UC Davis
UC Davis Previously Published Works

Title
Multiplatform metabolomic interlaboratory study of a whole human stool candidate reference material from omnivore and vegan donors.

Permalink
https://escholarship.org/uc/item/8nj8r6r8

Journal
Metabolomics, 20(6)

Authors
Cruz, Abraham
Alves, Marina
Andresson, Thorkell
et al.

Publication Date
2024-11-04

DOI
10.1007/s11306-024-02185-0

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

HHS Public Access

Author manuscript

Metabolomics. Author manuscript; available in PMC 2025 March 13.

Published in final edited form as:

Metabolomics. ; 20(6): 125. doi:10.1007/s11306-024-02185-0.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Multiplatform metabolomic interlaboratory study of a whole human stool candidate reference material from omnivore and vegan donors

A full list of authors and affiliations appears at the end of the article.

Abstract

Introduction—Human metabolomics has made significant strides in understanding metabolic changes and their implications for human health, with promising applications in diagnostics and treatment, particularly regarding the gut microbiome. However, progress is hampered by issues with data comparability and reproducibility across studies, limiting the translation of these discoveries into practical applications.

Objectives—This study aims to evaluate the fit-for-purpose of a suite of human stool samples as potential candidate reference materials (RMs) and assess the state of the field regarding harmonizing gut metabolomics measurements.

Methods—An interlaboratory study was conducted with 18 participating institutions. The study allowed for the use of preferred analytical techniques, including liquid chromatography-mass

*Corresponding author: sandra.dasilva@nist.gov.

Author contributions

All authors contributed to the study by analyzing, comparing, and curating the data provided by the participants. All authors have read, edited, and agreed to the final manuscript.

Supplementary information

The data associated with this work is available at https://doi.org/10.18434/mds2-3104

Once the link is accessed, there will be two folders: ILS_participants and Data. ILS_participants folder contains the reporting sheet provided by each participant. Eighteen laboratory reporting sheets can be found in this folder. The files contain sample extraction methods, analytical methods, instrumentation used in the analysis, data analysis, and a list of metabolites provided by each participant, while the data folder contains the compilation result from each laboratory and per each technique, including the data analysis. The files are separated into "data combined," which is the list of all metabolites detected by a given technique, and the "top 20 annotation," which is the list of the 20 most abundant metabolites detected by a given technique. In addition, it contains a file "LCMS_GCMS_NMR – Final data combined," which contains all the metabolites observed across all three techniques. The goal of this file was to identify compounds that were detected by all three techniques.

NIST Disclaimer

All opinions expressed in this report are the authors' and do not necessarily reflect the policies and views of NIST or affiliated venues. Certain pieces of commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

Conflict of interest

PCD consulted for DSM animal Health in 2023, is an advisor and holds equity in Cybele and scientific co-founder and holds equity in Enveda, Arome and Ometa with prior approval by UC-San Diego. All other authors declared no financial or proprietary interests in any material discussed in this article.

Informed consent

Informed consent was obtained from all individual participants who donated samples to generate the candidate RM used in this study. The ILS was reviewed and approved by the U. S. National Institute of Standards and Technology Research Protections Office. This study was determined to be "not human subjects research" (often referred to as research not involving human subjects) as defined in U. S. Department of Commerce Regulations, 15 CFR 27, also known as the Common Rule (45 CFR 46, Subpart A), for the Protection of Human Subjects by the NIST Human Research Protections Office and therefore not subject to oversight by the NIST Institutional Review Board.

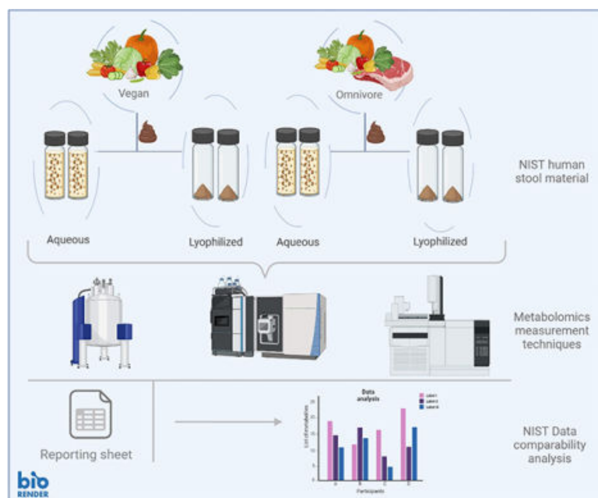
Author Manuscript Author Manuscript Author Manuscript Author Manuscript

spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR).

Results—Different laboratories used various methods and analytical platforms to identify the metabolites present in human stool RM samples. The study found a 40% to 70% recurrence in the reported top 20 most abundant metabolites across the four materials. In the full annotation list, the percentage of metabolites reported multiple times after nomenclature standardization was 36% (LC-MS), 58% (GC-MS) and 76% (NMR). Out of 9,300 unique metabolites, only 37 were reported across all three measurement techniques.

Conclusion—This collaborative exercise emphasized the broad chemical survey possible with multi-technique approaches. Community engagement is essential for the evaluation and characterization of common materials designed to facilitate comparability and ensure data quality underscoring the value of determining current practices, challenges, and progress of a field through interlaboratory studies.

Graphical Abstract



Keywords

Human stool; Fecal Matter; Gut metabolomics; Reference Materials; Multiplatform analysis; Metabolomics; Lipidomics

1 Introduction

Metabolomics is a discipline focusing on the systematic study of endogenous and exogenous small molecules that are intermediates and end products of metabolism. Metabolites are a downstream expression of the various changes that occur in the genome, transcriptome, and proteome, and are continuously influenced by external factors. As a result, the metabolome represents a phenotypic fingerprint by providing a snapshot of the biochemical status within a biological system, and is used to detect changes related to diet, environmental exposure, disease, etc. (Sun & Hu, 2016). Qualitative, semi-quantitative, and sometimes quantitative approaches are all used to obtain information about the collection of small molecules

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

found in a biological sample that can then inform about the status of a biological system. Untargeted metabolomics presents significant opportunities for chemical discovery, as it encompasses a wide spectrum of analytes (Sindelar & Patti, 2020). In metabolomics studies conducted in humans, biological samples such as blood, sweat, urine, breast milk, skin extracts, saliva, and cerebrospinal fluid have been used, each one of these biospecimens has specific metabolomes that can provide unique insight about the status of the respective organs (Bang et al., 2022; Brunmair et al., 2021; Elpa et al., 2021; Gardner et al., 2020; Kondoh et al., 2021; Langenau et al., 2020; Moreau et al., 2020; Rodríguez-Morató et al., 2018; Poulsen et al., 2022; Wawrzyniak et al., 2018).

