Supplementary Material

Effect of Prebiotic on Host-Microbial Co-metabolism in Peritoneal Dialysis Patients: A Pilot Study

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Supplementary Methods

Study Design

In a non-randomized, crossover study, 8 PD patients were followed during 3 sequential 8-week phases of no intervention, p-inulin (16 g/day) administration, and a post-intervention phase with no intervention. This study design permitted us to account for variability in microbiome and metabolome, provided adequate time for change in microbiome composition and their metabolic products and to determine sustainability of the intervention during the post-treatment phase (1). Furthermore, self-controlled cross-over studies could generate findings that are statistically and clinically valid with a smaller number of patients (2). The study participants were instructed not to make any significant changes in their diet during the study period. Dietary intake was assessed using the Block Food Frequency Questionnaire (https://nutritionquest.com). Stool samples were self-collected by the participants (3). Participants were provided with a commercial “toilet hat” stool specimen collection kit (specimen container, shipping box, and cold packs; Fisherbrand Commode Specimen Collection System) at the screening visit and as needed for resampling. Participants brought the stool samples in the closed container to the clinical trial center within 24 hours of collection. Stool samples were also collected in 7 individuals without kidney disease for the profiling of the microbiome. Blood samples were collected in a fasting state for metabolomic studies and routine labs. All study participants received the standard of care as recommended by Kidney Disease Outcomes Quality Initiative guidelines (4). Dialysis adequacy information was extracted from the dialysis unit electronic medical records. This study was approved by George Washington university IRB and registered at https://clinicaltrials.gov/ct2/show/NCT03265639.

Study Participants

Patients on PD for more than 3 months, aged ≥ 18 years were eligible for the study. The exclusion criteria were the use of prebiotics or probiotics during the past 8 weeks; consumption of probiotic yogurt during the past 2 weeks; use of antibiotics within the past 8 weeks; presence of HIV infection, chronic wound infection, osteomyelitis, inflammatory bowel disease, chronic diarrhea, *C. difficile* infection, liver disease, anticipated kidney transplantation, pregnancy or breastfeeding, inability to provide informed consent and severe anemia defined as hemoglobin <9.0 g/dL within the past 4 weeks. We also recruited 7 subjects without kidney disease.

We identified 13 eligible PD subjects, but only 10 consented to participate in this non-randomized, crossover study. Among the 10 patients, one patient transferred to another clinic, one patient received kidney transplant at week 7 of the study, so a total of 8 patients completed the study. There were no significant differences in demographics or comorbidities between PD and controls at baseline, but sodium and hemoglobin levels were significantly lower, and creatinine and blood urea nitrogen (BUN) significantly higher, in PD patients compared to controls.

The mean weekly Kt/V was 2.16 ± 0.25 and normalized protein catabolic rate (nPCR) was 0.99 ± 0.16 g/kg/day. About 82% of the dispensed p-inulin was consumed by patients with 75% of the participants exhibiting at least 70% compliance. One patient temporarily discontinued p-inulin due to heart burn and vomiting but resumed full dose from week 11. Another patient took only 4 g of p-inulin/day citing gastrointestinal (GI) intolerance. Analysis of GI Symptom Rating Scale (GSRS) showed that patients experienced significantly more flatus at weeks 8 and 12, borborygmi at week 24, and reduced hardness of stool at week 12. Total fat intake decreased during intervention but increased significantly at post-intervention. Other dietary factors showed no significant change over time.

Study Intervention

All participants received oligofructose-enriched p-inulin (Prebiotin™, **Jackson GI Medical,** Camp Hill, PA), a prebiotic at a dose of 8 g twice daily (5). Adherence was assessed by counts of returned packets at the Weeks 9, 12 and 16 visits and from participant self-report. Nonadherence was defined as failure to take ≥ 70% of prescribed p-inulin packets by the participant. GI symptoms were assessed using the GSRS (6, 7). The symptoms were rated in a scale of 0-3 by the patients.

