

# An Ambient-temperature Collection and Stabilization Device Performs Comparably to Flash-frozen Collection for Stool Metabolomics in Infants

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## Methodology

**Keywords:** metabolomics, stool, microbiome, infants, ambient temperature

**Posted Date:** October 26th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-94154/v1>

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# Abstract

**Background:** Stool metabolites provide essential insights into the function of the gut microbiome. The current gold standard for collection and storage of stool samples for metabolomics is flash-freezing at  $-80^{\circ}\text{C}$  which can be inconvenient and expensive. Ambient temperature collection of stool is more practical, however no available methodologies adequately preserve the metabolomic profile of stool. A novel sampling kit (OMNImet.GUT; DNA Genotek, Inc.) was introduced for ambient temperature collection and stabilization of feces for metabolomics; we aimed to test the performance of this kit vs. flash-freezing.

**Methods:** Stool collected from an infant's diaper was divided into two aliquots: 1) flash-frozen and 2) stored in an OMNImet.GUT tube at ambient temperature for 3-4 days. Samples from the same infant were collected at 2 different time points to assess metabolite changes over time. Subsequently, all samples underwent metabolomic analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS).

**Results:** Paired fecal samples (flash-frozen and ambient temperature) from 16 infants were collected at 2 time points ( $n=64$  samples). Similar numbers of metabolites were detected in both the frozen and ambient temperature samples (1126 in frozen, 1107 in ambient temperature, 1064 shared between sample types). Metabolite abundances were strongly correlated between collection methods (median Spearman correlation  $R_s=0.785$  across metabolites). Hierarchical clustering analysis and principal component analysis showed that samples from the same individuals at a given time point clustered closely, regardless of the collection method. Repeat samples from the same individual were compared by paired t-test, separately for the frozen and OMNImet.GUT. The number of metabolites in each biochemical class that significantly changed ( $p<0.05$ ) at timepoint 2 relative to timepoint 1 was similar in flash-frozen versus ambient temperature collection. Changes in microbiota modified metabolites over time were also consistent across both methodologies.

**Conclusion:** Ambient temperature collection and stabilization of stool in the OMNImet.GUT device yielded comparable metabolomic results to flash freezing in terms of 1) the identity and abundance of detected biochemicals 2) the distinct metabolomic profiles of subjects and 3) the biochemical signature of microbiome development over time. This method potentially provides a more convenient, less expensive home collection option for stool metabolomic analysis.

## Background

Small-molecule biochemicals, or metabolites, are critical signaling molecules that mediate host-microbiome communication and dynamics [1]. Bioactive metabolites produced by gut microbiota, such as short-chain fatty acids, aromatic amino acid metabolites, and bile acids, have been implicated in numerous human health conditions, including obesity [2], insulin resistance [3], cardiovascular disease [4], autism-spectrum disorder [5], and Parkinson's disease [6]. Due to the heterogeneity of human genetics, microbiome composition, and nutrient and other exposures, these studies have made only limited inroads

into the richness and diversity of human gut metabolites. In addition to providing fundamental insight, these molecules may offer avenues to therapeutic strategies and biomarker discovery. Consequently, attention in the field is increasingly turning to metabolomics – the discipline of measuring metabolites in a comprehensive and unbiased manner [7] – as a powerful tool to elucidate how the gut microbiome exerts its effects on human health and disease [8,9,10].

Despite this growing interest, the practical challenge of collecting fecal samples from human volunteers has constrained the application of metabolomics in gut microbiome research. In general, immediately freezing samples at -80°C has long been viewed as the optimal approach for preserving metabolites by quenching enzymatic activity, hydrolysis, oxidation, and other degradative processes ([11]. Yet, in the case of human feces, obvious considerations of donor privacy and convenience have driven a strong demand for at-home collection. However, storage and shipping of frozen at-home collected samples can be inconvenient for participants and prohibitively expensive for researchers, pushing the need for ambient-temperature storage options. To fill this need, several research groups have recently sought to repurpose currently available sampling devices, such as DNA-stabilizing tubes and fecal immunochemical test (FIT) tubes [12,13], for metabolomic analysis. However, the abundant detergents, buffers, salts, and other additives in these tubes render them incompatible with liquid chromatography and mass spectrometry (LC/MS), the technique of choice for metabolomic analysis. As a result, use of these devices significantly distorted the metabolomic profile of stool samples relative to the gold-standard flash-freezing methodology [12, 13].

Recently, OMNImet.GUT tubes (DNA Genotek, Inc.) were launched onto the market as a stool collection and stabilization kit developed specifically for metabolomics. We aimed to test the fidelity with which samples collected and stored in OMNImet.GUT tubes recapitulate the metabolomic profile of those collected by the gold-standard method, i.e., flash-freezing at -80°C. To accomplish this, we conducted a unique hospital-based field test in which matched samples (n=16 donors, each donor sampled at two timepoints) were both flash-frozen and stored in OMNImet.GUT tubes. This study design enabled us to compare the two sample types directly (within-donor comparisons) as well as to compare groups of donors within each sample type (inter-donor comparisons) to assess whether the OMNImet.GUT samples would yield the same biochemical findings as the flash-frozen samples.