Similarly, the investigation of the human microbiome has increasingly drawn interest due to its role in human health and disease. Specifically, the gut microbiome has been shown to have a critical impact on the modulation of human homeostasis and to have an influence on conditions such as depression, diabetes, obesity, metabolic syndrome, allergies and cancer (Barandouzi et al., 2022; Cunningham et al., 2021; De Filippis et al., 2021; Pantazi et al., 2023; Parekh et al., 2015; Radjabzadeh et al., 2022). Evaluating these associations in depth poses a set of new challenges that require probing a different type of biospecimen: human stool or fecal matter.

Fecal matter is primarily composed of water and bacterial biomass (Stephen & Cummings, 1980) but it also contains proteins, lipids (i.e., fats), and carbohydrates. This composition reflects the net result of nutrient ingestion, digestion, and absorption, offering a comprehensive sample type to understand the impact of the gut microbiome on human health. The gut microbiota is a collection of microorganisms, including bacteria, archaea, fungi, and viruses found in the gastrointestinal tract, which plays a role in digestion, immune function, disease, metabolism and hormone regulation (Neuman & Koren, 2016). Its composition is influenced by factors such as diet, use of antibiotics, inflammation status, physical activity, and body weight (Erlandson et al., 2021; Journey et al., 2020; Monda et al., 2017; Vandeputte et al., 2016; Wang et al., 2023). Therefore, fecal matter can provide valuable information about microbial activity and can be considered a non-invasive approach to studying host–microbiome interactions (Zierer et al., 2018).

The gut microbiota, which is considered the largest microbiome in the human body, is a complex and ever-changing ecosystem that plays a vital role in human health and disease, as shown in the literature. Although there have been promising advancements in using it as a diagnostic tool, there is still much to learn about this intricate system. Moreover, the field faces significant challenges in ensuring data reproducibility and comparability due to the complexity of the analytical approaches used in the analysis of stool samples. These challenges, widely acknowledged as critical bottlenecks, hinder the progression of metabolomics research, which is fundamental for understanding the intricate relationship between the gut microbiome and human host and the translation into clinical application and biotherapeutic development.

Given this context, the National Institute of Standards and Technology (NIST) is working to address these needs by developing a Human Fecal Reference Material (RM 8048), characterized for metagenomics and metabolomics, to support the gut microbiome

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

community. NIST history of developing successful reference materials in the biology space, such as the NISTmAb monoclonal antibody (De Leoz et al., 2020) and the SRM 1950 Metabolites in frozen plasma (Simón-Manso et al., 2013) sets a precedent for the potential impact of the human fecal reference material. These two examples have become a benchmark in their community by facilitating method development, validation, and setting quality control. Similarly, the human fecal reference material is expected to have the same impact in a field where the variability inherent in human gut microbiome poses significant analytical challenges. This reference material aims to serve as a critical tool for the community to achieve reliable and robust measurements. As part of this effort, NIST has conducted an interlaboratory exercise to evaluate the suitability of a candidate reference material as a standard in a diverse laboratory setting. This study had two primary goals: (1) to evaluate the fitness-for-purpose of a whole stool reference material that could support metabolomic measurement assurance and (2) to assess the state of the field with respect to data comparability given a common, complex material. To achieve these goals, the study was conducted on a suite of four stool samples prepared as lyophilized and aqueous materials, collected from donors who followed vegan and omnivorous diets. Samples were analyzed by eighteen participants from academic, industrial, and government institutions and results were reported to NIST as a list of annotated metabolites. Moreover, members of the scientific community have contributed to this effort by analyzing the candidate reference materials and publishing their findings, which further support the development and validation of these reference materials (Cumeras et al., 2023; Nam et al., 2023; Aristizabal-Henao et al., 2021; Gauglitz et al., 2022).

2 Materials and Methods

2.1 Human stool production

NIST launched a Gut Microbiome Metabolomics Interlaboratory Program in August 2020 to obtain consensus characterization of candidate human whole stool materials. The stool material was a candidate RM obtained from The BioCollective (Denver, CO, USA) through volunteer donors. Stool samples were collected from eight donors, including two vegan females, two vegan males, two omnivore females, and two omnivore males. Donors were matched by age, sex, and body mass index (BMI) and were all surveyed for health and diet to ensure they were healthy. Samples were collected after informed consent under approved Institutional Review Board (IRB) protocols at The BioCollective. After donation, the samples were deposited into a BioCollector™ device and shipped overnight on ice bricks, maintaining a temperature of approximately 4°C. Upon receipt, each sample was segmented into 30 g to 50 g portions, stored in specimen collection jars, and placed at −80°C until processing. Initial screening for pathogens including HIV, Hepatitis B, and Hepatitis C was performed using rapid diagnostic tests. During manufacturing, stool samples were homogenized, filtered, and aliquoted into aqueous and lyophilized forms. Further details are available in the NIST Internal Report 8451: Multi-omic Characterization of Human Whole Stool Research Grade Test Materials (RGTMs) (Bayless et al., 2023). Samples consisted of vegan and omnivore donors (self-identified) stored in aqueous and lyophilized conditions (i.e., vegan-aqueous, vegan-lyophilized, omnivore-aqueous, and omnivore-lyophilized) at −80°C until shipped on dry ice to participants.

Author Manuscript

2.2 Participants

Twenty-six participants enrolled in the study and only 18 participants returned data, representing a 69.2% response rate. The demographic distribution of participants included 14 from the United States, 10 from Europe and two from Canada. Regarding their affiliations, 20 were associated with academic institutions, five from governmental organizations, and one from industry.

2.3 Study design

Vegan-aqueous, vegan-lyophilized, omnivore-aqueous and omnivore-lyophilized samples were shipped in triplicate (twelve samples in total) on dry ice to the participants. A detailed breakdown of the analytical techniques used by each participant is provided in Table 1. The participants analyzed the samples and provided a report containing all annotated metabolites. In addition, participants were instructed to rank the top 20 metabolites by abundance (signal intensity, peak height, peak area and/or concentration — no instruction was applied). Participants were allowed to use the analytical technique and sample preparation protocol of their choice. This approach was chosen to reflect the diverse methodologies used across laboratories, aiming to evaluate the comparability among results without the constraints of a standardized protocol. A summary of the methods used by participants is provided in the Supplementary Information (Tables 2–4), and further details can be accessed through the DOI provided in the Supplementary Information section.

