OMNImet™•GUT

Methods appendix: *OMNImet™•GUT (ME-200) enables at-home collection and ambient temperature transport of fecal samples for metabolomics* (PD-WP-00066)

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2020-06-16

Introduction

This methods appendix describes the study design, wet lab procedure and data analysis process that led to the findings and interpretations within PD-WP-00066.

Materials and methods

Study design

The main study design is shown in Table 1. A fresh fecal sample was collected from 7 adult volunteers and stored in n=3 technical replicates (3 cryovials for the flash-frozen (FF) condition; 3 OMNImet™•GUT devices for the remaining conditions) per donor. For each donor, the same 3 OMNImet•GUT devices were sampled at each of the indicated timepoints (immediately frozen (T0), room temperature for 1, 4, or 7 days (T1, T4, T7)). For the Freeze-Thaw (FT) condition, an aliquot from each OMNImet•GUT device was held at -20°C for 7 days, followed by 4 hours at 30°C. After the indicated storage condition, all samples were held at -80°C until analysis.

Collection method	Timepoint	Technical replicates per donor (donor 1 to 7)
Cryovial	T0 (FF)	n=3
OMNImet•GUT	T0	n=3
	T1	n=3
	T4	n=3
	T7	n=3
	FT	n=3

***Table 1*: Study design for validation of fecal sample stability in OMNImet•GUT.** *T0 (FF) represents flash-frozen samples; T0, T1, T4 and T7 represent samples stored in OMNImet•GUT tubes at room temperature for 0, 1, 4, or 7 days; FT represents storage at -20°C for 7 days, followed by 4 hours at 30°C.*

In a second experiment, the performance of OMNImet•GUT device in stabilizing short-chain fatty acids (SCFAs) in fecal sample at room temperature was evaluated relative to unstabilized storage. A fecal sample was collected from a different group of 7 adult volunteers, pooled and mixed thoroughly and stored in n=4 technical replicates per condition as shown in Table 2. In this experiment, independent replicates (cryovials or OMNImet•GUT devices) were used at each timepoint. After the indicated storage timepoint, all samples were held at -80°C until analysis.

Collection method	Timepoint	Technical replicates (pooled sample)
Cryovial	T0 (FF)	n=4
	T1	n=4
	T4	n=4
OMNImet•GUT	T0	n=4
	T1	n=4
	T4	n=4
	T7	n=4
	T13	n=4

***Table 2*:** *Study design for evaluation of SCFA stability in fecal samples stored at room temperature in OMNImet•GUT devices vs. unstabilized samples. Timepoints are labeled as in Table 1.*

Sample preparation, data acquisition and data processing: Global metabolomics

To prepare fecal samples for metabolomic analysis, unstabilized samples were lyophilized while OMNImet•GUT samples were dried in a Genevac evaporator. All dried samples were weighed and then resuspended at a 50:1 (50 µL deionized water for every 1 mg of fecal sample) ratio for homogenization[1].

The homogenates were subjected to automated biochemical extraction and analysis by liquid chromatography and high-resolution tandem mass spectrometry (LC-MS/MS) on Metabolon's global metabolomics platform[2, 3]. Raw data were extracted, peak-identified and processed by Metabolon using proprietary software[4, 5, 6]. In brief, compounds of exogenous, human and microbial origin were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a dynamic and proprietary biochemical reference library of more than 4,500 known metabolites (based on authenticated standards) and more than 2,000 novel metabolites (without an identified chemical structure); each library entry contains the retention time/index (RI), mass to charge ratio (m/z) and chromatographic data (including MS/MS spectral data). Biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ±10 ppm and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. Integrated ion peaks were quantified using area-under-the-curve and scaled to a median of 1 for each biochemical. Missing values, if any, were imputed with the observed minimum for that particular compound. The data were natural log-transformed prior to statistical analyses including Welch's two-sample t-test, principal component analysis (PCA) and hierarchical clustering analysis (HCA) using complete clustering with Euclidean distance.

Sample preparation, data acquisition and data processing: Short-chain fatty acid (SCFA) assay

For unstabilized samples, each fecal sample was weighed and extracted in organic solvent in the presence of isotopically labeled internal standards; the extracts were then derivatized with 2,4-difluorophenyl hydrazine. For OMNImet•GUT samples, each tube was centrifuged and an aliquot of the supernatant was derivatized in the presence of the same internal standards under the same conditions as the unstabilized samples. For both sample types, the derivatization reaction mixture was diluted and an aliquot was injected onto an Agilent 1290/AB Sciex QTrap® 5500 LC-MS/MS system equipped with a C18 reversed phase UHPLC column. The mass spectrometer was operated in negative mode using electrospray ionization (ESI). The peak area of each individual analyte product ion was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least squares regression analysis generated from calibration standards fortified with isotopically labeled internal standards, prepared immediately prior to each run. For unstabilized samples, SCFA concentrations were normalized by wet weight. OMNImet•GUT samples were normalized by the average wet weight collected per tube as established in preliminary experiments.

References:

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