## Methods

As part of a longitudinal microbiome study conducted at the Inova Health System (IRB# 15-1945), infants who were admitted in the neonatal intensive care unit (NICU) were enrolled shortly after birth after written consent from their parent and had serial stool samples collected. Stool was collected from the infant's diaper and divided into two ~ 500 mg aliquots: 1) flash-frozen, i.e., immediately stored in an Eppendorf tube without preservatives at -80 °C, and 2) stored in an OMNImet.GUT tube (DNA Genotek, Inc.) at ambient temperature for 3–4 days prior to freezing at -80 °C. Samples from the same infant were collected at 2 different time points, at a minimum of 5 days apart, to assess changes in the metabolomic

profile over time. OMNImet.GUT devices (product number ME-200) were obtained from DNA Genotek (Ottawa, ON).

To prepare feces for metabolomic analysis, frozen 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  $\mu$ L deionized water for every 1 mg of feces weight) ratio for homogenization as previously described [13]. 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 Platform, as previously described [14,15,16]. Raw data were extracted, peak-identified, and processed by Metabolon using proprietary software [17,18,19]. In brief, metabolites - 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 spectral data (including MS/MS fragmentation). Biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library  $\pm$  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. Three types of controls were included: a pool of small portions of each experimental sample serving as a technical replicate throughout the platform run; extracted water samples (process blanks); and a cocktail of standards spiked into every analyzed sample allowing instrument performance monitoring.

After identification, metabolites were quantified by peak integration. To analyze the Frozen and OMNImet.GUT sample sets separately, the data were scaled to a median of 1 for each biochemical in that sample set. Missing values, if any, were imputed with the observed minimum for that particular metabolite in that sample set. The data were natural log-transformed prior to statistical analyses including paired t-test and principal component analysis (PCA). To directly compare the Frozen and OMNImet.GUT sample sets, each set was normalized to a bridging control sample, which was prepared by pooling the extracts from the Frozen samples and was analyzed in n = 4 technical replicates in parallel with both of the experimental sets. In each set, metabolites were retained if present in at least 3 of the 4 bridging samples; for each metabolite, its raw peak areas were divided by the median of the raw peak areas across the bridging samples. After combining the two sample sets, only metabolites present in both sets were retained; for each metabolite, its missing values were imputed with its minimum value. The resulting merged dataset was natural log-transformed prior to statistical analyses including paired t-test, PCA, hierarchical clustering analysis (HCA) using complete clustering with Euclidean distance and Spearman correlation.

The datasets generated and/or analyzed during the current study are available in the Metabolights repository, (<https://www.ebi.ac.uk/metabolights/MTBLS201>).

## Results

A total of 16 infants provided paired fecal samples (flash frozen and ambient) collected at 2 different time points (mean time between samples = 23 days, range 5–44 days), for a total of 64 samples. See Table 1 for demographic and clinical data for subjects.

Table 1  
Characteristics of study cohort

<b>Variable</b>	<b>Mean (range) or Frequency % n=16</b>
<b>Gender</b>	
Male	5/16 (31%)
Female	11/16 (69%)
<b>Ethnicity</b>	
Hispanic or Latino	2/16 (13%)
Not Hispanic or Latino	5/16 (31%)
Unknown	9/16 (56%)
<b>Gestational age at birth (weeks)</b>	28.1 (24-35)
<b>Delivery mode</b>	
Cesarean Section	13/16 (81%)
Vaginal Delivery	3/16 (19%)

Similar numbers of metabolites were detected in both the frozen and ambient temperature samples, with a total of 1126 metabolites in frozen samples and 1107 in ambient temperature samples, of which 1064 were shared between the sample types, i.e. 94.5% of the metabolites detected in flash-frozen samples were also detected in the OMNI<sub>met</sub>.GUT samples (Fig. 1). These biochemicals were classified into 10 super-pathways (all shared between both sample types) and 121 sub-pathways, of which 117 were shared between both sample types. 62 metabolites from 7 super-pathways and 38 sub-pathways were only found in frozen samples; 43 metabolites from 8 super-pathways and 24 sub-pathways were only found in ambient temperature samples (Supplemental table 1). Metabolite abundances were strongly correlated between collection methods, with a median Spearman correlation  $R_s = 0.785$  across metabolites that were detected in at least 50% of samples of each type (Fig. 2, Supplemental Table 2).

PCA and HCA of the 1064 metabolites detected in both frozen and OMNI<sub>met</sub>.GUT samples showed that the samples from the same individuals at a given time point clustered closely, regardless of the collection method (Figs. 3 and 4), suggesting that the effect of OMNI<sub>met</sub>.GUT collection on the individual metabolomic profiles was small compared to the differences among individual patients. Both HCA and

PCA also showed a partial separation between samples from the two timepoints, also regardless of collection method, consistent with time-dependent metabolomic changes that occurred across multiple patients and were captured by both collection methods. To further investigate this time-dependent signature, repeat samples from the same individual were compared by paired t-test, separately for the frozen and OMNImet.GUT collections. The number of metabolites in each biochemical class that significantly changed ( $p < 0.05$ ) at timepoint 2 relative to timepoint 1 was similar in flash frozen versus ambient temperature collection (Fig. 5).

To gain better insight into how fecal sample collection and storage for the frozen and OMNImet.GUT samples impacted biological changes related to timepoint 1 and timepoint 2 in the same individual, PCA analysis was performed separately for the frozen and OMNImet.GUT dataset. PCA showed major separation of samples from the two time points on Component 1 for both frozen (Fig. 6A) and OMNImet.GUT samples (Fig. 6B), suggesting substantial changes in metabolomic profiles between the time points mediated by developmental changes in the infants. Together, these observations suggest that time point might be a prominent contributor to variation in metabolomic profile in both the frozen and OMNImet.GUT tube samples. Importantly, regardless of collection method, the frozen samples and OMNImet.GUT tube-collected samples yielded strikingly similar PCA results, suggesting that the method of fecal collection did not greatly influence the biochemical signature.