2.4 Data Analysis

The full list of annotated metabolite data provided by each participant was organized in Excel files with one spreadsheet per participant and a separate file per technique (LC-MS, GC-MS, and NMR). In a subsequent spreadsheet, all metabolites reported across participants by a given technique were combined to count the number of total metabolites. Metabolite nomenclature standardization was performed via RefMet (Fahy & Subramaniam, 2020). All lists provided by the participants were then combined and sorted in alphabetical order. Each metabolite was color-coded by each participant to identify its source. Finally, the combined list was screened to reduce duplicate metabolites to one entry per participant across diets and storage conditions. Some of the metabolites were not able to be converted by RefMet, and the original laboratory annotation was kept (Fig. 1).

The top 20 metabolites by abundance reported by each participant were analyzed following almost the same steps applied to the full list of metabolites. The list was partially combined (only across participants) but separated by diet (omnivore and vegan) and storage conditions (aqueous and lyophilized) to assess the results of each analytical method. It should be mentioned that six participants used more than one method to analyze their samples. The metabolite annotations and peak areas or peak heights, as provided by participants 4 and 6, were not listed in descending order of intensity and had to be reorganized by NIST to display the correct top 20 metabolites. In two other cases, participants 5 and 12 submitted data incorrectly or in an unusable format, and the raw publicly available data was located and processed by NIST. Participant 10 provided partially processed data with no annotations, only the m/z result, and was excluded from the initial top 20 analysis. As shown in Table 1, the ordering and reprocessing was only applied to the top 20 metabolites

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

by abundance, except for participants 5 and 12 where NIST was able to process and include the top 20 and the whole list of metabolites. Further details on the data analysis process can be accessed through the DOI provided in the Supplementary Information.

3. Results

3.1 LC-MS

Fourteen participants provided LC-MS data (full annotated list and top 20 most abundant annotated metabolites). A compilation of the experimental approaches is described in Supplementary Table 1, which reports the ionization mode, reported annotation, and the type of analysis (targeted or untargeted) performed. Most participants used both positive and negative ionization, two participants used only positive mode and two did not report which ionization mode was used. In addition, most participants did not report the type of analysis performed on the samples (targeted or untargeted). Participants largely classified the annotated analytes in the broad term of *metabolites*. Several categorized identifications regarding specific chemical classes, such as vitamins, bile acids, lipids, amino acids, short-chain fatty acids (SCFAs) and per- and polyfluoroalkyl substances (PFAS).

Details about the experimental conditions such as chromatographic columns, solvents, temperature, and the general analytical workflow are available in the Supplementary Table 2. Participants used different solvent combinations to extract analytes from samples, including acetonitrile, water, methanol, sulfadimethoxine, isopropanol, chloroform, ethanol, methyl tert-butyl ether (MTBE), 1-cyclohexyl ureido dodecanoic acid (CUDA), and 1-phenylureido 3-hexanoic acid (PUHA). Chromatographic separation of extracts covered multiple strategies; eleven participants used C18 columns, three used amide columns, two used hydrophilic interaction liquid chromatography (HILIC) columns and the remaining three participants used amino, phenyl and C8 columns. Mobile phases were acetonitrile, water, methanol and isopropanol and were used with modifiers. The modifiers included acetic acid, formic acid, ammonium formate, ammonium hydroxide, and ammonium acetate at varying volume fractions. Chromatography temperatures ranged from 20 to 60°C, and flow rates were between 250 and 500 μL/min.

In the case of the top 20 most abundant metabolites, several participants provided data for more than one LC-MS method (i.e., varying columns, extraction methods and solvents), resulting in 25 total datasets. Initially, 14,448 metabolites were reported by LC-MS. Upon RefMet standardization, the number decreased to 8,510 unique metabolites. A comparison of all reported metabolites was performed to assess the percentage of participants observing the same metabolites independent of the experimental approach. In a separate analysis of the data, a given metabolite reported multiple times by the same participant was reduced to a single report. This was noticed for participants who utilized different experimental approaches (e.g., different LC column/gradient systems) or analytical techniques to analyze the stool samples. As a result of this analysis, only 36% of metabolites were reported more than once (Fig. 2). The list of metabolites is in the Supplementary Information. For instance, tyrosine, isoleucine, hypoxanthine and deoxycholic acid were reported by 12 participants (Supplementary Fig. 1).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

For the top 20 most abundant metabolites, 25 datasets from 14 participants resulted in 499 metabolites per each diet and storage condition. A few participants used more than one analytical technique to analyze the samples. Thus, there were 399 unique metabolites reported for vegan-lyophilized stool, 396 metabolites reported for vegan-aqueous stool, and 408 metabolites each for omnivore-lyophilized and omnivore-aqueous stools. Before RefMet nomenclature standardization, an average of 38.9% of metabolites were reported more than once, with a relative standard deviation (relSD) of 4.9%, while after normalization, this number increased to 54.4% (4.4% relSD) (Fig. 3). The list with the commonly reported metabolites for each sample is available in Supplementary Fig. 2.

3.2 GC-MS results

Six participants conducted GC-MS analysis on the supplied materials; five participants provided data and the sixth dataset was acquired from published results (Aristizabal-Henao et al., 2021). A summary of GC-MS experimental approaches is shown in Supplementary Table 3. All participants used helium as a carrier gas, and, while there were some common extraction solvents, the methods used were unique. Five participants used silylation as derivatization method, four used 2-stage trimethylsilylation with methoxylamine hydrochloride (MeOX) and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and one used N-(tert.-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA). Only one participant used solid phase microextraction (SPME). For this analytical technique, 544 metabolites were obtained after RefMet nomenclature standardization in the same manner as the LC-MS data. A comparison across participants revealed that 58% of these metabolites were reported more than once (Fig. 4). Examples of reported metabolites can be found in Supplementary Fig. 3.

Similarly, to the LC-MS data, the top 20 most abundant metabolites by GC-MS were used to assess the number of times that a metabolite was reported by participants. A total of 120 metabolites were provided, with 98 unique metabolites reported for vegan-lyophilized stool, 88 metabolites for vegan-aqueous stool, 99 metabolites for omni-lyophilized stool and 91 metabolites for omnivore-aqueous stool after nomenclature standardization using RefMet. Before standardization, an average of 38.2 % (relSD 11.9%) of metabolites were recorded more than once. The percentage increased to 59.6 % (relSD 7.3 %) after standardization (Supplementary Fig. 4).

