DFI-HMMF Targeted Metabolomics: General and Detailed Methods

Methods for the targeted metabolite panels
(1) PFBBr
(2) Bile Acid
(3) Indole/Tryptophan
(4) TMS-MOX

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A. Metabolomics Overview

The fecal metabolome will be analyzed across four mass spectrometry platforms to capture quantitative and qualitative levels of gut-derived metabolites with varying physiochemical properties such as hydrophobicity, size, and charge. The DFI Host-Microbe Metabolomics Facility (DFI-HMMF) routinely studies fecal material with the proposed methods and analysis pipelines. Previously, all 304 compounds have been validated by the DFI-HMMF through retention time and fragmentation comparison to standards and available databases. Compounds were chosen based on known host-microbe mechanisms or the compound level in fecal material was shown to significantly vary across patient populations indicating a potential role in health status.

B. General Methods for Panels 1-4

(1) PFBBr Panel: Gas chromatography-mass spectrometry (GC-MS) will be used to detect 51 compounds following derivatization with pentafluorobenzyl bromide (PFBBr).[1] SCFAs (acetate, butyrate, propionate) will be quantitatively analyzed following PFB derivatization and detection by negative collision induced-gas chromatography-mass spectrometry ((-)CI-GC-MS, Agilent 8890). An additional 47 compounds will be reported by normalized relative abundance and include tryptophan catabolites, indoles, amino acids, branched chain fatty acids, phenolic aromatic, and aromatic compounds.

(2) Bile Acid Panel: With the use of negative mode liquid chromatography-electrospray ionization-quadrupole time-of-flight-MS ((-)LC-ESI-QTOF-MS, Agilent 6546), 49 bile acids from the primary, secondary and glyco/tauro-conjugated subclasses will be analyzed from fecal material.[2] In addition to retention time validation, the standard intact and fragment masses are routinely detected with differences < 5 ppm compared to calculated values. In addition to the 49 compounds reported with qualitative information, quantitative values will be calculated for 17 bile acids and include: cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), isodeoxycholic acid (isoDCA), 3-oxolithocholic acid (3-oxoLCA), allolithocholic acid (alloLCA), isolithocholic acid (isoLCA), isoallolithocholic acid (isoalloLCA), allocholic acid (alloCA), ursodeoxycholic acid (UDCA), β-muricholic acid (βMCA), α-muricholic acid (αMCA). To accurately quantify the wide range of concentrations often detected in fecal material, samples with high concentrations of bile acids whose peak area is outside of the linear range of the instrument will be run as concentrated (ex. standard dilution) and diluted (ex. 100x dilution from concentrated).

(3) Indole/Tryptophan Panel: Positive mode LC-triple quadrupole-MS ((+)LC-ESI-QQQ-MS, Agilent 6547) will be used to analyze 35 indole and tryptophan catabolites. Tryptophan is an essential amino acid that is ingested in the host diet. Conversion by microbes and the host result in biologically active compounds such as serotonin, kynurenine, and melatonin. These metabolic products impact the local gut environment as well as systemically with many capable of crossing the blood-brain-barrier.

(4) TMS-MOX Panel: Gas chromatography-mass spectrometry (GC-MS) will be used to detect 169 following derivatization with trimethylsilyl-methoxamine (TMS-MOX).[3,4] Positive ion electron impact-GC-MS ((+)EI-GC-MS, Agilent 7890B) will be used to detect 169 molecules in the organic acid, carbohydrate, TCA intermediate, sterol, amino acid, indole and fatty acid subclasses following TMS-MOX derivatization.

C. Detailed Methods

Metabolite Extraction from Fecal/Cecal Material

Extraction solvent (80% methanol spiked with internal standards and stored at -80 °C) was added at a ratio of 100 mg of material/mL of extraction solvent in beadruptor tubes (Fisherbrand; 15-340-154). Samples were homogenized at 4 °C on a Bead Mill 24 Homogenizer (Fisher; 15-340-163), set at 1.6 m/s with 6 thirty-second cycles, 5 seconds off per cycle. Samples were then centrifuged at -10 °C, 20,000 x *g* for 15 min and the supernatant was used for subsequent metabolomic analysis.