Inflammatory biomarker assays

Plasma levels of interleukin (IL)-6, high sensitive c-Reactive protein (CRP), tumor necrosis factor (TNF)-α and soluble CD14 (sCD14) were measured at week 1, 8, 10, 12, 16, 17 and 24 using high-sensitivity ELISA assay kits. All measurements were performed in a blinded fashion, in duplicates and the mean used. All assays were subjected to standardization and validation using internal controls prior to the commencement of sample analysis.

Shotgun metagenomic sequencing

The microbiome profiles of stool samples were determined using a shotgun DNA sequencing-based approach, as previously described (8). Samples were processed using Qiagen's DNeasy PowerSoil extraction plates. Extraction plates were shipped on dry ice to CoreBiome for downstream extraction and library preparation. Extracts were quantified using the Quant-iT PicoGreen dsDNA assay (Thermo Fisher). Libraries were prepared using the NexteraXT kit and a HiSeq 1 x 150-cycle v3 kit (Illumina) was used to sequence samples. Generation of taxa count tables from raw sequencing data used an approach previously described (8). The database used was generated by selecting up the first 20 strains per species in RefSeq v87 by first choosing genomes with assembly level annotated as "Complete Genome", then "Chromosome", then "Scaffold", then "Contig".

Metabolomic Analyses

Metabolomic studies were performed at the West Coast Metabolomics Center at the University of California Davis. Three platforms were performed including GC-TOF MS for profiling of plasma primary metabolites, HILIC-QTOF MS for profiling of plasma biogenic amines and CSH-QTOF MS for profiling of plasma complex lipids. Targeted metabolomics was performed to quantify the concentration of plasma TMAO, indoxyl sulfate, p-cresol sulfate and bile acids.

Gas chromatograph and mass spectrometry analysis

Metabolites were extracted from a 20 microL plasma aliquot using 1 ml of degassed and cold (at −20°C) acetonitrile:isopropanol:water (3:3:2, v/v/v) mixture. Samples were vortexed for 10 seconds, shaken for 5 minutes and then centrifuged for 2 minutes at 14,000 rcf. Dried samples were cleaned with 0.5 mL of an acetonitrile:water (1:1, v/v) mixture, decanted and evaporated. For derivatization, a 10 microL of methoxyamine hydrochloride in pyridine (40 mg/mL) was added to each sample. Then 90 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich) was added for trimethylsilylation. C8–C30 fatty acid mthyl esters (FAMEs) were added as internal standard for retention time correction. These derivatized samples were analyzed by GCMS.

An Agilent 6890 gas chromatography instrument equipped with a Gerstel automatic linear exchange systems (ALEX) which included a multipurpose sample dual rail and a Gerstel cold injection system. A Rtx-5Sil MS column (30m length, 0.25 mm i.d, 0.25 µM 95% dimethyl 5% diphenyl polysiloxane film) was used. Mobile phase was 99.9999% pure helium gas with a flow rate of 1mL/min.

A Leco Pegasus IV time of flight mass spectrometer was used for acquiring the mass spectral data. The spectrometer was operated using the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI). The transfer line temperature between gas chromatograph and mass spectrometer was set to 280°C. Electron impact ionization at 70V was employed with an ion source temperature of 250°C. Acquisition rate was 17 spectra/second, with a scan mass range of 85-500 Da. Raw data files were preprocessed by ChromaTOF followed by BinBase algorithm for metabolite annotation and reporting.

Hydrophilic interaction liquid chromatography (HILIC) qTOF mass spectrometry for polar metabolites

Metabolites were extracted from a 20 microL plasma aliquot using 1 ml of degassed and cold (at −20°C) acetonitrile:isopropanol:water (3:3:2, v/v/v) mixture. Samples were vortexed for 10 seconds, shaken for 5 minutes and then centrifuged for 2 minutes at 14,000 rcf. Sample were re-suspended in acetonitrile.