3.3 NMR results

Five participants provided NMR data, and the experimental conditions were similarly consistent across all participants, including the software used to identify the metabolites (Supplementary Table 4). The full list of metabolites was analyzed in the same manner as the other two analytical techniques. Across the five participants (total number of participants using NMR to analyze the stool samples), 246 metabolites were generated after RefMet normalization. This number includes repeated metabolites, indicating that 77% of the metabolites were reported multiple times. Out of 246 metabolites, 60 metabolites were reported a single time, while nine metabolites were reported across all five participants (Fig. 5).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acetic acid, alanine, butyric acid, fumaric acid, isoleucine, lactic acid, propionic acid, uracil and xanthine were metabolites reported across all five participants (Supplementary Fig. 5). In the list of the top 20 most abundant metabolites, 93 metabolites were counted across all five participants, with one participant reporting only 13 metabolites. Nomenclature standardization via database was unnecessary, as only a few metabolites were named differently due to the software conventions. Only a few metabolites were reported multiple times including acetate, butyrate, propionate, and alanine. Out of 93 metabolites, about 40 metabolites were unique according to diet and storage conditions. Prior to harmonization, 70.1% (relSD 1.9 %) of the metabolites were detected by multiple participants. Following harmonization, this percentage increased to 80% (Supplementary Fig. 6).

3.4 Metabolites from all methods

A comprehensive comparison using the full metabolite list was conducted using the datasets from all participants registered in this ILS to assess metabolites commonly recorded independent of diet, storage condition or technique. The dataset consolidation included 8510 metabolites from LC-MS, 544 metabolites from GC-MS and 246 metabolites from NMR. Twenty-eight metabolites were reported across all three analytical techniques, which included mostly amino acids, dicarboxylic acids, hexoses, and saturated fatty acids (FA) (Table 2).

4 Discussion

In the rapidly evolving field of gut metabolomics, the complexity of the gut microbiome presents both opportunities and challenges in confidently correlating a metabolic signature with human health and disease. To better understand the current challenges, NIST conducted an interlaboratory study. The first goal of the study was to evaluate the fitness-for-purpose of a candidate human whole stool reference material to support metabolomic measurement assurance. The second goal was to assess the state of the field with respect to data comparability given a common, complex material. Notably, rather than prescribe specific methods, this ILS asked participants to use their preferred protocols.

Overall, the materials developed were amenable to multiple metabolomic extraction methods and analytical techniques; we received data back from all four sample types. Two primary characteristics were evaluated when considering if this material was fit-for-purpose for metabolomic measurements: (1) do the two dietary cohorts present unique profiles? and (2) what is the effect of preservation method? A reference material with two cohorts can be useful when evaluating the ability of a workflow to distinguish between distinct populations. A clear separation in gut microbiomes based on diet, has been reported by several studies, making this a useful option for obtaining two distinct materials (; Prochazkova et al., 2022; Sun et al., 2023; Xing et al., 2023). Some of the laboratory participants published reports on the identified differences between the vegan and omnivore samples presented here (Bayless et al., 2023; Cumeras et al., 2023; Gauglitz et al., 2022) supporting that the two dietary cohorts result in distinguishable microbiome samples. While the top 20 metabolite profiles reported for the two dietary cohorts were distinct on a lab-by-lab basis, more global analysis was not practical given that reported metabolite difference between materials would

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

be confounded by methodological differences and the disproportionately small number of metabolites requested for a given sample. Similarly, with respect to preservation method, data could be compared on a lab-by-lab basis, but it was difficult to distinguish reported metabolites that were solely attributable to a specific preservation method. While some literature suggests lyophilization is a better method for preserving fresh stool samples (De Spiegeleer et al., 2020), given that both preservation methods appeared suitable for analysis, NIST has decided to preserve the upcoming homogenized human fecal material in aqueous condition to represent a fresh sample more closely. This decision was also made to avoid any potential losses of volatile compounds, such as SCFAs, which could potentially occur during the lyophilization process. It is important to note that the type of reference material used in this study is designed to support comparability and reproducibility or measurement precision. This type of material does not represent ground truth, in that the actual chemical constituents have not been confirmed, and therefore is not amenable for evaluating a preservation method (or analytical method) to measure what is present in the sample.

With respect to the second goal, this study provided a comprehensive overview of the diverse methodologies in the field. All reported sample preparation protocols for each analytical approach and technique were distinct, despite some similarities in extraction solvents and general steps. This study aimed to evaluate the comparability of results across diverse analytical methods, allowing participants to use their own sample preparation protocols to reflect real-world variability. For example, by comparing different approaches, such as silylation for GC-MS and SPME (Fiehn, 2016), the study focused on identifying which metabolites were consistently detected across laboratories, providing insights into the comparability of metabolite identification. The goal was to compile a comprehensive list of metabolites and assess the consistency of detection across a variety of analytical platforms and distinct methodologies.

Methodologies are usually determined by fit-for-purpose of the specific aims of a study; for example, specific extraction methodology and analytical protocols can be developed to optimize metabolite recovery in stool for specified applications (Deda et al., 2017; Gray et al., 2022; Moosmang et al., 2019). It is unlikely that metabolomics will adopt standardized methodologies and developing a standardized protocol may stifle scientific advances; however, this inhibits data and study comparability. Rather than a prescribed method, these instances highlight where a reference material such as NIST RM 8048, would increase the transferability of application-driven methods across laboratories. Given the range of analytical tools with varying levels of throughput, coverage, and sample preparation used to address metabolomics-related hypotheses (not limited to LC-MS, GC-MS, and NMR as presented in this study), a reference material would promote some interchangeability between platforms, then comparisons can be made when results are considered in the context of the methods used. A RM would also highlight relevant strengths and weaknesses of each technique beyond the well-known strengths of greater coverage in mass spectrometry and the inherently quantitative and non-destructive nature of NMR.

To this effect, an interlaboratory study is an important community collaboration effort to gauge the state of the field. Ideally, this is a repetitive occurrence, evaluating the

same reference materials, to understand the current state and the progression of the field, identify best methodologies or reveal practices that show promise as useful techniques, and demonstrate the bottlenecks and challenges that should be addressed to advance metabolomics. This particular effort expanded the analysis of a candidate reference material through community engagement and diverse workflows. The paradigm of reference material development has shifted with the advent of omics research (Lippa et al., 2022), where standards development organizations, like NIST, cannot fully characterize a material to suit the extensive needs of the community, thus requiring collaboration from community expertise. Currently, the Reference and Test Material Working Group of the Metabolomics Quality Assurance and Quality Control (mQACC) consortium (https://www.mqacc.org/) is spearheading such an effort to more fully characterized the widely-used NIST SRM 1950 – Metabolites in Frozen Plasma. If successful, perhaps this pioneering endeavor will be considered for other highly valuable reference materials such as NIST RM 8048. In this interlaboratory exercise, we showcased broad chemical coverage of candidate human whole stool reference materials using multiple methods and multiple platforms.