With a clear distinction of metabolomic profiles between timepoint 1 and timepoint 2, we next determined if metabolites known to be modified by the gut microbiome were contributing to the time dependent changes. Consistent with an altered gut microbiome, changes in specific aromatic amino acids metabolites, which are derived from microbial metabolism of these amino acids, were observed between timepoint 1 and timepoint 2 in both frozen and OMNImet.GUT tube samples. Altered levels of phenyllactate (PLA), tyramine, indole and 3-indoxyl sulfate between timepoint 1 and timepoint 2 were seen (Fig. 7A). Furthermore, differences in primary and secondary bile acids (which are also modified by the gut microbiota) were seen between timepoint 1 and 2 in both collection methods (Fig. 7B). Overall, these results may represent altered microbial activity between the two developmental time points, captured by both collection methods.

## Discussion

This is the first study to our knowledge to directly compare the fidelity of the new OMNImet.GUT device, designed for ambient temperature collection and stabilization of stool metabolites, with the current gold standard of flash freezing of stool. We found that metabolite analysis from the OMNImet.GUT device yielded comparable results to flash freezing in terms of 1) the identity and abundance of detected biochemicals, 2) the distinct metabolomic profiles of subjects, and 3) the biochemical signature of gut microbiome development over time.

While the optimal method for metabolic profiling of stool is likely extraction within 1 hour of collection [20], this method is out of reach in the vast majority of circumstances. It is therefore accepted that the

next best method and more practical “gold standard” is flash-freezing of stool below  $-20\text{ }^{\circ}\text{C}$  [21]. However, flash-freezing of samples is inconvenient, expensive and not practical for large epidemiological studies. A handful of prior studies have attempted to address the feasibility of ambient temperature collection and storage for metabolite analysis using different collection methods. Loftfield et al. [13] performed metabolomic analysis of samples collected into 95% ethanol, fecal occult blood test cards (FOBT) and fecal immunochemical test tubes (FIT) relative to matched flash-frozen samples from 18 healthy adult volunteers. The 95% ethanol tubes performed the best, providing detection of 89% of the metabolites detected in flash-frozen samples, while collection into either FIT or FOBT led to a significant decline in both metabolite coverage and correlation to flash-frozen samples. Another study [12] of three healthy adults investigated whether a DNA-stabilizing device intended for ambient storage of stool for metagenomic analysis (OMNIgene.GUT) would also be suitable for metabolomics. These authors found a median Spearman correlation  $< 0.5$  between flash-frozen samples and samples immediately frozen in the metagenomic analysis tubes (i.e., without room temperature storage), indicating that this collection method significantly distorted the metabolomic profile relative to flash-freezing. In addition to these low correlations, Lim et al. [12] also found that the differences among a single individual's samples across collection methods were greater than those among the three individuals' samples when the collection method was held constant, which precludes the use of these metagenomic tubes for discovery of metabolomic distinctions among individuals. Finally, Wang et al. [22] also compared 95% ethanol and OMNIgene.GUT with flash-freezing. Consistent with Lim et al., in this second study OMNIgene.GUT also performed poorly, providing detection of only 34.3% of the metabolites detected in flash-frozen samples and with a median interclass correlation coefficient (ICC) to flash-frozen of only 0.21. Surprisingly, however, in the study by Wang et al. [22], 95% ethanol also showed a median ICC of only 0.35, possibly due to practical difficulties inherent in using a “homemade” sampling methodology rather than a commercially available kit. Together, these studies demonstrated the lack of a commercially available, metabolomics-compatible methodology for ambient fecal collection and storage prior to the development of OMNImet.GUT collection device. In contrast, our study found that ambient temperature collection in the OMNImet.GUT device maintained both the identity and abundance of detected biochemicals (94.5% overlapping coverage, median Spearman  $R = 0.795$ ) and the distinct metabolomic profiles of the subjects.

Moreover, we examined the metabolic signature reflective of microbiome development over time including microbiome modified aromatic amino acid metabolites and bile acids [23,24]. We found that sample collection and storage in the OMNImet.GUT device accurately retained the changes in gut-microbial metabolites such as phenyllactate (PLA) and indole over time compared to the gold standard methodology of flash freezing.

## Limitations

Although this field testing of the OMNImet.GUT device showed highly comparable results to the gold standard of flash freezing, this is not precisely the same as home collection of stool as trained lab staff conducted the aliquoting of samples. Therefore, further validation of the OMNImet.GUT device may be needed in samples collected at home in the by lay study participants. All subjects in this study were

infants and the metabolite content of stool likely differs in older children and adults due to microbiome and diet differences. Further validation may be valuable in stool collected from adults.

## **Conclusions**

Ambient temperature collection and stabilization of stool in the OMNImet.GUT device yielded comparable metabolomic results to flash freezing in terms of 1) the identity and abundance of detected biochemicals 2) the distinct metabolomic profiles of subjects and 3) the biochemical signature of microbiome development over time. This potentially provides a more convenient and less expensive home collection option for stool metabolite analysis, with many potential uses, including in longitudinal childhood microbiome research.

## **List Of Abbreviations**

PCA: Principal Component Analysis

HCA: Hierarchical Clustering Analysis

## **Declarations**

## **Ethics approval and consent to participate.**

This study was approved by the Inova Health System's Human Research Protections Office (IRB# 15-1945). Written consent was obtained from the parents of enrolled infants.