A 5 µL sample was injected on a Waters Acquity UPLC BEH Amide column (150 mm length × 2.1 mm id; 1.7 µm particle size) maintained at 45°C. A Waters Acquity VanGuard BEH Amide pre-column (5 mm × 2.1 mm id; 1.7 µm particle size) was used as guard column. Mobile phase A was 100% LC-MS grade water with 10 mM ammonium formate and 0.125% formic acid and mobile phase B was 95:5 v/v acetonitrile:water with 10 mM ammonium formate and 0.125% formic acid. Gradient was started at 100% (B) for 2 min, 70% (B) at 7.7 min, 40% (B) at 9.5 min, 30% (B) at 10.25 min, 100% (B) at 12.75 min and isocratic until 16.75 min. The column flow was 0.4 mL/min. Agilent 1290 infinity UHPLC instrument was used.

The MS data were acquired using an Agilent 6530 mass spectrometer equipped with an ion funnel (iFunnel) electro spray ionization source. Data were acquired in both ESI (-) and ESI (+)

modes. Source parameters were gas temperature 325 °C, drying gas flow 8l/min, nebulizer 35 psig, sheath gas temp 350 °C, sheath gas flow 11 L/min and Fragmentor 175V. Mass scan range was 50-1700 and acquisition rate was 2 spectra per second. Reference masses 121.050 and 922.009 in ESI (+) and 119.036 and 966.0007 in ESI(-) mode were used for continuous calibration of the mass accuracy. Data were acquired in the centroid mode.
Raw data files (.d) format were converted to .abf using the ABF converter (https://www.reifycs.com/AbfConverter/index.html). MS-DIAL software was used for peak detection, alignment, gap filling and annotations. Adducts were grouped and duplicate peaks were removed using the MS-FLO software.

CSH-qTOF liquid chromatography mass spectrometry for lipidomics

Lipids were extracted from a 20 microL plasma aliquot. A 225 microL of cold methanol containing a mixture of odd chain and deuterated lipid internal standards were added and samples were vortexed for 10 seconds. Then 750 microL of MTBE was added. Samples were vortexed for 10 seconds and shaked for 5 mins at 4°C. Next, 188 microL water was added and samples were vortexed for 10 seconds and centrifuged for 2 mins at 14000 rcf. Two 350 microL aliquots from the non-polar layer were prepared. One is stored at -20ºC as a backup and the other is evaporated to dryness in a SpeedVac. Dried extracts are resuspended using a mixture of methanol/toluene (9:1, v/v) (60 µL) containing an internal standard [12-[[(cyclohexylamino) carbonyl]amino]-dodecanoic acid (CUDA)] used as a quality control. Method blanks and pooled human plasma (BioreclamationIVT) were included as quality control samples and prepared along with the study samples.

The LC/QTOFMS analyses are performed using an Agilent 1290 Infinity LC system (G4220A binary pump, G4226A autosampler, and G1316C Column Thermostat) coupled to either an Agilent 6530 (positive ion mode) or an Agilent 6550 mass spectrometer equipped with an ion

funnel (iFunnel) (negative ion mode). Lipids are separated on an Acquity UPLC CSH C18 column (100 x 2.1 mm; 1.7 µm) maintained at 65°C at a flow rate of 0.6 mL/min. Solvent pre-heating (Agilent G1316) was used. The mobile phases consist of 60:40 acetonitrile:water with 10 mM ammonium formate and 0.1% formic acid (A) and 90:10 propan-2-ol:acetonitrile with 10 mM ammonium formate and 0.1% formic acid. The gradient is as follows: 0 min 85% (A); 0–2 min 70% (A); 2–2.5 min 52% (A); 2.5–11 min 18% (A); 11–11.5 min 1% (A); 11.5–12 min 1% (A); 12–12.1 min 85% (A); 12.1–15 min 85% (A). A sample volume of 3 µL is used for the injection. Sample temperature is maintained at 4°C in the autosampler. Samples are injected (1.7 ml in positive mode and 5 ml in negative ion mode) with a needle wash for 20 seconds (wash solvent is isopropanol).