In addition to providing a survey of materials and current methodologies, this study also revealed some challenges posed by data reporting. Significant effort was required to standardize the nomenclature and enable an accurate representation of the frequency distinct metabolites was reported. One source of the lack of comparability was attributable to the various metabolite identification databases used, a challenge well known to the community. Collecting full annotation lists for analysis by individual participants improved the detection of similar metabolites across labs. Preceding the guidance on metabolite naming conventions is the important step of metabolite identity authentication. Metabolite reporting should, at a minimum, include the Metabolomics Standards Initiative (MSI) level (Sumner et al., 2007) at which the metabolite was annotated. Even for qualitative studies, verifying the identification of a metabolite using an authentic standard is increasingly recognized as important, especially when claims are made regarding the biological significance of said compound. While this would be ideal; it is not financially realistic to obtain and run standards for all putative annotations in a metabolic phenotyping, comprehensive study. Therefore, materials like NIST RM 8048, which include a list of confident annotations, can provide the community with greater assurance in their results, especially as the community continues to build on and report the metabolic composition of this and similar materials. The community is aware of the urgent need for updated guidelines for metabolite identification and nomenclature standardization and efforts are currently underway (Alseekh et al., 2021; Köfeler et al., 2021; McDonald et al., 2022; Salek et al., 2013; Wilson et al., 2021).

Across various scientific disciplines, but specifically in the microbiome field, metadata, and data reporting standards are an active area of data harmonization. Notably for metabolomics, minimum reporting standards have been proposed by the MSI (Fiehn et al., 2007; Sumner et al., 2007), mQACC (Kirwan et al., 2022), Non-targeted Analysis (NTA) community (Peter et al., 2021), toxicologists (Viant et al., 2019) and others. Reporting standards adoption will ultimately facilitate data comparability including the use and reuse of data submitted to repositories. In this study, many laboratories reported an untargeted analysis, but upon further investigation, it was revealed that searches were conducted within a specific mass range or for particular classes of compounds. If this information had been reported, the data

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

could have been less ambiguous and could have simplified and expedited the alignment of results for meaningful comparisons. Findings from this study will help inform future interlaboratory study design including a more complete standard reporting format using guidelines available to the community, requests for full annotation lists rather than a subset, nomenclature reporting guidelines or protocols for standardization, and clearly defined benchmarks.

5. Conclusions

Different laboratories employed various methods and analytical platforms to identify the metabolites present in human stool RM samples. This collaborative venture created a more defined chemical fingerprint for each specimen than any single method alone. Community engagement in reference material characterization is crucial. Continuous reference material use (and reporting of) and data evaluation can reveal progress or change as a field evolves, can aid in defining best practices, or uncover challenges like the difficulties in harmonizing datasets due to metabolite nomenclature discrepancies that occurred here.

This study highlights the complexity associated with metabolomic measurements and provides an opportunity to reflect on the challenges and insights that arise from this type of measurement. It also serves as a prelude to establishing communication between NIST, the gut microbiome, and metabolomics communities to come up with strategies to improve data reproducibility and comparability through the development of standards. The results of this study have been used to inform the design of two microbiome materials. RM 8048 Human Fecal Material, a reference material similar to the material used for this study, is aimed at improving data reproducibility in metagenomics and metabolomics research. Measurements will include metagenomics sequences with relative abundances and highly confident metabolite annotations with associated reference datasets. This material will be comprised of a set of samples derived from two cohorts, vegetarian and omnivore, and delivered as an aqueous slurry stored at −80°C. In addition, the need for instrument harmonization led to the development of RGTM 10212 Fecal Metabolite Mixture which is intended to provide ground truth measures of instrument performance both in system suitability and across-batch/lab precision with known quantitative values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Authors

Abraham Kuri Cruz[1], Marina Amaral Alves[2,3], Thorkell Andresson[4], Amanda L. Bayless[5], Kent J. Bloodsworth[6], John A. Bowden[7], Kevin Bullock[8], Meagan C. Burnet[6], Fausto Carnevale Neto[9], Angelina Choy[8], Clary B. Clish[8], Sneha P. Couvillion[6], Raquel Cumeras[10,11], Lucas Daily[8], Guido Dallmann[12], W. Clay Davis[5], Amy A. Deik[8], Alex M. Dickens[2,13], Danijel Djukovic[9], Pieter C. Dorrestein[14], Josie G. Eder[6], Oliver Fiehn[10], Roberto Flores[15], Helen Gika[16,17], Kehau A. Hagiwara[5], Tuan Hai Pham[12], James J. Harynuk[18], Juan, J. Aristizabal-Henao[7,25], David

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

W. Hoyt[6], Focant Jean-François[19], Matilda Kråkström[2], Amit Kumar[4], Jennifer E. Kyle[6], Santosh Lamichhane[2], Yuan Li[20], Seo Lin Nam[18], Rupasri Mandal[21], A. Paulina de la Mata[18], Michael J. Meehan[14], Thomas Meikopoulos[16,17], Thomas O. Metz[6], Thomai Mouskeftara[16,17], Nathalie Munoz[6], G. A. Nagana Gowda[9], Matej Orešic[2,22], Morgan Panitchpakdi[14], Stefanuto Pierre-Hugues[19], Daniel Raftery[9], Blake Rushing[20], Tracey Schock[5], Harold Seifried[4], Stephanie Servetas[23], Tong Shen[10], Susan Sumner[20], Kieran S. Tarazona Carrillo[18], Dejong Thibaut[19], Jesse B. Trejo[6], Lieven Van Meulebroek[24], Lynn Vanhaecke[24], Christina Virgiliou[16,17], Kelly C. Weldon[14], David S. Wishart[21], Lu Zhang[21], Jiamin Zheng[21], Sandra Da Silva[23,*]

Affiliations

[1]Chemical Sciences Division, National Institute of Standards and Technology (NIST), 100 Bureau Dr. Gaithersburg, MD, 20899 United States.

[2]Turku Bioscience Centre, University of Turku and Åbo Akademi University, Tykistökatu 6, 20520 Turku, Finland.