(1) Metabolite Analysis using GC-nCI-MS and PFBBr Derivatization

Short chain fatty acids were derivatized as described by Haak *et al.* with the following modifications.[1] The metabolite extract (100 µL) was added to 100 µL of 100 mM borate buffer (pH 10) (Thermo Fisher, 28341), 400 µL of 100 mM pentafluorobenzyl bromide (Millipore Sigma; 90257) in Acetonitrile (Fisher; A955-4), and 400 µL of *n*-hexane (Acros Organics; 160780010) in a capped mass spec autosampler vial (Microliter; 09-1200). Samples were heated in a thermomixer C (Eppendorf) to 65 °C for 1 hour while shaking at 1300 rpm. After cooling to RT, samples were centrifuged at 4°C, 2000 x *g* for 5 min, allowing phase separation. The hexanes phase (100 µL) (top layer) was transferred to an autosampler vial containing a glass insert and the vial was sealed. Another 100 µL of the hexanes phase was diluted with 900 µL of n-hexane in an autosampler vial. Concentrated and dilute samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in negative chemical ionization mode, using a HP-5MSUI column (30 m x 0.25 mm, 0.25 µm; Agilent Technologies 19091S-433UI), methane as the reagent gas (99.999% pure) and 1 µL split injection (1:10 split ratio). Oven ramp parameters: 1 min hold at 60 °C, 25 °C per min up to 300 °C with a 2.5 min hold at 300 °C. Inlet temperature was 280 °C and transfer line was 310 °C. A 10-point calibration curve was prepared with acetate (100 mM), propionate (25 mM), butyrate (12.5 mM), and succinate (50 mM), with 9 subsequent 2x serial dilutions. Data analysis was performed using MassHunter Quantitative Analysis software (version B.10, Agilent Technologies) and confirmed by comparison to authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

(2) Bile Acid Analysis

Bile acids were analyzed using LCMS. The metabolite extract (75 µL) was added to prelabeled mass spectrometry autosampler vials (Microliter; 09-1200) and dried down completely under a nitrogen stream at 30 L/min (top) 1 L/min (bottom) at 30 °C (Biotage SPE Dry 96 Dual; 3579M). Samples were resuspended in 50:50 Water:Methanol (750 µL). Vials were added to a thermomixer C (Eppendorf) to resuspend analytes at 4 °C, 1000 rpm for 15 min with an infinite hold at 4 °C. Samples were then transferred to prelabeled microcentrifuge tubes and centrifuged at 4 °C, 20,000 x *g* for 15 min to remove insoluble debris. The supernatant (700 µL) was transferred to a fresh, prelabeled mass spectrometry autosampler vial. Samples were analyzed on a liquid chromatography system (Agilent 1290 infinity II) coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (Agilent 6546), operating in negative mode, equipped with an Agilent Jet Stream Electrospray Ionization source. The sample (5 µL) was injected onto an XBridge© BEH C18 Column (3.5 µm, 2.1 x 100 mm; Waters Corporation, PN) fitted with an XBridge© BEH C18 guard (Waters Corporation, PN) at 45 °C. Elution started with 72% A (Water, 0.1% formic acid) and 28% B (Acetone, 0.1% formic acid) with a flow rate of 0.4 mL/min for 1 min and linearly increased to 33% B over 5 min, then linearly increased to 65% B over 14 min. Then the flow rate was increased to 0.6 mL/min and B was increased to 98% over 0.5 min and these conditions were held constant for 3.5 min. Finally, re-equilibration at a flow rate of 0.4 mL/min of 28% B was performed for 3 min. The electrospray ionization conditions were set with the capillary voltage at 3.5 kV, nozzle voltage at 2 kV, and detection window set to 100-1700 *m/z* with continuous infusion of a reference mass (Agilent ESI TOF Biopolymer Analysis Reference Mix) for mass calibration. A ten-point calibration curve was used

for quantitation. Data analysis was performed using MassHunter Profinder Analysis software (version B.10, Agilent Technologies) and confirmed by comparison with authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

