An optimized sample handling strategy for metabolic profiling of human feces

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ABSTRACT: Fecal metabolites are being increasingly studied to unravel the host-gut microbial metabolic interactions. However, there are currently no guidelines for fecal sample collection and storage based on a systematic evaluation of the effect of time, storage temperature, storage duration and sampling strategy. Here we derive an optimized protocol for fecal sample handling with the aim of maximizing metabolic stability and minimizing sample degradation. Samples obtained from five healthy individuals were analyzed to assess topographical homogeneity of feces, and to evaluate storage duration-, temperature- and freeze-thaw cycle-induced metabolic changes in crude stool and fecal water using a ^{1}H NMR spectroscopy-based metabolic profiling approach. Inter-individual variation was much greater than that attributable to storage conditions. Individual stool samples were found to be heterogeneous and spot sampling resulted in a high degree of metabolic variation. Crude fecal samples were remarkably unstable over time and exhibited distinct metabolic profiles at different storage temperatures. Microbial fermentation was the dominant driver in time-related changes observed in fecal samples stored at room temperature and this fermentative process was reduced when stored at 4°C. Crude fecal samples frozen at -20°C manifested elevated amino acids and nicotinate and depleted short chain fatty acids compared to crude fecal control samples. The relative concentrations of branched-chain and aromatic amino acids significantly increased in the freeze-thawed crude fecal samples, suggesting a release of microbial intracellular contents. The metabolic profiles of fecal water samples were more stable compared to crude samples. Our recommendation is that intact fecal samples should be collected, kept at 4°C or on ice during transportation, and extracted ideally within 1 h of collection, or a maximum of 24 h. Fecal water samples should be extracted from a representative amount (~15 g) of homogenized stool sample, aliquoted and stored at < -20°C, avoiding further freeze-thaw cycles.

Metabolic profiling of biofluids and tissues generates data on a wide range of metabolites and provides extensive metabolic information on multiple biological processes in complex superorganisms such as mammals. Although urine and blood are often used to investigate systemic responses of animals and humans to various environmental stimuli or therapeutic interventions[1], the search for disease biomarkers in fecal samples and studies on host-microbial interactions have intensified over the last decade. The human intestinal tract harbors >100 trillion microbial cells[2] and these microbes exert their influences on the human host primarily by metabolic signaling and therefore optimized methodologies for the study of microbial metabolic footprint is crucial to this field. Mounting evidence shows that the microbial composition and its collective metabolic activity profoundly impacts host physiology and modulates the disease risk of the host[3]. To investigate this metabolic crosstalk between the host and its gut microbiota, a fecal sample provides the most direct information, since feces carries numerous biochemical compounds derived from the host, microbiota and food residuals, but also captures a metabolic output resulted from the interactions among the microbiota. Previous studies have reported decreased fecal levels of short chain fatty acids (SCFAs) after a high animal protein and fat and low-fiber diets compared to a high-fiber and low-fat diet[4], and increased proportions of fecal branched-chain fatty acids after high protein consumption[5]. Metabolic changes were also found in patients with colorectal cancer[6,7] or inflammatory bowel disease (IBD)[8], inferring a strong association between diet and gut health. Hence, fecal metabolite profiles hold a huge potential for fecal biomarker discovery and deepening our understanding of host-microbial interactions.

Metabolic profiling of fecal samples has been carried out using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-MS (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy[9,10,11]. Published studies so far have focused on developing various fecal extraction and sample preparation methods for metabolic profiling[12,13]. However, a standard protocol for fecal sample handling has yet to be established based on a systematic evaluation of experimental conditions. Ideally, biological samples should be analyzed or prepared as soon as they are obtained to quench further biological processes that occur within the samples post collection. Unlike urine and blood samples, the added practical difficulties in obtaining fecal samples introduces an unavoidable delay between obtaining the sample and sample processing in the clinic. Although fecal sample collection is non-invasive, it is rarely done at outpatient clinics. Often patients are asked to collect fecal samples at home and bring it into the clinic on the following day to be processed and analyzed. Stool contains both microbial and mammalian cells with numerous enzymes, and biological processes continue during the sample collection, storage and transport. These processes may be influenced by storage temperature, oxygen exposure and time, thus introducing bias into a study.

A collection protocol of fecal samples for microbiome analysis has been proposed by Cardona et al.[14], who recommended that samples should be immediately frozen (-20°C) if the samples cannot be brought to the laboratory within 24 h after collection, and transported in a freezer pack in order to prevent the sample from thawing. Another microbial study