[3]Walter Mors Institute of Research on Natural Products, Federal University of Rio de Janeiro, 21941-599 Rio de Janeiro, Brazil.

[4]Division of Cancer Protection, National Institutes of Health, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892 United States.

[5]Chemical Sciences Division, National Institute of Standards and Technology (NIST), 331 Fort Johnson Rd. Charleston, SC, 29412 United States.

[6]Pacific Northwest National Laboratory, 902 Battelle Blvd, Richland, 99354 WA, United States.

[7]University of Florida, Gainesville, FL, 32611 United States.

[8]Broad Institute of MIT and Harvard, Merkin Building, 415 Main St. Cambridge, MA 02142 United States.

[9]Northwest Metabolomics Research Center, University of Washington, Seattle, Gerberding Hall G80 Box 351202 Seattle, WA 98195, United States.

[10]West Coast Metabolomics Center, University of California Davis, One Shields Ave, Davis, CA 95616, United States.

[11]Institut d'Investigació Sanitària Pere Virgili (IISPV), CERCA, 43204 Reus, Spain.

[12]Biocrates Life Sciences AG, Eduard-Bodem-Gasse 8, 6020 Innsbruck, Austria.

[13]Department of Chemistry, University of Turku, 20014 Turku, Finland.

[14]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, 9500 Gilman Dr, La Jolla, 92093 CA, United States.

[15]Division of Program Coordination, Planning and Strategic Initiatives, Office of Nutrition Research, Office of the Director, National Institutes of Health (NIH), 9000 Rockville Pike, Bethesda, MD 20892 United States.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[16]Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece.

[17]Biomic AUTh, Center for Interdisciplinary Research and Innovation (CIRI-AUTH). Balkan Center B1.4, 10th km Thessaloniki-Thermi Rd, 57001 Thessaloniki, Greece.

[18]Department of Chemistry, University of Alberta, Edmonton, AB T6G 2G2, Canada.

[19]Organic and Biological Analytical Chemistry Group, MolSys Research Unit, University of Liège, Pl. du Vingt Août 7, 4000 Liège, Belgium.

[20]UNC Chapel Hill's Nutrition Research Institute, 500 Laureate Way, Kannapolis, NC 28081, United States.

[21]Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2G2, Canada.

[22]School of Medical Sciences, Faculty of Medicine and Health, Örebro University, 70281 Örebro, Sweden.

[23]Biosystems and Biomaterials Division, National Institute of Standards and Technology (NIST), 100 Bureau Dr. Gaithersburg, MD, 20899, United States.

[24]Laboratory of Integrative Metabolomics, Department of Translational Physiology, Infectiology and Public Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium.

[25]BPGbio Inc., 500 Old Connecticut Path, Framingham, MA, 01701, United States.

Acknowledgments

The authors thank The BioCollective for the collection of frozen pooled human whole stool samples, the Biosystems and Biomaterials and Chemical Sciences Divisions at NIST for conducting an ILS and all the institutions that participated in this study.

The authors also thank IAFNS. This work was supported partially by the Institute for the Advancement of Food and Nutrition Sciences (IAFNS) (through an ILSI North America Gut Microbiome Committee grant). IAFNS is a nonprofit science organization that pools funding from industry and advances science through in-kind and financial contributions from private and public sector members. *IAFNS had no role in the design, analysis, interpretation, or presentation of the data and results.*

Metabolomics and lipidomics measurements at PNNL were performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy Office of Biological and Environmental Research and located on the campus of PNNL in Richland, Washington. PNNL is a multiprogram national laboratory operated by Battelle for the DOE under Contract DE-AC05-76RLO 1830.

Funding

P.C.D. would like to acknowledge support from NIH U19 AG063744, and support for the collaborative microbial metabolite center to P.C.D (1U24DK133658). Mass spectrometry-based metabolomics and lipidomics data generated at PNNL were supported by National Center for Complementary and Integrative Health grant R01 AT010271 (T.O.M.) and National Institute of Environmental Health Sciences grant U2CES030170 (T.O.M.).

K.T., S.L.N., A.P.d.l.M., and J.J.H. would like to thank MITACS, DNA Genotek, Inc., and The Natural Sciences and Engineering Research Council of Canada (NSERC) for support. The support of The Canada Foundation for Innovation (CFI), Genome Canada, and Genome Alberta to The Metabolomics Innovation Center (TMIC) is also acknowledged.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Metabolomics assays performed at the University of Alberta and The Metabolomics Innovation Centre (TMIC) were funded, in part, by Genome Canada and the Canada Foundation for Innovation.

R.C. acknowledges that "This project received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. (798038)".

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

References

Alseekh S, Aharoni A, Brotman Y, Contrepois K, D'Auria J, Ewald J, Ewald CJ, Fraser PD, Giavalisco P, Hall RD, Heinemann M, Link H, Luo J, Neumann S, Nielsen J, Perez de Souza L, Saito K, Sauer U, Schroeder FC, … Fernie AR (2021). Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. Nature Methods (Vol. 18, Issue 7, pp. 747–756). Nature Research. 10.1038/s41592-021-01197-1 [PubMed: 34239102]

Aristizabal-Henao JJ, Lemas DJ, Griffin EK, Costa KA, Camacho C, & Bowden JA (2021). Metabolomic Profiling of Biological Reference Materials using a Multiplatform High-Resolution Mass Spectrometric Approach. Journal of the American Society for Mass Spectrometry, 32(9), 2481–2489. 10.1021/jasms.1c00194 [PubMed: 34388338]

Bang G, Park JH, Park C, Kim K. joong, Kim JK, Lee SY, Kim JY, & Park YH (2022). High-resolution metabolomics-based biomarker discovery using exhaled breath condensate from patients with lung cancer. Journal of Analytical Science and Technology, 13(1). 10.1186/s40543-022-00347-0

Barandouzi ZA, Lee J, del Carmen Rosas M, Chen J, Henderson WA, Starkweather AR, & Cong XS (2022). Associations of neurotransmitters and the gut microbiome with emotional distress in mixed type of irritable bowel syndrome. Scientific Reports, 12(1). 10.1038/s41598-022-05756-0

Bayless A, Da Silva S, Davis WC, Kuri Cruz A, Piotrowski P, Schock T, & Servetas S (2023). Multi'omic Characterization of Human Whole Stool RGTMs. NIST Internal Report (IR) 8451. 10.6028/NIST.IR.8451