## **Consent for publication.**

Not Applicable

## **Availability of data and materials.**

The datasets generated and/or analyzed during the current study are available in the Metabolights repository, (<https://www.ebi.ac.uk/metabolights/MTBLS201>).

## **Competing interests.**

As part of this research collaboration, authors Suchitra Hourigan and Sean Moore received discounted materials from Metabolon. Authors Sivapriya Ramamoorthy, Anne Evans, Luke Miller and Elizaveta Freinkman were employed by Metabolon at the time of analysis.

## Funding.

Research reported in this publication was supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR003015 (Moore, Hourigan) and the National Institute of Child Health and Human Development under Award Number K23HD099240 (Hourigan). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Authors' contributions.

AE, LM, SM, EF and SKH designed the study. SL, MM and AA collected clinical samples and data. LM performed laboratory work on samples. SR, and EF performed analysis of data. SR, MM, AA, EF and SKH drafted the initial manuscript and all authors edited the manuscript. All authors read and approved the final manuscript.

## Acknowledgements.

The authors thank Kristy and Roger Crombie for their generous philanthropic donation toward this project in loving memory of their daughter Anna Charlotte.

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## Figures

Metabolites	Frozen	OMNImet.GUT
<b>Total</b>	<b>1126</b>	<b>1107</b>
Named/Identified	936	919
Unnamed	190	188

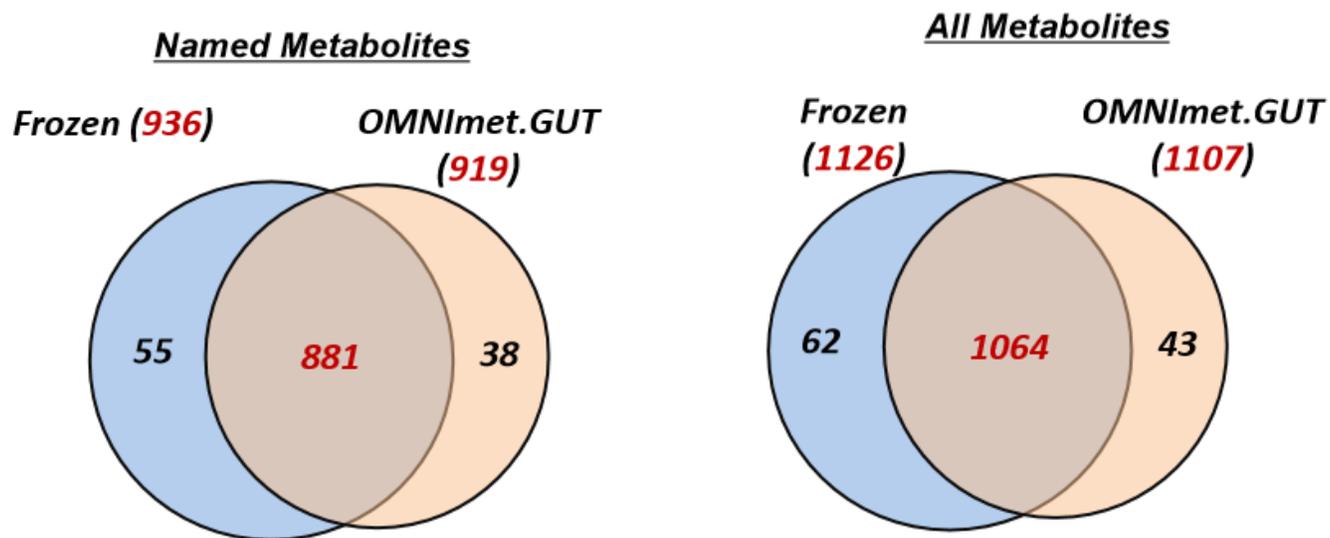
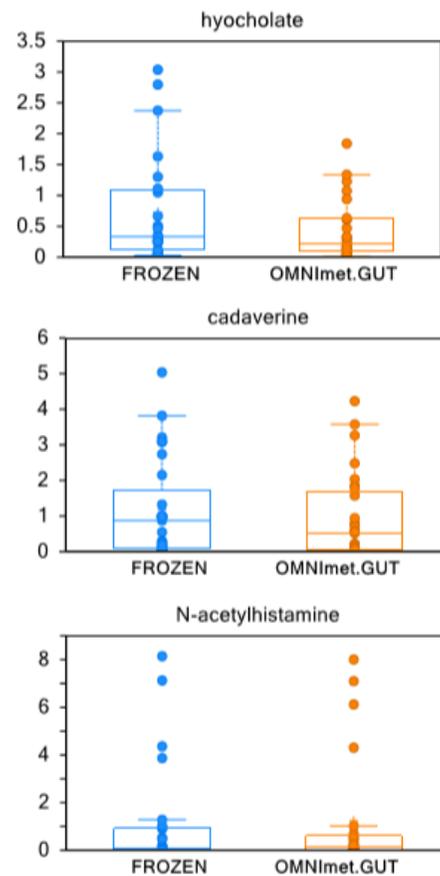
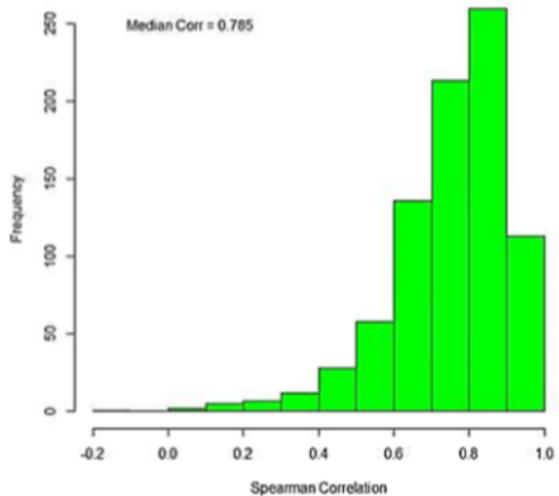