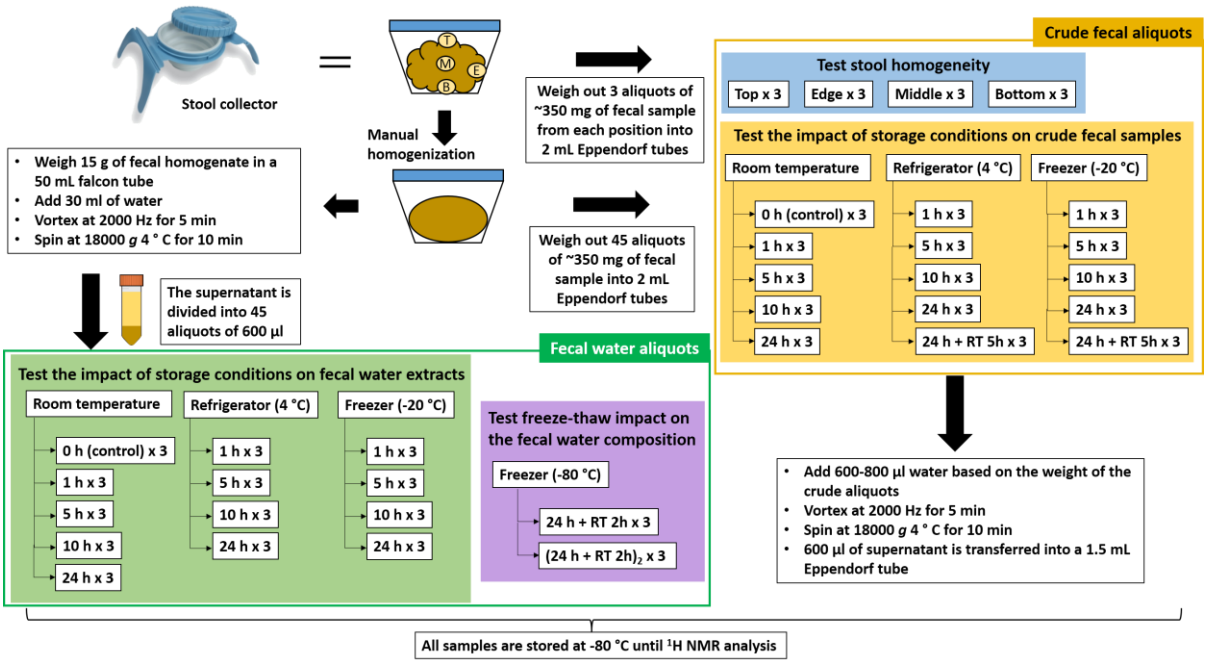

Figure 1. Schematic of the study design showing the workflow: Key: B, bottom; E, edge; M, middle; RT: room temperature; and T, top.

showed that the bacterial diversity and the total number of bacteria were significantly reduced in human fecal samples stored at either room temperature or 4°C for 8 h and 24 h, with the greatest variation observed after 24 h at room temperature[15], indicating extensive temperature- and time-dependent changes in the microbial community of fecal samples. However, the impact of these storage conditions on fecal metabolite profiles remains unknown. As such, guidelines for fecal sample storage and transportation become imperative, if we are to capture a realistic snapshot of the fecal metabonome.

In the current work, we investigated the topographical homogeneity of feces, the effects of storage duration and temperature on the metabolic profiles of crude stool and fecal water, and characterize freeze-thaw cycle-induced metabolic alterations using a ^{1}H NMR spectroscopy-based metabolic profiling approach. Based on the metabolic stability of fecal samples, we derived and optimized a standard protocol for human fecal sample collection and handling.

■ MATERIALS AND EXPERIMENTS

Study Design. A total of five healthy individuals were recruited under the approval from the UK National Research Ethics Centre approval (13/LO/1867) and consent forms were obtained. These five individuals were Caucasian (2 male, 1 female) and British-Indian (1 male, 1 female), all, non-smokers, and between 27 and 33 years of age. None of them had been taking regular medication or antibiotics for at least 6 weeks prior to the fecal sample collection. Each volunteer provided one complete stool specimen in a feces collector (FECOTAINER®, AT Medical BV, The Netherlands) in the early morning at St Mary's Hospital, London, UK. The stool specimens were put on ice immediately after collection, transferred to the laboratory in the same hospital and processed according to the study design shown in Figure 1 in a hood cleaned with 70% ethanol followed by sterile water to avoid potential contaminants.

To investigate stool homogeneity, three aliquots of ~350 mg of crude fecal samples were obtained from four locations (e.g. top, edge, middle and bottom of the stool specimens in the fecal collector) as shown in Figure 1, followed by fecal water extraction described below. These locations do not necessarily reflect the same locations in the original stool. The aim of this part of the study was to demonstrate the heterogeneity of the stool samples rather than examining the metabolic difference between different locations of the original stool sample. The remaining stool specimen was immediately and thoroughly homogenized manually on ice with combined 3 disposable and sterile high-impact polystyrene sticks (Fisher Scientific) for approximately 3 min. The homogenized stool samples were used for the subsequent collection of crude fecal aliquots and fecal water aliquots.

To investigate the effects of storage conditions on the metabolite composition of crude fecal samples, a total of 45 aliquots from each fecal homogenate (~350 mg per aliquot) were weighed (weights were recorded) and transferred into 2 mL microcentrifuge tubes, which were subjected to a range of experimental conditions including three storage temperatures (RT: room temperature approx. 20°C; FG: 4°C; and FZ: freezer -20°C on manual-defrost mode) and four storage durations at each of these temperatures (1, 5, 10 and 24 h). Three crude fecal control aliquots were immediately extracted and resulting fecal water were frozen at -80°C, and six aliquots were subjected to a combined storage condition (refrigerator or freezer for 24 h followed by 5 h storage at room temperature) to mimic a typical clinical scenario whereby outpatients store samples in the refrigerator/freezer at home and bring the samples to the hospital on the following day. The exact weights of the aliquots were recorded for subsequent fecal water extraction. For each

sample, the fecal water extraction process was undertaken as described below.

