Sample receipt and storage: A portion of each stool sample (40-100 mg) and the entire volume of ethanol preservative were stored in 15 mL centrifuge tubes at -80 °C until all samples were collected.

Sample processing: Samples were thawed on ice and then centrifuged (4 ˚C, 5,000 x g) for 5 minutes. Ethanol was evaporated using a gentle stream of nitrogen gas using a nitrogen evaporator (TurboVap LV; Biotage, Charlotte, NC) and stored at -80 ˚C until all samples in the study had been dried. Aqueous homogenates were generated by sonicating each sample in 900 µl of H_2O using an ultrasonic probe homogenizer (Branson Sonifier 250) set to a duty cycle of 25% and output control of 2 for 3 minutes. Samples were kept on ice during the homogenization process. The homogenate for each sample was aliquoted into two 10 µL and two 30 µL in 1.5mL centrifuge tubes for LC-MS sample preparation and 30 µL of homogenate from each sample were transferred into a 50 mL conical tube on ice to create a pooled reference sample. The pooled reference mixture was mixed by vortexing and then aliquoted (100 µL per aliquot) into 1.5 mL centrifuge tubes. Aliquots and reference sample aliquots were stored at -80 °C until LC-MS analyses were conducted.

LC-MS analyses: A combination of four LC-MS methods were used to profile metabolites in the fecal homogenates; two methods that measure polar metabolites, a method that measures metabolites of intermediate polarity (e.g. fatty acids and bile acids), and a lipid profiling method. For the analysis queue in each method, participants were randomized and longitudinal samples from each participant were randomized and analyzed as a group. Additionally, pairs of pooled reference samples were inserted into the queue at intervals of approximately 20 samples for QC and data standardization. Samples were prepared for each method using extraction procedures that are matched for use with the chromatography conditions. Data were acquired using LC-MS systems comprised of Nexera X2 U-HPLC systems (Shimadzu Scientific Instruments; Marlborough, MA) coupled to Q Exactive/Exactive Plus orbitrap mass spectrometers (Thermo Fisher Scientific; Waltham, MA). The method details are summarized below:

LC-MS Method 1 – HILIC-pos: positive ion mode MS analyses of polar metabolites. LC-MS samples were prepared from stool homogenates (10 µL) via protein precipitation with the addition of nine volumes of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8, Isotec; and phenylalanine-d8, Cambridge Isotope Laboratories; Andover, MA). The samples were centrifuged (10 min, 9,000 x g, 4°C), and the supernatants injected directly onto a 150 x 2 mm Atlantis HILIC column (Waters; Milford, MA). The column was eluted isocratically at a flow rate of 250 µL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over m/z 70-800 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings are: ion spray voltage, 3.5 kV; capillary temperature, 350°C; probe heater temperature, 300 °C; sheath gas, 40; auxiliary gas, 15; and S-lens RF level 40.

LC-MS Method 2 – HILIC-neg: negative ion mode MS analysis of polar metabolites. LC-MS samples were prepared from stool homogenates (30 µL) via protein precipitation with the addition of four volumes of 80% methanol containing inosine-15N4, thymine-d4 and glycocholate-d4 internal standards (Cambridge Isotope Laboratories; Andover, MA). The samples were centrifuged (10 min, 9,000 x g, 4°C) and the supernatants were injected directly onto a 150 x 2.0 mm Luna NH2 column (Phenomenex; Torrance, CA). The column was eluted at a flow rate of 400 µL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 100% mobile phase A. MS analyses were carried out using electrospray ionization in the negative ion mode using full scan analysis over m/z 60-750 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings are: ion spray voltage, -3.0 kV; capillary temperature, 350°C; probe heater temperature, 325 °C; sheath gas, 55; auxiliary gas, 10; and S-lens RF level 40.

LC-MS Method 3 – C18-neg: negative ion mode analysis of metabolites of intermediate polarity (e.g. bile acids and free fatty acids). Stool homogenates (30 µL) were extracted using 90 µL of methanol

containing PGE2-d4 as an internal standard (Cayman Chemical Co.; Ann Arbor, MI) and centrifuged (10 min, 9,000 x g, 4°C). The supernatants (10 µL) were injected onto a 150 x 2.1 mm ACQUITY BEH C18 column (Waters; Milford, MA). The column was eluted isocratically at a flow rate of 450 µL/min with 20% mobile phase A (0.01% formic acid in water) for 3 minutes followed by a linear gradient to 100% mobile phase B (0.01% acetic acid in acetonitril) over 12 minutes. MS analyses were carried out using electrospray ionization in the negative ion mode using full scan analysis over m/z 70-850 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings are: ion spray voltage, -3.5 kV; capillary temperature, 320°C; probe heater temperature, 300 °C; sheath gas, 45; auxiliary gas, 10; and S-lens RF level 60.

LC-MS Method 4 – C8-pos: Lipids (polar and nonpolar) were extracted from stool homogenates (10 µL) using 190 µL of isopropanol containing 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphocholine as an internal standard (Avanti Polar Lipids; Alabaster, AL). After centrifugation (10 min, 9,000 x g, ambient temperature), supernatants (10 µL) were injected directly onto a 100 x 2.1 mm ACQUITY BEH C8 column (1.7 µm; Waters; Milford, MA). The column was eluted at a flow rate of 450 µL/min isocratically for 1 minute at 80% mobile phase A (95:5:0.1 vol/vol/vol 10 mM ammonium acetate/methanol/acetic acid), followed by a linear gradient to 80% mobile-phase B (99.9:0.1 vol/vol methanol/acetic acid) over 2 minutes, a linear gradient to 100% mobile phase B over 7 minutes, and then 3 minutes at 100% mobile-phase B. MS analyses were carried out using electrospray ionization in the positive ion mode using full scan analysis over m/z 200-1100 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings are: ion spray voltage, 3.0 kV; capillary temperature, 300°C; probe heater temperature, 300 °C; sheath gas, 50; auxiliary gas, 15; and S-lens RF level 60.

Data processing. Raw LC-MS data were acquired to the data acquisition computer interfaced to each LC-MS system and then stored on a robust and redundant file storage system (Isilon Systems) accessed via the internal network at the Broad Institute. Data processing was conducted using one of five Dell Precision T7600 workstations, each equipped with eight core XEON E5-2687W processors, 32 GB of DDR3 RAM, and 2 TB of storage in RAID 0 array of four 600 GB SAS hard drives. Nontargeted data were processed using Progenesis CoMet software (v 2.0, Nonlinear Dynamics) to detect and de-isotope peaks, perform chromatographic retention time alignment, and integrate peak areas. Peaks of unknown ID were tracked by method, m/z and retention time. Identification of nontargeted metabolite LC-MS peaks were conducted by i) matching measured retention times and a masses to mixtures of reference metabolites analyzed in each batch, ii) matching an internal database of >600 compounds that have been characterized using the Broad Institute methods, and iii) matching exact masses only to an external database of >40000 metabolites (Human Metabolome Database v3; [Wishart et al, 2013; PMID: 23161693]). Compounds matched to the external database were confirmed by analyzing reference standards if they are available. Temporal drift was monitored and normalized with the intensities of features measured in the pooled reference samples.

Reference

Wishart, et al., (2013) HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acid Res. 41:D801-7.*