Figure 1

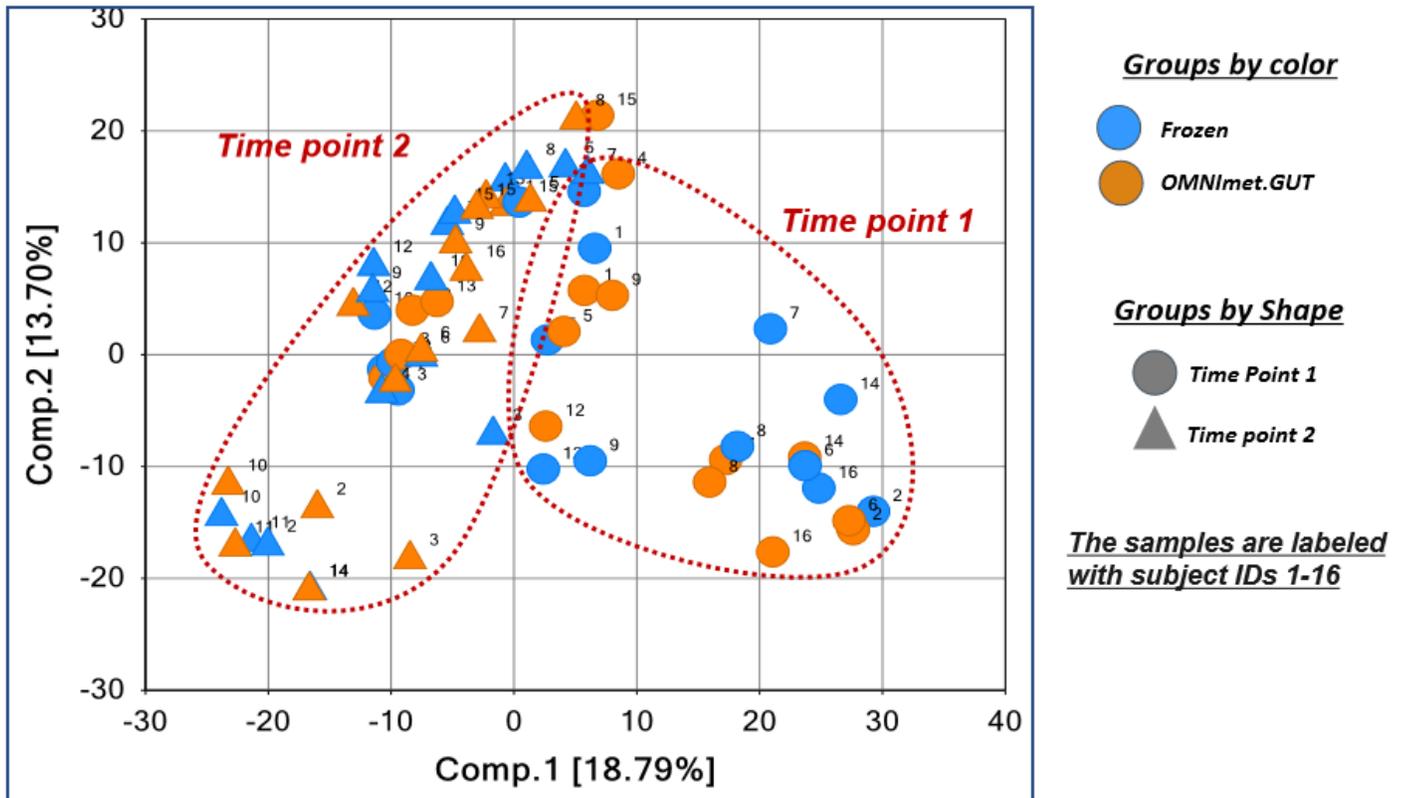
Venn Diagram Comparing all biochemicals detected in Frozen and OMNImet.GUT tube fecal samples.



COMPOUND	Correlation	P-value	Percent Fill		SUPER_PATHWAY	SUB_PATHWAY
			Frozen	OMNImet.GUT		
hyocholate	0.978	4.97E-22	75%	72%	Lipid	Secondary Bile Acid Metabolism
N-acetylhistamine	0.973	1.12E-20	81%	75%	Amino Acid	Histidine Metabolism
cadaverine	0.971	3.39E-20	84%	84%	Amino Acid	Lysine Metabolism
N-acetyl-cadaverine	0.967	2.76E-19	91%	88%	Amino Acid	Lysine Metabolism