Brunmair J, Gotsmy M, Niederstaetter L, Neuditschko B, Bileck A, Slany A, Feuerstein ML, Langbauer C, Janker L, Zanghellini J, Meier-Menches SM, & Gerner C (2021). Finger sweat analysis enables short interval metabolic biomonitoring in humans. Nature Communications, 12(1). 10.1038/s41467-021-26245-4

Cumeras R, Shen T, Valdiviez L, Tippins Z, Haffner BD, & Fiehn O (2023). Differences in the Stool Metabolome between Vegans and Omnivores: Analyzing the NIST Stool Reference Material. Metabolites, 13(8). 10.3390/metabo13080921

Cunningham AL, Stephens JW, & Harris DA (2021). Gut microbiota influence in type 2 diabetes mellitus (T2DM). Gut Pathogens (Vol. 13, Issue 1). BioMed Central Ltd. 10.1186/s13099-021-00446-0

De Filippis F, Paparo L, Nocerino R, Della Gatta G, Carucci L, Russo R, Pasolli E, Ercolini D, & Berni Canani R (2021). Specific gut microbiome signatures and the associated pro-inflamatory functions are linked to pediatric allergy and acquisition of immune tolerance. Nature Communications, 12(1). 10.1038/s41467-021-26266-z

De Leoz MLA, Duewer DL, Fung A, Liu L, Yau HK, Potter O, Staples GO, Furuki K, Frenkel R, Hu Y, Sosic Z, Zhang P, Altmann F, Grunwald-Grube C, Shao C, Zaia J, Evers W, Pengelley S, Suckau D, … Stein SE (2020). NIST interlaboratory study on glycosylation analysis of monoclonal antibodies: Comparison of results from diverse analytical methods. Molecular and Cellular Proteomics, 19(1), 11–30. 10.1074/mcp.RA119.001677 [PubMed: 31591262]

De Spiegeleer M, De Graeve M, Huysman S, Vanderbeke A, Van Meulebroek L, & Vanhaecke L (2020). Impact of storage conditions on the human stool metabolome and lipidome: Preserving the most accurate fingerprint. Analytica Chimica Acta, 1108, 79–88. 10.1016/j.aca.2020.02.046 [PubMed: 32222247]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Deda O, Chatziioannou AC, Fasoula S, Palachanis D, Raikos N, Theodoridis GA, & Gika HG (2017). Sample preparation optimization in fecal metabolic profiling. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 1047, 115–123. 10.1016/j.jchromb.2016.06.047 [PubMed: 27423778]

Elpa DP, Chiu HY, Wu SP, & Urban PL (2021). Skin Metabolomics. Trends in Endocrinology and Metabolism (Vol. 32, Issue 2, pp. 66–75). Elsevier Inc. 10.1016/j.tem.2020.11.009 [PubMed: 33353809]

Erlandson KM, Liu J, Johnson R, Dillon S, Jankowski CM, Kroehl M, Robertson CE, Frank DN, Tuncil Y, Higgins J, Hamaker B, & Wilson CC (2021). An exercise intervention alters stool microbiota and metabolites among older, sedentary adults. Therapeutic Advances in Infectious Disease, 8. 10.1177/20499361211027067

Fahy E, & Subramaniam S (2020). RefMet: a reference nomenclature for metabolomics. Nature Methods (Vol. 17, Issue 12, pp. 1173–1174). Nature Research. 10.1038/s41592-020-01009-y [PubMed: 33199890]

Fiehn O (2016). Metabolomics by gas chromatography-mass spectrometry: Combined targeted and untargeted profiling. Current Protocols in Molecular Biology, 2016. 10.1002/0471142727.mb3004s114

Fiehn O, Robertson D, Griffin J, vab der Werf M, Nikolau B, Morrison N, Sumner LW, Goodacre R, Hardy NW, Taylor C, Fostel J, Kristal B, Kaddurah-Daouk R, Mendes P, van Ommen B, Lindon JC, & Sansone SA (2007). The metabolomics standards initiative (MSI). Metabolomics, 3(3), 175–178. 10.1007/s11306-007-0070-6

Gardner A, Carpenter G, & So PW (2020). Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function. Metabolites (Vol. 10, Issue 2). MDPI AG. 10.3390/metabo10020047

Gauglitz JM, West KA, Bittremieux W, Williams CL, Weldon KC, Panitchpakdi M, Di Ottavio F, Aceves CM, Brown E, Sikora NC, Jarmusch AK, Martino C, Tripathi A, Meehan MJ, Dorrestein K, Shaffer JP, Coras R, Vargas F, Goldasich LDR, … Dorrestein PC (2022). Enhancing untargeted metabolomics using metadata-based source annotation. Nature Biotechnology, 40(12), 1774–1779. 10.1038/s41587-022-01368-1

Gray J, Guo B, Bearden R, & Manka J (2022). A fast, fully validated GC-MS method using a simplified pretreatment for the quantification of short and branched chain fatty acids in human stool. Journal of Mass Spectrometry, 57(4). 10.1002/jms.4817

Journey EN,K, Ortega-Santos CP, Bruening M, & Whisner CM (2020). Changes in Weight Status and the Intestinal Microbiota Among College Freshman, Aged 18 Years. Journal of Adolescent Health, 66(2), 166–171. 10.1016/j.jadohealth.2019.06.005

Kirwan JA, Gika H, Beger RD, Bearden D, Dunn WB, Goodacre R, Theodoridis G, Witting M, Yu LR, & Wilson ID (2022). Quality assurance and quality control reporting in untargeted metabolic phenotyping: mQACC recommendations for analytical quality management. Metabolomics (Vol. 18, Issue 9). Springer. 10.1007/s11306-022-01926-3

Köfeler HC, Eichmann TO, Ahrends R, Bowden JA, Danne-Rasche N, Dennis EA, Fedorova M, Griffiths WJ, Han X, Hartler J, Hol apek M, Jirásko R, Koelmel JP, Ejsing CS, Liebisch G, Ni Z, O'Donnell VB, Quehenberger O, Schwudke D, Shevchenko A, Wakelam M, Wenk MR, Wolrab D, Ekroos K (2021). Quality control requirements for the correct annotation of lipidomics data. Nature Communications 12, 4771. 10.1038/s41467-021-24984-y

Kondoh H, Kameda M, & Yanagida M (2021). Whole blood metabolomics in aging research. In International Journal of Molecular Sciences (Vol. 22, Issue 1, pp. K., 1–13). MDPI AG. 10.3390/ijms22010175

