and then 25 cycles of the following: 95 °C for 20 seconds, 55 °C for 15 seconds, 72 °C for 2 min. Final extension was conducted at 72 °C for 10 minutes. Following completion of PCR reaction, all samples were pooled in equal volumes and purified with Ampure XP beads (Beckman Coulter, Indianapolis, IN) according to manufacturer’s specifications. The purified library was quantified, loaded, and sequenced in a 600 cycle run on an Illumina MiSeq using V3 chemistry according to manufacturer's specifications (Illumina, San Diego, CA) and protocols described previously [5].

16SV4 sequence analysis

Sequences were demultiplexed using Illumina MiSeq Control Software 3.1.0.13 (Illumina, San Diego, CA). Adapter sequences were trimmed using cutadapt [6]. The following steps were performed using the R package DADA2 [7] mostly in accordance with a published workflow [8]. Briefly, (1) forward reads were trimmed at positions 15 and 245; reverse reads were trimmed at

positions 10 and 206, (2) reads were de-noised and mate-pairs merged, (3) unique sequences were identified, and (4) chimeric sequences removed. Unique sequences were taxonomically classified using SINTAX [9] with the RDP v16 training set [10]. Alpha diversity, beta diversity, and ordinations were performed using phyloseq [11].

Short-Chain Fatty Acids

Short-Chain Fatty Acids were measured via an acidified-water method of fecal SCFA extraction described previously [12]. Briefly, for each subject stool, a 720 µL aliquot of stool homogenate was thawed and 80 µL of 10 mM 2-ethylbutyric acid (Millipore Sigma, Burlington, MA) was spiked into each homogenate as an internal standard. Homogenate was then centrifuged and passed through a 0.2 µM PTFE syringe filter (ThermoFisher, Waltham, MA) to remove solid material and most cells. 100 µL of the filtrate volume was transferred into 150 µL autosampler vials, and 10 µL of 3 N HCl was used to acidify the filtrate immediately prior to sealing the vial (typical pH < 1, ensuring that equilibrium overwhelmingly favored the protonated form of each acid). 1 µL was then run on a Shimadzu 2010 Plus Capillary GC equipped with a flame ionization detector.

Concentration of 2-ethylbutyric acid, acetic acid, propionic acid, and butyric acid were determined for each sample by transforming GC-reported areas according to an analyte specific calibration curve determined by a robust linear regression of GC-reported areas on concentrations of Volatile Free Acid Mix analytical standards (Millipore Sigma, Burlington, MA). A 10mM stock solution was spiked with 10 mM 2-ethylbutyric acid and then diluted to the following six concentrations: 10, 5, 2, 1, 0.5, and 0.1 mM. These standards were run for every batch of samples.

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