To investigate the effects of storage conditions on the metabolite composition of fecal water extracts, ~15 g of fecal homogenate was transferred into a 50 mL Falcon tube for fecal water extraction as described below. The resulting fecal water samples were divided into 45 aliquots of 600 µL each and subjected to various experimental conditions including RT, FG and FZ at 1, 5, 10 and 24 h. Three aliquots of fecal water samples per individual were immediately stored at -80°C and served as fecal water controls. To test the impact of freeze-thaw cycles on the metabolite composition of fecal water, three aliquots were subjected to either one or two freeze-thaw cycle whereby aliquots were frozen at -80°C for 24 h and thawed at room temperature for 2 h. Once the respective experimental conditions were met, the fecal water aliquots were then stored at -80°C until ^{1}H NMR spectroscopic analysis.

Samples were uniquely labelled. For example, D1CF_RT5h_c represents an aliquot (c) from donor 1 (D1) and it is a crude fecal aliquot (CF) subjected to a condition at room temperature for 5 h (RT5h). D3FW_FZ24h_a represents an aliquot (a) from donor 3 (D3) and it is a fecal water aliquot (FW) subjected to a condition in a -20°C freezer for 24 h (FZ24h). The complete list of labels is shown in Table S1.

Fecal Water Extraction. Fecal water was extracted from each sample with a 2:1 ratio of water (HPLC-grade, Sigma Aldrich) : net weight of fecal sample (µL : mg). The mixture was vortexed for 5 min and centrifuged at 4°C at 18,000 *g* for 10 min. For the fecal water aliquots, the supernatant from the 15 g fecal material was aliquoted as aforementioned.

Proton NMR spectroscopic analyses were performed on 510 samples and 506 spectra were included into the statistical analyses. Spectrum exclusion, spectral processing and data modelling are described in the Supporting Information (SI).

■ RESULTS AND DISCUSSION

Overview of the ^{1}H NMR Fecal Profiles. The dominant source of variation in the dataset was attributed to inter-individual differences in the fecal metabolite profiles, as demonstrated in the PCA scores plot of the ^{1}H NMR fecal spectral data (Figure 2A), which shows a clear clustering of samples according to the donors. This is consistent with published literature showing that the fecal metabonome is host-specific[11]. Donor 2, a male Caucasian, is separated from the rest of donors along the first principal component (PC1), whereas donor 5, a female British-Indian, is separated from the other 4 donors along PC3 (Figure S1A). Along the second PC, a clear linear separation among donors 1-4 was observed (Figure S1B). The mean ^{1}H NMR spectra (Figure S1C) and the corresponding loadings plots of PCA (not shown) indicate donor 2 was characterized by higher levels of lactate whereas higher levels of nicotinate and uracil were observed in donor 5 compared to the other donors. The metabolites assigned from the ^{1}H NMR spectra are summarized in Table S2. High interpersonal variations have been previously reported not only in the metabolic profiles of urine[16] and plasma[17], but also in the gut microbial composition[18] and fecal metabolites[11], implicating that the genetic background and environmental diversity significantly contribute to the metabolic phenotype of the host. Aside from being requested to abstain from alcohol for 24 h prior to fecal sample donation, the donors enrolled in the study were not subjected to any dietary restrictions, which could enhance the interpersonal variations observed. Although the metabolic variations of fecal metabolites have been reported to be influenced by gender[11], unsurprisingly, given the small number of study participants, gender- and ethnicity-dependent grouping was not observed in the current study, most likely due to the heavy influence of environmental factors such as lifestyle and diet.

To visualize the storage duration- and temperature-dependent effects without the interference of inter-donor variation, the mean-subtracted spectral data was analyzed using PCA. The resulting scores plot (Figure 2B) showed an overlap of the donors, which indicates that the inter-individual differences were successfully reduced. To effectively visualize the data distribution in relation to the experimental conditions, the same PCA scores plot (Figure 2C) was color-coded based on a

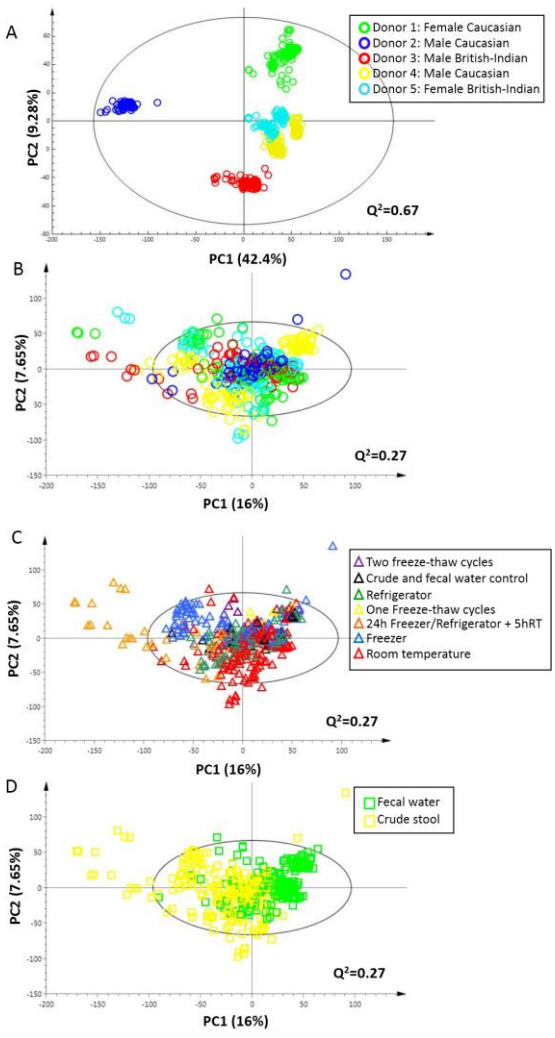

Figure 2. PCA scores plot of the 506 samples showing inter-individual variation (PC1 (first principal component) *vs.* PC2) (A). PCA scores plots of the NMR fecal profiles where the mean spectrum of a given individual is subtracted for every spectrum for that particular individual in order to reduce the influence of inter-individual variation (B). The same PCA scores plots color-coded based on the storage temperatures (C) and type of samples (D).