The quadrupole/time-of-flight (QTOF) mass spectrometers are operated with electrospray ionization (ESI) performing full scan in the mass range m/z 65–1700 in positive (Agilent 6530, equipped with a JetStreamSource) and negative (Agilent 6550, equipped with a dual JetStream Source) modes producing both unique and complementary spectra. Instrument parameters are as follows (positive mode) Gas Temp 325°C, Gas Flow 8 l/min, Nebulizer 35 psig, Sheath Gas 350°C, Sheath Gas Flow 11, Capillary Voltage 3500 V, Nozzle Voltage 1000V, Fragmentor 120V, Skimmer 65V. Data (both profile and centroid) are collected at a rate of 2 scans per second. In negative ion mode, Gas Temp 200°C, Gas Flow 14 l/min, Fragmentor 175V, with the other parameters identical to positive ion mode.

Raw data files (.d) format were converted to .abf using the ABF converter (https://www.reifycs.com/AbfConverter/index.html). MS-DIAL software was used for peak detection, alignment, gap filling and annotations. Adducts were grouped and duplicate peaks were removed using the MS-FLO software.

Targeted metabolomics method

Bile acids were measured as described previously (9). TMAO, indoxyl sulfate and p-cresol sulfate standards were prepared in the 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/mL concentrations for making the calibration curve. Betaine, choline, and TMAO were measured using HILIC-qTOF mass spectrometry as described above. Molar concentrations for Betaine, choline, and TMAO, indoxyl sulfate and p-cresol sulfate were computed using their calibration curves and linear regression fitting.

Statistics

Measurement periods included baseline, during-intervention, and post-intervention (**Table 1**). Baseline demographics, comorbidities, vital signs, and laboratory values were compared between PD and control groups using 2-tailed between-groups t-tests or the Kruskal-Wallis test for continuous variables, and chi square or the Fisher Exact test for categorical variables. Treatment effects on bacterial taxonomy, KEGG modules and plasma metabolites were examined using random effects mixed models with robust standard errors, while controlling for within-subject autocorrelation by nesting observations within patient (SAS version 9.4, SAS version 9.4, Cary, NC). The random effects mixed model was coded by period where time points were grouped into time periods as baseline (weeks 1-7), intervention (weeks 8-16), and post-intervention (weeks 17-24), with baseline as the reference group. P-inulin treatment effects were considered significant only if both overall p-value < 0.05 and intervention vs. baseline p-value < 0.05. For TMAO, IS and PCS, the random effects mixed model was coded by both period and by week.

Supplementary Tables

Table S1. Study design

Tests	Baseline (0-8 weeks)	P-inulin Intervention (9-16 weeks)	Post-Intervention (17-24 weeks)
Metagenomics	1, 4, 7 and 8 weeks	9, 10, 12, 13, 15 and 16 weeks	17, 18, 21, 23 and 24 weeks
Metabolomics	0, 1, 4, 7 and 8 weeks	9, 10, 11, 12, 13, 15 and 16 weeks	17, 18, 21, 23 and 24 weeks
Biomarkers	1 and 8 weeks	10, 12 and 16 weeks	17 and 24 weeks
Food Frequency	0 and 8	16 week	24 week
GI symptoms assessment	0, 4 and 8 weeks	12 and 16 weeks	20 and 24 weeks
Compliance*		10, 12 and 16 weeks	

Note: * Sachet count

Table S2. Baseline participant characteristics by treatment group

Variable	PD (n=9)	CON (n=7)	P-value
Age	68 (14)	61 (10)	0.34
Sex F	4 (44%)	3 (43%)	0.95
Race			0.69
Black	5 (56%)	3 (43%)	
White	3 (33%)	2 (29%)	
Other	1 (11%)	2 (28%)	
BMI	28 (3)	30 (4)	0.45
DM	4 (44%)	3 (43%)	0.99
HTN	9 (100%)	5 (71%)	0.18
OSA	1 (11%)	1 (14%)	0.99
HLD	1 (11%)	3 (43%)	0.26
CHF	1 (11%)	0 (0%)	0.99
Blood pressure			
SBP	137 (23)	142 (18)	0.63
DBP	76 (11)	75 (5)	0.81
Laboratory values			
Sodium	138 (3)	143 (2)	**0.02**
Creatinine	9.4 (3.9)	0.9 (0.2)	**0.0002**
BUN	59 (21)	15 (6)	**0.0002**
Potassium	4.4 (0.5)	4.2 (0.2)	0.19
Hemoglobin	10.7 (1.6)	13.4 (1.9)	**0.021**