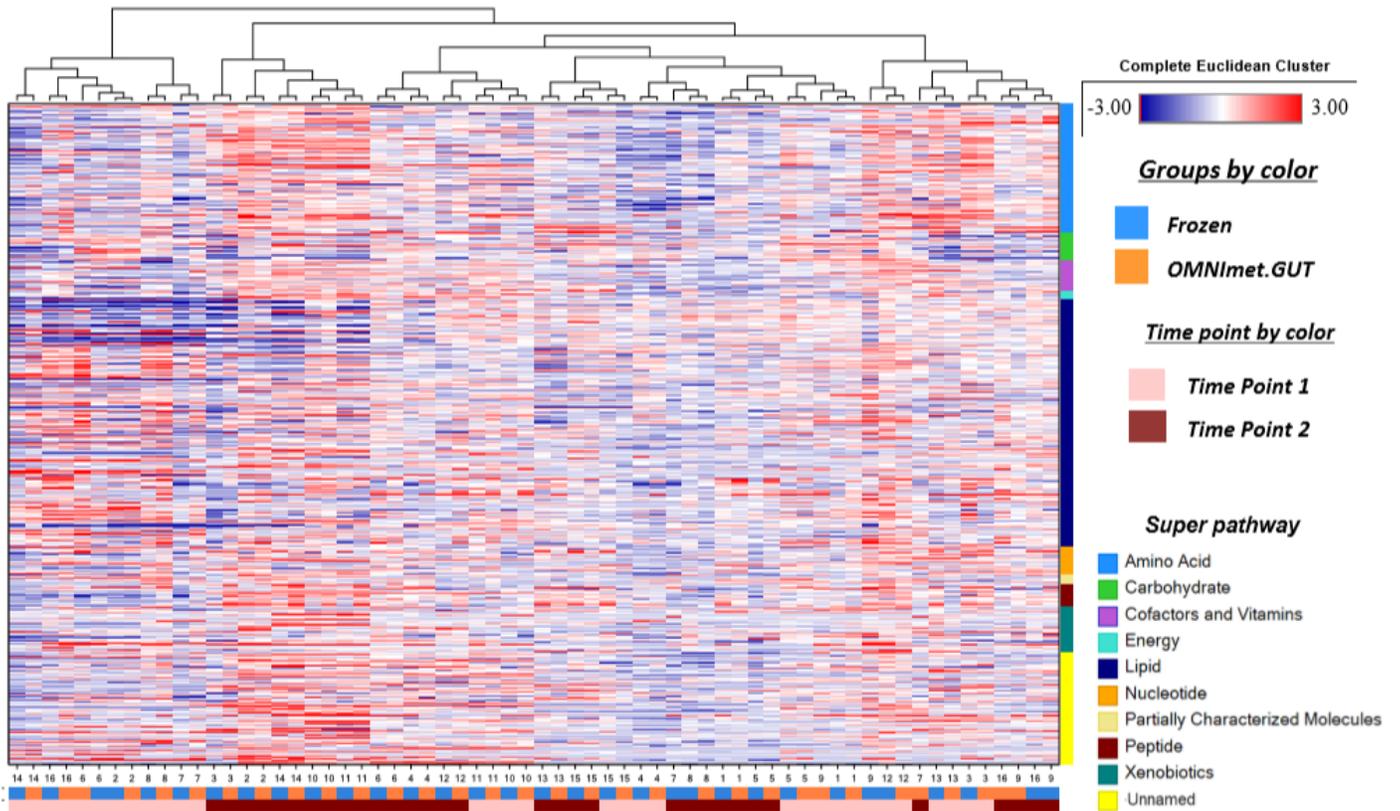
**Figure 2**

Correlation of metabolite abundance between in Frozen and OMNImet.GUT tube fecal samples. Hyocholate, N-acetylhistamine, cadaverine and N-acetyl-cadaverine were metabolites with high correlation values and were detected in more than 70% of the samples. A table including examples of the top metabolites that correlated between Frozen and OMNImet.GUT tube samples with their corresponding correlation value, p-value, percent fill, superpathway and subpathway are also shown. Representative plots for the top correlating metabolites show the level of the metabolite in Frozen and OMNImet.GUT tube samples.