(3) Indole/Tryptophan Analysis

Indole-containing metabolites, B-vitamins and other targeted metabolites were analyzed by LC-MS/MS. The metabolite extract (400 µL) was added to pre-labeled microcentrifuge tubes. Samples were dried down completely using a Genevac EZ-2 Elite. Samples were resuspended in 100 µL of 50:50 Water:Methanol and added to an Eppendorf thermomixer® C at 4 ºC, 1000 rpm for 15 min to resuspend analytes. Samples were then centrifuged at 4 ºC, 20,000 x *g* for 15 min to remove insoluble debris. The supernatant (80 µL) was transferred to a fresh, prelabeled MS vial with inserts or 96 deep-well plate (Agilent 5065-4402). Samples were analyzed on an Agilent 1290 infinity II liquid chromatography system coupled to an Agilent 6470 triple quadrupole mass spectrometer, operating in positive mode, equipped with an Agilent Jet Stream Electrospray Ionization source. Each sample (2 µL) was injected into a Acquity UPLC HSS PFP column, 1.8 µm, 2.1 x 100 mm (Waters; 186005967) equipped with a Acquity UPLC HSS PFP VanGuard Pre-column, 100Å, 1.8 µm, 2.1 mm X 5 mm (Waters; 186005974) at 45 ºC. Mobile phase A was 0.35% formic acid in Water and mobile phase B was 0.35% formic acid in 95:5 Acetonitrile:Water. The flow rate was set to 0.5 mL/min starting at 0% B held constant for 3 min, then linearly increased to 50% over 5 min, then linearly increased to 95% B over 1 min, and held at 100% B for the next 3 min. Mobile phase B was then brought back down to 0% over 0.5 min and held at 0% for re-equilibration for 2.5 min. The QQQ electrospray conditions were set with capillary voltage at 4 kV, nozzle voltage at 500 V, and Dynamic MRM was used with cycle time of 500 ms. Transitions were monitored in positive mode for 46 analytes (table on next slide). An 11-point calibration curve (ranging from 0.88 nM to 909 µM) was prepared for tryptophan, tyrosine, phenylalanine, serotonin, 5-HIAA, melatonin, tryptamine, kynurenine, kynurenic acid, anthranilic acid, and niacin. Data analysis was performed using MassHunter Quant software (version B.10, Agilent Technologies) and confirmed by comparison with authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

(4) TMS-MOX Analysis

Metabolites were analyzed using GC-MS with electron impact ionization. The metabolite extract (100 µL) mass spec autosampler vials (Microliter; 09-1200) and dried down completely under nitrogen stream at 30 L/min (top) 1 L/min (bottom) at 30 °C (Biotage SPE Dry 96 Dual; 3579M). To dried samples, 50 µL of freshly prepared 20 mg/mL methoxyamine (Sigma; 226904) in pyridine (Sigma; 270970) was added and incubated in a thermomixer C (Eppendorf) for 90 min at 30 °C and 1400 rpm. After samples are cooled to room temperature, 80 µL of derivatizing reagent (BSTFA + 1% TMCS; Sigma; B-023) and 70 µL of ethyl acetate (Sigma; 439169) were added and samples were incubated in a thermomixer at 70 °C for 1 hour and 1400 rpm. Samples were cooled to RT and 400 µL of Ethyl Acetate was added to dilute samples. Turbid samples were transferred to microcentrifuge tubes and centrifuged at 4 °C, 20,000 x *g* for 15 min. Supernatants were then added to mass spec vials for GCMS analysis. Samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in electron impact ionization mode, using a HP-5MSUI column (30 m x 0.25 mm, 0.25 µm; Agilent Technologies 19091S-433UI) and 1 µL injection. Oven ramp parameters: 1 min hold at 60 °C, 16 °C per min up to 300 °C with

a 7 min hold at 300 °C. Inlet temperature was 280 °C and transfer line was 300 °C. Data analysis was performed using MassHunter Quantitative Analysis software (version B.10, Agilent Technologies) and confirmed by comparison to authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

E. Quality Control

Quality Control Samples

Control biological samples are used to evaluate all HMMF processes. Specifically, metabolite extraction efficiency and instrument performance. The samples are extracted with solvent containing known internal standard (IS) concentrations. These samples are processed and analyzed alongside project runs. Recovery, retention time and %CV are calculated for ISs. Method blanks are run, and these samples do not contain any metabolites and are included in datasets to indicate instrument noise for the *m/z* at the retention time extracted. A small aliquot of submitter samples are combined to create a pooled quality control (PooledQC) sample that is run to determine if there are matrix impacts on HMMF processes.

Quality Control Ions

All submitted samples are extracted with solvent that includes deuterated or heavy atom standards with known concentrations to evaluate metabolite extraction efficiency and instrument performance. Quality control ions are tracked in all control samples and the submitted samples and analyzed for batch impact and intra-project variability.

References

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