classification of the experimental conditions (e.g. control, room temperature, refrigerator, freezer and freeze-thaw cycle). It is obvious that samples clustered based on the storage temperatures, with a group of moderate outliers (orange triangles in Figure 2C) from crude fecal aliquots that underwent 24-h cold storage followed by 5-h storage at room temperature. The PCA scores plot (Figure 2D) was recoded based on assignment of samples as fecal water extracts versus crude fecal samples. Fecal water extracts formed a tighter cluster compared to the crude fecal samples, indicating that the storage conditions exert a greater impact on the metabolic composition of the crude fecal samples than that of the fecal water extracts. This impact will be discussed below.

Homogeneity of Fecal Samples. Since it is impractical to collect, homogenize and store a whole stool evacuation, the typical fecal sampling method is to scoop a small portion of feces into a 30 mL container, which could introduce analytical biases due to the heterogeneity of fecal samples. We compared control samples derived from homogenized feces (of the entire fecal evacuation) with localized samples collected from the top, middle, bottom or edge positions of the specimen prior to homogenization. The PCA scatter plots (Figure S2) show that the crude fecal samples collected from different positions are widely distributed in the metabolic space and this distribution pattern is inconsistent across all individuals. OPLS-DA models comparing the homogenized crude fecal control samples with each sampling position of all five individuals were constructed, and the sample composition was found to be significantly different between the whole sample homogenate and the topographical positions based on the p-values of these OPLS-DA models (middle, $p = 0.003$; top, $p = 7.4 \times 10^{-4}$; edge, $p = 0.007$; and bottom, $p = 2.6 \times 10^{-4}$). In contrast, the differences of microbial species composition between the inner and outer layers of the stool have been reported to be insignificant[19]. As it is common that a study involves both metabolic and microbial profiling, homogenizing fecal samples prior to extraction is strongly recommended.

We also note that the fecal water control samples extracted from 15 g of fecal material were much more reproducible than the crude fecal control samples obtained by spot sampling (350 mg) from a homogenized fecal specimen (Figure S2). The OPLS-DA loadings plot (Figure S3) shows that fecal water extracted from 15 g of feces contained higher concentrations of butyrate, *N*-acetyl compounds and aromatic amino acids such as tyrosine and phenylalanine and lower concentrations of glutamate and fumarate, compared with fecal water extracted from 350 mg of fecal samples. The results indicate that stool samples are highly heterogeneous and homogenization of feces prior to fecal water extraction is crucial in obtaining a representative metabolite profile. In addition, fecal water