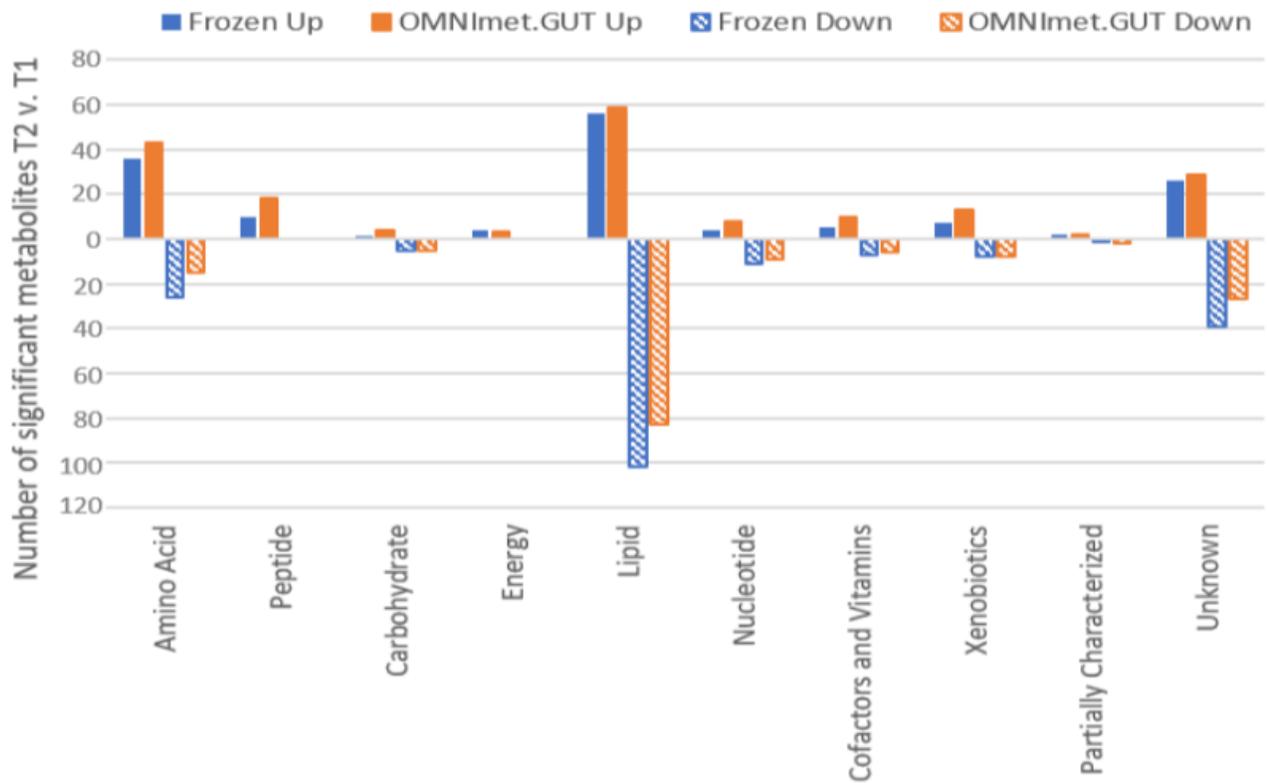
**Figure 3**

Principal Component Analysis (PCA) of Frozen vs. OMNImet.GUT fecal samples collected at 2 timepoints. The PCA was generated using the merged data including the 1064 metabolites detected in both the Frozen and OMNImet.GUT tubes.



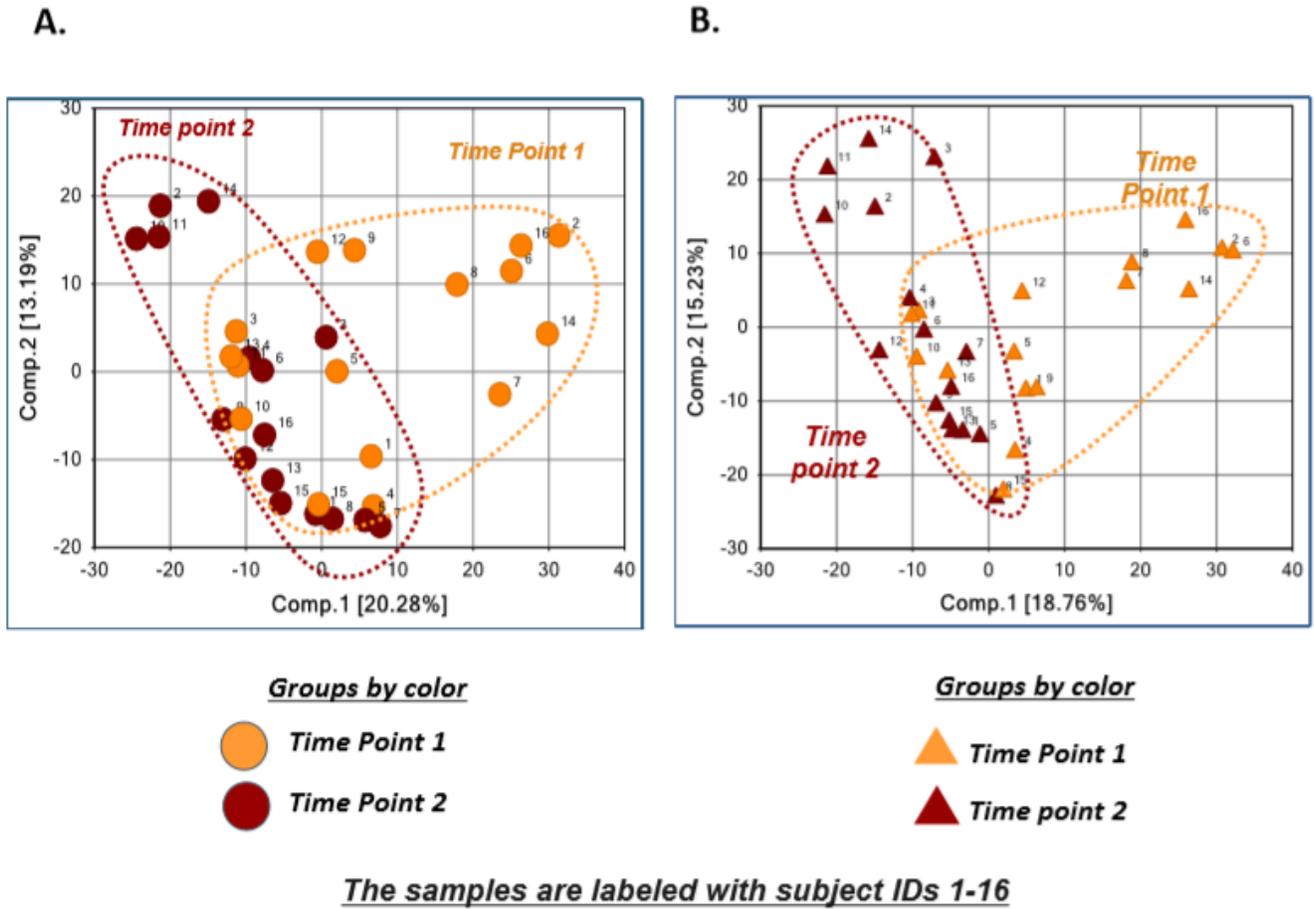
**Figure 4**

Hierarchical clustering analysis (HCA) of the relative abundance of metabolites in Frozen vs. OMNImet.GUT fecal samples collected at 2 timepoints. The HCA was generated using the merged data including the 1064 metabolites detected in both the Frozen and OMNImet.GUT tubes.