Note: N(%) or mean (sd) are shown; P-values < 0.05 are shown in bold.

Table S3. Dietary intake assessed by food frequency questionnaire

Variable	Baseline	Intervention	Post-Intervention	P-value
Calories (Kcal)	1134.7±404.0	1082.2±423.8	1103.0±444.8	0.93
Total carbohydrate (g)	113.0±37.3	109.5±54.2	92.5±35.3	0.30
Protein (g)	47.0±20.4	47.8±18.6	49.0±21.6	0.93
Total Fat (g)	55.9±24.3	50.7±21.8	60.6±26.1	**0.04**
Calcium (mg)	405.3±194.4	358.9±180.2	409.0±182.0	0.21
Phosphorous (mg)	765.1±252.8	703.9±223.8	727.1±236.0	0.78

Note: Mean ± sd; p-value < 0.05 is shown in bold

Table S4. Gastrointestinal Symptom Rating Scale during the study

Symptom	Baseline	8 week	12 week	16 week	24 week	P-value
Abdominal Distension	0.375±0.744	0.375±0.74	0.375±0.744	0.167±0.408	0.333±0.81	0.08
Flatus	0.371±0.744	0.625±0.744*	0.750±0.707*	0.375±0.518	0.333±516	**0.005**
Borborygmi	0.375±0.518	0.500±0.534	0.375±0.744	0.250±0.463	0.667±0.408*	**0.001**
Eruction	0.125±0.353	0.125±0.354	0.375±0.518	0.125±0.354	0.333±0.516	0.30
Hard stool	0.751±1.165	0.250±0.707	0.125±0.354*	0.125±0.351*	0.833±0.753	**0.001**

Note: P-values < 0.05 are shown in bold.

Table S5. MaAsLin2 analysis of dietary intake and gut microbes

Dietary	Genus	Coef	Stderr	N	N.not.0	P-value	Q-value
DT_SFAT	Sutterella	0.004289	7.41E-05	11	3	0	0
DT_FIBE	Sutterella	0.001582	7.55E-05	11	3	4.29E-82	8.41E-80
DT_KCAL	Sutterella	-0.0128	0.001124	11	3	1.96E-28	2.56E-26
DT_TFAT	Sutterella	0.004006	0.000516	11	3	1.92E-14	1.88E-12
DT_CARB	Sutterella	0.002646	0.000468	11	3	2.08E-08	1.63E-06
DT_TFAT	Bacteroides	-0.6586	0.006278	11	11	1.91E-06	0.000125
DT_CARB	Bacteroides	-0.53845	0.005699	11	11	2.61E-06	0.000146
DT_KCAL	Bacteroides	1.191135	0.01368	11	11	3.34E-06	0.000164
DT_FIBE	Bacteroides	0.075334	0.000918	11	11	3.99E-06	0.000174
DT_PROT	Bacteroides	-0.20046	0.00289	11	11	6.61E-06	0.000259
Period	Bacteroides	0.05834	0.000868	11	11	7.27E-06	0.000259

Note:
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Sutterellaceae.g__Sutterella
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides