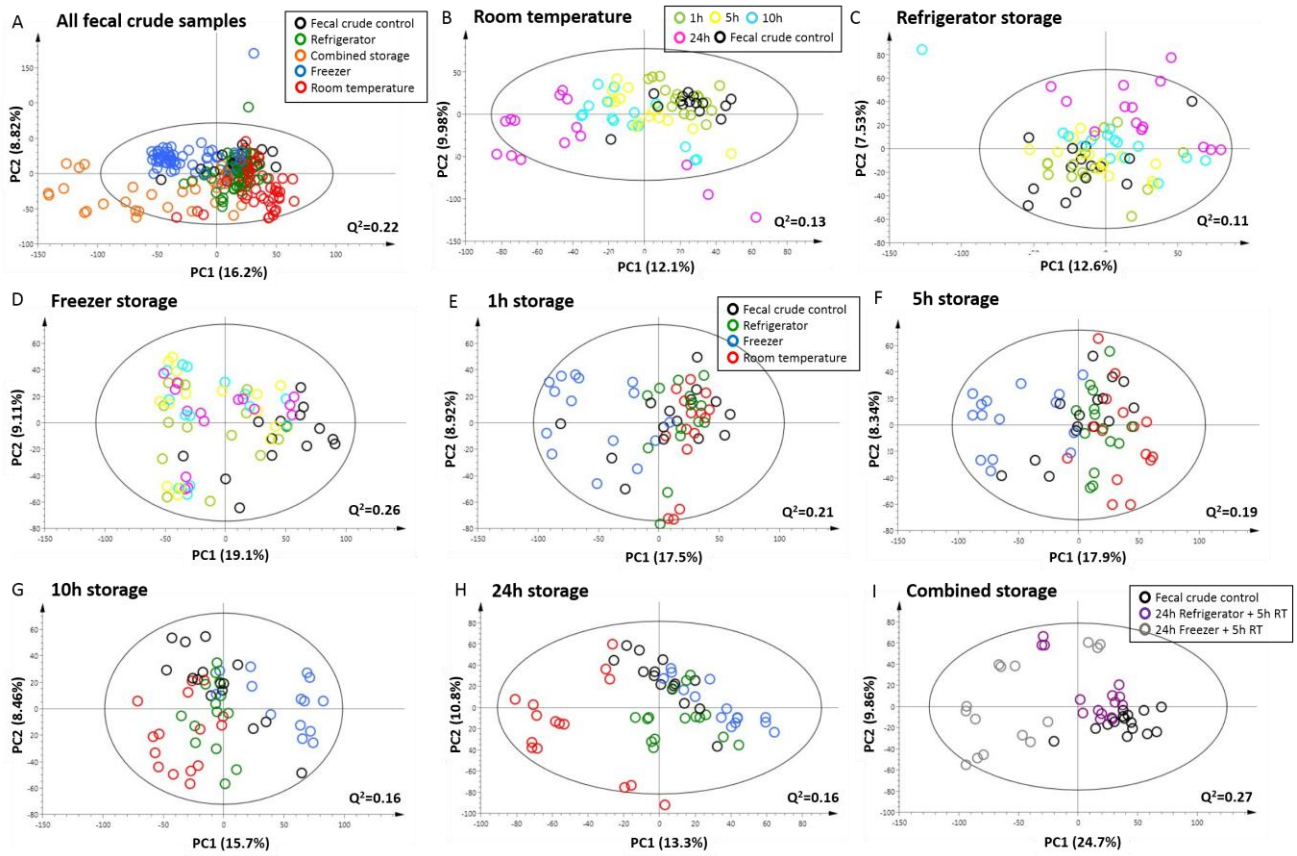

Figure 3. PCA scores plots of the mean subtracted fecal ^{1}H NMR spectral data obtained from controls and crude samples stored at different storage conditions (A), room temperature (B), refrigerator storage (C) and freezer storage (D) with different storage durations. PCA scores plots of the mean subtracted fecal ^{1}H NMR spectral data obtained from controls and crude fecal samples stored at room temperature (RT), refrigerator temperature (FG) and -20°C (FZ) for 1h (E), 5h (F), 10h (G) and 24 h (H). The PCA scores plots of fecal samples subjected to a combined storage condition (I).

extraction from a relatively large quantity of stool (e.g. 15 g) is strongly recommended in order to compensate for the inefficiency of fecal homogenization due to the significant variation of the types of human fecal matter (as classified by the Bristol stool scale) provided by volunteers and patients.

The Impact of Storage Conditions on Crude Feces. The impact of storage temperature and duration on the crude fecal samples was evaluated in order to elucidate metabolites that are affected by various active processes (e.g. enzymatic and chemical). The PCA scores plot of ^{1}H NMR spectra of fecal water obtained from crude feces after various storage condition challenges (Figure 3A) shows that the major metabolic variation along the PC1 axis is most strongly influenced by 24-h storage at 4°C, or -20°C followed by 5-h storage at room temperature. Samples stored at room temperature were mostly separated from the controls along the PC2 axis. Samples stored at 4°C exhibit a tighter cluster and are closer, metabolically, to the controls, suggesting that fridge storage introduces less metabolic disruption in contrast to room temperature and freezer storage. We subsequently investigated the temperature- and time-dependent metabolic effects in feces, separately.

To study the effects of time before processing the samples, PCA analysis was applied to samples stored at room temperature, 4°C and -20°C for various durations. The metabolic profiles of crude fecal samples at 1-h room temperature storage clustered together with controls (Figure 3B) suggesting no real impact on sample integrity, but samples stored for a longer time prior to processing (5, 10 and 24 h) gradually shifted along PC1, with the exception of donor 2 (three purple dots in the bottom right quadrant). Distinct grouping of donors at 24 h was also noted. A similar trend was observed, to a lesser extent, during storage at 4°C (Figure 3C), indicating that lower temperature slows down the biological processes in the crude samples, which results in fewer alterations to the overall metabolic pattern. However, marked metabolic shifts were observed after 24-h storage at 4°C (Figure 3C). Unlike room temperature and 4°C storage, freezing samples at -20°C immediately altered the metabolic composition of the crude fecal samples (Figure 3D). Pair-wise comparisons between control samples and each storage condition were carried out using OPLS-DA. The corresponding parameters of the models are summarized in Table S3. The Q^2Y (predictive ability of the model) and R^2X (fitness of the model) values of these models increased with storage time from 1 h to 24 h, which indicate increased metabolic changes in crude fecal samples with time at any storage temperature. At room temperature, an increase in acetate and valerate levels and a decrease in fumarate, succinate and glutamate were observed after just 1-h storage (Table S3). After 5 h, SCFAs including acetate, butyrate, propionate and valerate, methanol and phenylalanine were elevated, whereas glutamate and fumarate were depleted and these changes remained evident until the last observation time point of 24 h. Moreover, increased alanine was found following 24-h room temperature storage. Crude samples stored at 4°C had no significant metabolite changes at 1 h compared to control samples. However, levels of glutamate and fumarate were lower, while acetoacetate, *β*-xylose and *β*-arabinose increased in the crude fecal extracts at 5 h at 4°C. More changes were observed at the later time points, including elevated concentrations of short chain fatty acids (e.g. acetate and propionate), α-glucose, α-xylose, pyruvate, alanine and methanol, together with decreased levels of *N*6-acetyllysine. A rapid metabolic shift was observed in crude fecal samples frozen at -20°C for 1 h. These immediate changes include decreased propionate and increased fumarate, nicotinate and glucose levels. After 10-h freezing, additional alterations in concentrations of acetate, glutamate and tyrosine were also observed. As a general trend, the metabolic profile of fecal samples altered over the time regardless of the storage temperatures.