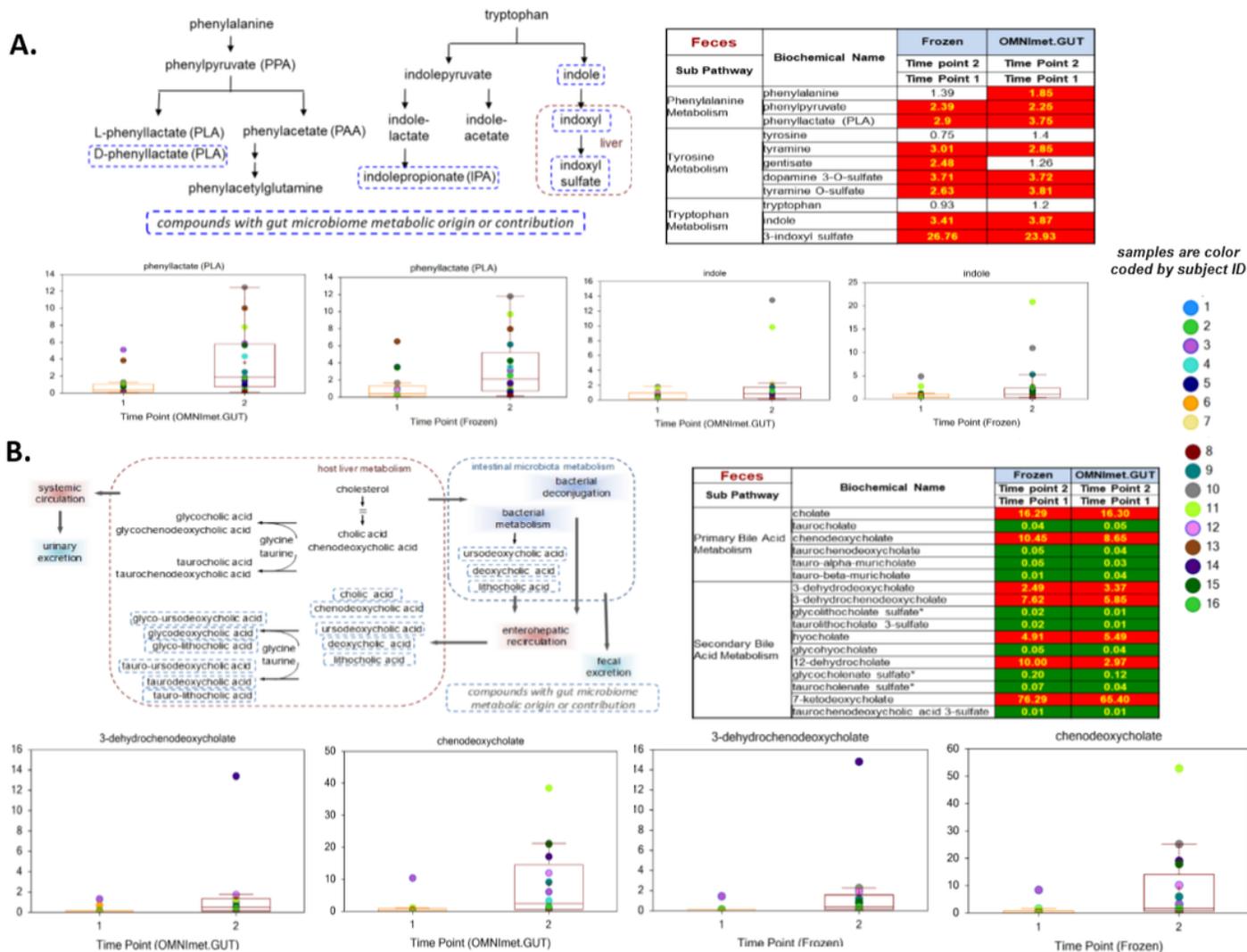
**Figure 5**

Repeat samples from the same individual were compared by paired t-test, separately for the Frozen and OMNImet.GUT collections. The number of metabolites in each biochemical class that increased (up; solid bars) or decreased (down; hatched bars) with  $p < 0.05$  at timepoint 2 relative to timepoint 1 is plotted for Frozen (blue) and OMNImet.GUT (orange) sample sets.



**Figure 6**

Principal Component Analysis (PCA) of samples collected at 2 timepoints. 6A: The PCA was generated using the 1126 metabolites detected in the Frozen samples. 6B: The PCA was generated using the 1107 metabolites detected in the OMNImet.GUT samples.



**Figure 7**

Changes in microbiome modified metabolites (7A: aromatic amino acids, 7B: primary and secondary bile acids) between time points in both Frozen and OMNImet.GUT tubes samples. Heat map representation of statically significant metabolites between Time Point 1 and Time Point 2 in frozen and OMNImet.GUT tube samples. Significant ( $p \leq 0.05$ ) increases are indicated by red, while significant reductions are represented by green.

## Supplementary Files

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- [Supplementaltable2101320.xlsx](#)