Supplemental Figure

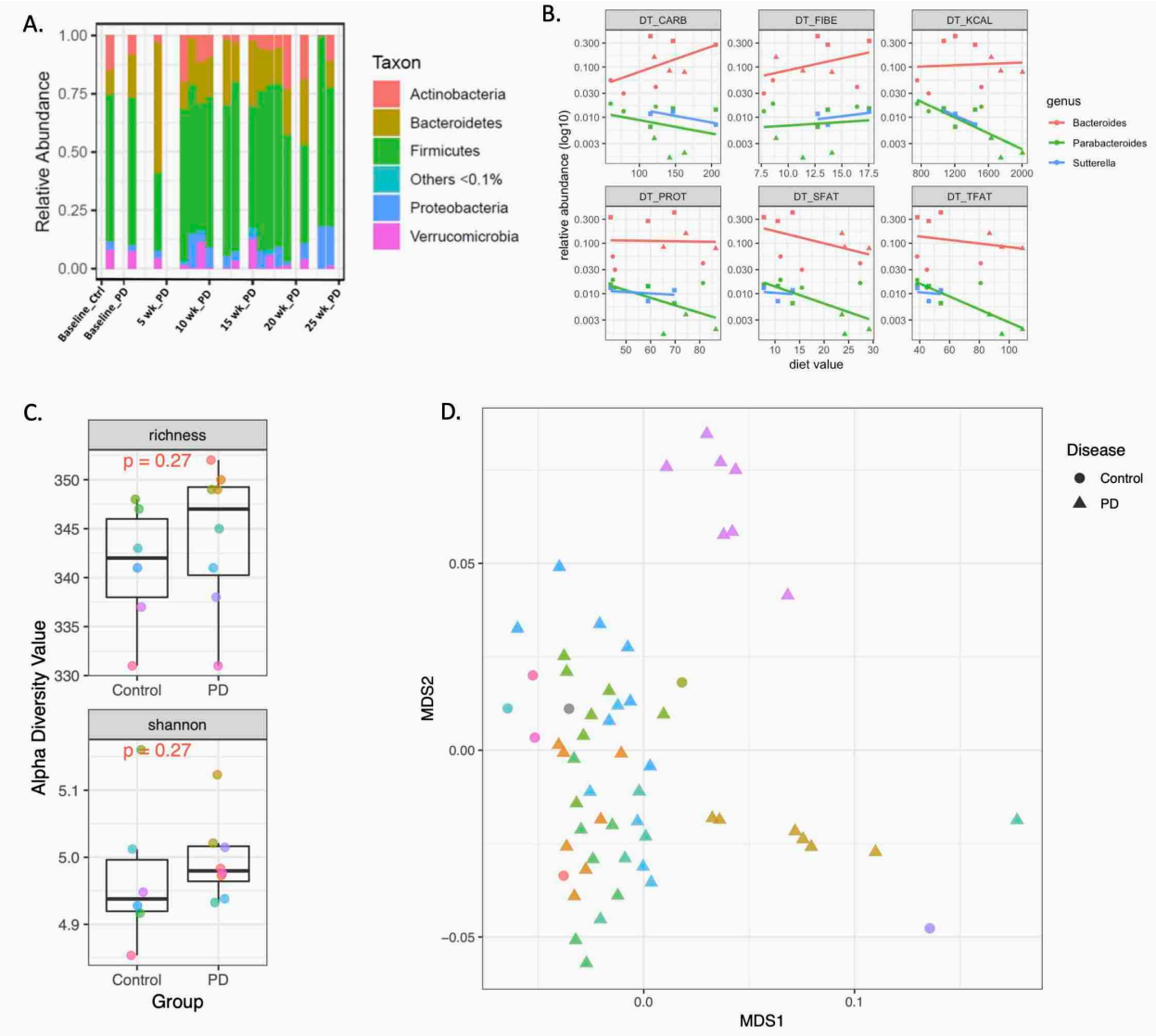

Figure S1. Gut microbiota in PD patients. (A) Detected microbiome at phylum level in PD patients and control subjects. (B) Association of diet with microbial abundance. Different shapes represent different subjects. (C) Alpha-diversity of functional capacity in PD patients and control subjects. Different colors represent different subjects. (D) Clustering of microbial functional capacity by individual in the NMDS ordination. Different colors represent different subjects.

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