Glutamate is one of the most abundant metabolites in bacterial cells and plays an important role in bacterial carbon and nitrogen metabolism[20]. Facultative anaerobic bacteria such as *Lactobacillus fermentum* contain $NADP^+$-specific glutamate dehydrogenase, an enzyme that converts glutamate to 2-oxoglutamate and vice versa[21]. SCFAs are produced *via* bacterial fermentation of indigestible dietary fibers[22]. Hence, it is expected that concentrations of SCFAs increased in crude fecal samples stored at room temperature across all time points due to the on-going microbial fermentative activity. Fumarate and succinate are the intermediates of propionate production from carbohydrates through the succinate pathway, which explains the reduced concentrations of these two metabolites. Human colonic bacteria also produce methanol *via* the degradation of pectins, which are a class of heterogeneous polysaccharides and can be found in the inter- and intra-cell walls of most fruits and vegetables[23]. Higher concentrations of methanol were found after 5-h room temperature storage, which indicate that fecal bacteria were able to further degrade indigestible dietary components when samples were stored at room temperature. D-alanine is present in both Gram-positive and Gram-negative bacterial cell walls[24], and increased alanine after 24-h storage at room temperature and 4°C may suggest bacterial cell lysis.

To investigate the effects of storage temperature, PCA analysis of ^{1}H fecal NMR spectra was used to demonstrate metabolic differences and similarities among controls and crude fecal samples stored at room temperature, 4°C and -20°C for the same storage duration (e.g. 1, 5, 10 or 24 h). The PCA scores plots derived from samples stored for 1, 5 or 10 h (Figure 3E-G) shared a similar pattern, whereas the samples stored at room temperature (Figure 3H) showed a clear separation from samples stored at 4°C or -20°C along the PC1. The clustering between the control samples and those stored at RT became more evident as the storage duration increased. At 10 h, room temperature samples separated from the controls along the PC2 axis (Figure 3G) and at 24 h the main variation was attributed to the marked metabolic shift in room temperature samples (Figure 3H). Despite the use of mean-subtracted spectra, the inter-person variations also became evident, suggesting that the metabolite changes caused by the biological processes (e.g. bacterial fermentation) during 24 h are highly individual-dependent. This observation may have further relevance when considering systematic metabolic differences introduced by various pathologies.

Freeze-thawing crude samples mimics the process of sample storage in either the refrigerator or freezer overnight (24 h), and subsequent transportation (i.e. thawing at room temperature for 5 h) to the laboratory until the samples are processed to obtain fecal water. In order to mimic the logistics of sample collection and transportation from patients' homes to the laboratories or hospitals, we studied the effects of 24-h refrigerator or freezer storage followed by 5 h at room temperature (abbreviated as FG24h_RT5h and FZ24h_RT5h). The FZ24_RT5h samples

were widely dispersed in the PCA scores plot (Figure 3I), whereas FG24h_RT5h samples clustered relatively tightly and located closer to the control group. As seen in Table S3, FZ24h_RT5h and FG24h_RT5h samples are significantly different from crude controls ($p = 6.4x10^{-10}$; $p = 7.7x10^{-10}$). FG24h_RT5h samples showed elevated concentrations of SCFAs (e.g. acetate and propionate), amino acids (valine, glycine, alanine, phenylalanine and tyrosine), lactate and uracil, whereas FZ24h_RT5h samples exhibited decreased levels of SCFAs and increased levels of fumarate, amino acids, branched chain amino acids (BCAAs), nicotinate, glucose and uracil in contrast to crude controls. Although exposing crude fecal samples to colder temperatures (refrigerator or freezer) followed by a 5-h storage at room temperature leads to marked metabolite changes, these changes are not consistent such as dramatic increases in BCAAs in FZ24h_RT5h samples, which could contribute to experimental bias in particular studies, such as IBD focusing on SCFAs and BCAAs. Stool samples from IBD patients may have a higher water content and a greater exposure to oxygen prior to sample preparation, which may in turn have an effect on the results. As suggested by Cardona et al.[14], if frozen fecal samples are to be transported, this should be done as quickly as possible ideally on ice as to avoid the thawing process. If samples are to be transported on ice like previously suggested, and if the process of fecal water extraction is carried out efficiently on ice, the thawing process at room temperature could be avoided. If the storage time of samples is less than 24 h, then according to the OPLS-DA models (Table S3), fecal samples are best preserved in the fridge. A previous study on SCFAs has also revealed that the best storage condition for studies pertaining to fecal fermentation was the refrigerator rather than the freezer[25].

The Impact of Storage Conditions on Fecal Water. Unlike crude fecal extracts, the PCA scores plot (Figure 4A) of fecal water extracts showed that samples stored in the freezer clustered in the center of the plot, whereas samples stored at room temperature or that had undergone freeze-thaw cycles were more widely scattered. This indicates that the metabolic composition of fecal water is relatively stable when stored in the freezer but that chemical and/or biological processes remain active at room temperature. We subsequently investigated the temperature and time effects on the fecal water composition, separately.

Fecal water composition was less affected by storage time compared to the crude fecal extracts. However, the individual-dependent and time-related metabolic effects remained evident at room temperature (Figure 4B). At 4°C and -20°C, inter-person variation became clearer, reflected by the tighter clusters of samples from the same individual. The metabolite composition of samples from donor 4 appeared to be more affected by time than samples from other donors (Figure 4B-D).

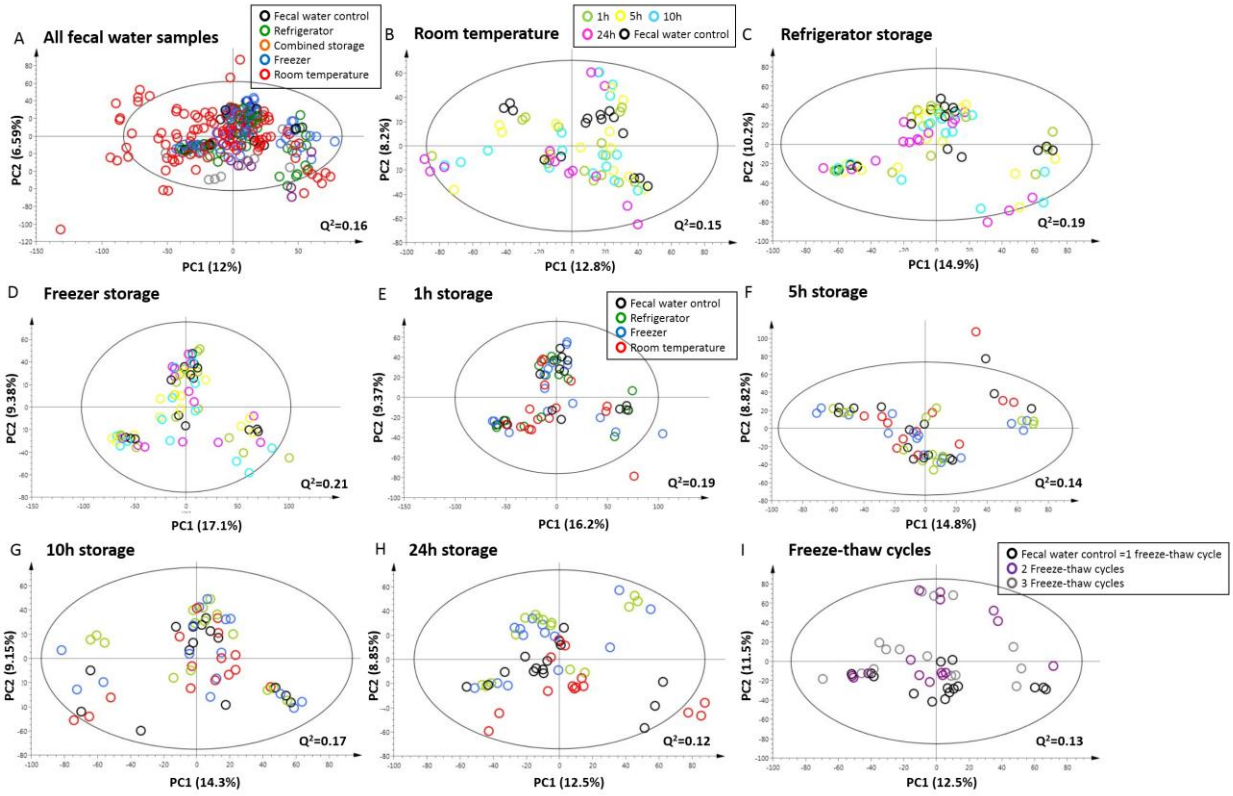

Figure 4. PCA scores plots of the mean subtracted fecal ^{1}H NMR spectral data obtained from controls and fecal water samples stored at different storage conditions (A), room temperature (B), refrigerator storage (C) and freezer storage (D) with different storage durations. PCA scores plots of the mean subtracted fecal ^{1}H NMR spectral data obtained from controls and fecal water samples stored at room temperature (RT), refrigerator (FG) and -20°C (FZ) for 1 h (E), 5 h (F), 10 h (G) and 24 h (H). The PCA scores plots of fecal water subjected to 1-3 freeze-thaw cycles (I).

The pair-wise comparison between the fecal water control and samples analyzed under each storage condition was carried out using OPLS-DA and model characteristics are summarized in Table S4. It should be noted that significant metabolic changes occurred after 5-h storage at room temperature and after 24 h at 4°C. Concentrations of SCFAs (formate and propionate) and TCA cycle intermediates (2-oxoglutarate and pyruvate) increased, whereas valine and N6-acetyllysine decreased over storage time. The metabolic profiles of samples stored at -20°C were stable and no significant difference between controls and samples stored at -20°C was observed.

The effects of temperature on the metabolic composition of fecal water samples became increasingly evident over storage time as seen from the PCA scores plots (Figure 4E-H), which showed a clear and closed donor clustering at 1 h, and gradual dispersion of the clusters over time. At 24 h, it was noticeable that the control samples clustered with samples stored at -20°C and separated from the samples stored at room temperature and 4°C (Figure 4H). The centrifugation step, which removes cellular components, significantly reduced various biological and chemical processes within the sample, hence minimizing their impact on the biochemical composition of the sample.

Fecal water samples are often stored in the freezer in laboratories, and defrosted before sample analysis. Refreezing samples is required when no spare aliquot is available. Hence, we investigated the effect of freeze-thaw cycles on the metabolic profiles of fecal water samples. Statistically significant models were obtained from controls (equivalent to one freeze-thaw cycle because of the -80°C storage prior to ^{1}H NMR analysis) and two or three freeze-thawed samples (Table S4). Alanine, lysine, leucine, isoleucine and uracil were elevated after two freeze-thaw cycles and these changes persisted after the third freeze-thaw cycle in addition to the increased levels of phenylalanine and decreased N6-acetyllysine compared to the fecal water control samples. This observation implies that if portions of fecal water are to be analyzed at different moments, fecal water should be aliquoted and stored separately as to avoid unnecessary freeze-thaw cycles.

Proposed Protocol for Fecal Sample Collection and Storage. Our results showed that some fecal metabolites were more stable than others – particular attention should be paid to the amino acids and volatile compounds such as SCFAs and methanol. Based on these results, we propose a sample handling protocol for the analysis of fecal samples (Figure 5). For inpatients who provide samples in hospitals, sample collection and extraction should be performed within 1 h of stool collection. For outpatients who are asked to provide stool samples, patients should try to collect them as close in time to their doctor's appointment as possible in order to minimize the metabolic changes that occur over time. Doctors should equip patients with a fecal collection kit as it facilitates the stool collection process, prevents contamination, and minimizes unpleasant odors. Patients should collect the entirety of their stool in the fecal collection kit, and place it in the refrigerator or cold pack as soon as possible. Samples should stay refrigerated to prevent the metabolic changes that occur during thawing, and they should be transported to the clinic in a cold pack or on ice. Once the clinic has received the patient's stool, laboratory technicians should follow the guidelines presented in the protocol above for fecal water extraction on ice. This protocol is more realistic for outpatients, and should therefore be considered as a guideline for the collection, storage and transportation of fecal samples for the purpose of metabolic profiling.

An entire stool sample should be collected from patients and kept on ice throughout the extraction process. The sample should be homogenized immediately post-collection, ideally within 1 h of collection, and a relatively large amount (e.g. >15 g) should be aliquoted for fecal water extraction in order to obtain a full representation of the fecal metabolic profile of the

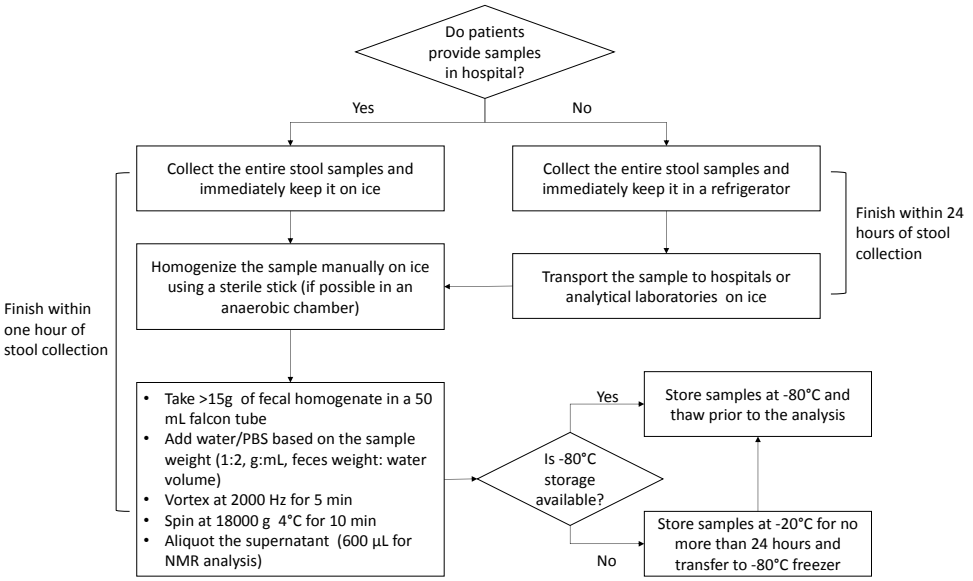

Figure 5. A proposed workflow for fecal sample collection from inpatients and outpatients and subsequent sample storage for metabolic profiling.

samples. If analysis is not immediately possible, the fecal water should be stored at -80°C. If such a freezer is not available, fecal water can also be stored in a -20°C freezer for no more than 24

h. More than one freeze-thaw cycle of fecal water is not recommended; hence aliquoting fecal water prior to freezing is essential for multiple-platform-based metabolic profiling. This also implies that fecal water samples should be transported on dry ice to the analytical laboratories. Although these steps might not be feasible for outpatients, they can be performed for inpatients at hospitals and other medical institutions.

■ CONCLUSIONS

This work provided metabolic evidence on the storage duration- and temperature-induced alterations in the biochemical composition of fecal samples, and proposes a human fecal sample collection protocol for the metabolic phenotyping of fecal water. In studies, where inter-individual differences outweigh variation that are caused by sample storage and processing, a standard protocol should also be applied to avoid any analytical biases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information Available: This section contains the methods for ^{1}H NMR analysis and spectral data processing methods. Sample labels, chemical shifts of the identified metabolites and metabolic changes due to storage conditions, together with supporting figures, are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declared no competing financial interest.

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