Langenau J, Oluwagbemigun K, Brachem C, Lieb W, Di Giuseppe R, Artati A, Kastenmüller G, Weinhold L, Schmid M, & Nöthlings U (2020). Blood metabolomic profiling confirms and identifies biomarkers of food intake. Metabolites, 10(11), 1–17. 10.3390/metabo10110468

Lippa KA, Aristizabal-Henao JJ, Beger RD, Bowden JA, Broeckling C, Beecher C, Clay Davis W, Dunn WB, Flores R, Goodacre R, Gouveia GJ, Harms AC, Hartung T, Jones CM, Lewis MR, Ntai I, Percy AJ, Raftery D, Schock TB, … Ubhi BK (2022). Reference materials for MS-based untargeted metabolomics and lipidomics: a review by the metabolomics quality assurance

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and quality control consortium (mQACC). Metabolomics (Vol. 18, Issue 4). Springer. 10.1007/s11306-021-01848-6

McDonald JG, Ejsing CS, Kopczynski D, Hol apek M, Aoki J, Arita M, Arita M, Baker ES, Bertrand-Michel J, Bowden JA, Brügger B, Ellis SR, Fedorova M, Griffiths WJ, Han X, Hartler J, Hoffmann N, Koelmel JP, Köfeler HC, … Ekroos K (2022). Introducing the Lipidomics Minimal Reporting Checklist. Nature Metabolism (Vol. 4, Issue 9, pp. 1086–1088). Nature Research. 10.1038/s42255-022-00628-3

Monda V, Villano I, Messina A, Valenzano A, Esposito T, Moscatelli F, Viggiano A, Cibelli G, Chieffi S, Monda M, & Messina G (2017). Exercise modifies the gut microbiota with positive health effects. Oxidative Medicine and Cellular Longevity (Vol. 2017). Hindawi Limited. 10.1155/2017/3831972

Moosmang S, Pitscheider M, Sturm S, Seger C, Tilg H, Halabalaki M, & Stuppner H (2019). Metabolomic analysis—Addressing NMR and LC-MS related problems in human feces sample preparation. Clinica Chimica Acta, 489, 169–176. 10.1016/j.cca.2017.10.029

Moreau R, Clària J, Aguilar F, Fenaille F, Lozano JJ, Junot C, Colsch B, Caraceni P, Trebicka J, Pavesi M, Alessandria C, Nevens F, Saliba F, Welzel TM, Albillos A, Gustot T, Fernández J, Moreno C, Baldassarre M, … Angeli P (2020). Blood metabolomics uncovers inflammation-associated mitochondrial dysfunction as a potential mechanism underlying ACLF. Journal of Hepatology, 72(4), 688–701. 10.1016/j.jhep.2019.11.009 [PubMed: 31778751]

Nam SL, Tarazona Carrillo K, de la Mata AP, & Harynuk JJ (2023). Untargeted Metabolomic Profiling of Aqueous and Lyophilized Pooled Human Feces from Two Diet Cohorts Using Two-Dimensional Gas Chromatography Coupled with Time-of-Flight Mass Spectrometry. Metabolites, 13(7). 10.3390/metabo13070828

Neuman H, & Koren O (2016). The Gut Microbiome. Encyclopedia of Cell Biology (Vol. 2, pp. 799–808). Elsevier Inc. 10.1016/B978-0-12-394447-4.20083-7

Pantazi AC, Mihai CM, Balasa AL, Chisnoiu T, Lupu A, Frecus CE, Mihai L, Ungureanu A, Kassim MAK, Andrusca A, Nicolae M, Cuzic V, Lupu VV, & Cambrea SC (2023). Relationship between Gut Microbiota and Allergies in Children: A Literature Review. Nutrients (Vol. 15, Issue 11). MDPI. 10.3390/nu15112529

Parekh PJ, Balart LA, & Johnson DA (2015). The influence of the gut microbiome on obesity, metabolic syndrome and gastrointestinal disease. Clinical and Translational Gastroenterology (Vol. 6, Issue 6). Nature Publishing Group. 10.1038/ctg.2015.16

Peter KT, Phillips AL, Knolhoff AM, Gardinali PR, Manzano CA, Miller KE, Pristner M, Sabourin L, Sumarah MW, Warth B, & Sobus JR (2021). Nontargeted Analysis Study Reporting Tool: A Framework to Improve Research Transparency and Reproducibility. Analytical Chemistry, 93(41), 13870–13879. 10.1021/acs.analchem.1c02621 [PubMed: 34618419]

Poulsen KO, Meng F, Lanfranchi E, Young JF, Stanton C, Ryan CA, Kelly AL, & Sundekilde UK (2022). Dynamic Changes in the Human Milk Metabolome Over 25 Weeks of Lactation. Frontiers in Nutrition, 9. 10.3389/fnut.2022.917659

Prochazkova M, Budinska E, Kuzma M, Pelantova H, Hradecky J, Heczkova M, Daskova N, Bratova M, Modos I, Videnska P, Splichalova P, Sowah SA, Kralova M, Henikova M, Selinger E, Klima K, Chalupsky K, Sedlacek R, Landberg R, … Cahova M (2022). Vegan Diet Is Associated With Favorable Effects on the Metabolic Performance of Intestinal Microbiota: A Cross-Sectional Multi-Omics Study. Frontiers in Nutrition, 8. 10.3389/fnut.2021.783302

Radjabzadeh D, Bosch JA, Uitterlinden AG, Zwinderman AH, Ikram MA, van Meurs JBJ, Luik AI, Nieuwdorp M, Lok A, van Duijn CM, Kraaij R, & Amin N (2022). Gut microbiome-wide association study of depressive symptoms. Nature Communications, 13(1). 10.1038/s41467-022-34502-3

Rodríguez-Morató J, Pozo ÓJ, & Marcos J (2018). Targeting human urinary metabolome by LC–MS/MS: a review. Bioanalysis (Vol. 10, Issue 7, pp. 489–516). Future Medicine Ltd. 10.4155/bio-2017-0285 [PubMed: 29561651]

Salek RM, Steinbeck C, Viant MR, Goodacre R, & Dunn WB (2013). The role of reporting standards for metabolite annotation and identification in metabolomic studies. GigaScience, 2(1). 10.1186/2047-217X-2-13

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Simón-Manso Y, Lowenthal MS, Kilpatrick LE, Sampson ML, Telu KH, Rudnick PA, Mallard WG, Bearden DW, Schock TB, Tchekhovskoi DV, Blonder N, Yan X, Liang Y, Zheng Y, Wallace WE, Neta P, Phinney KW, Remaley AT, & Stein SE (2013). Metabolite profiling of a NIST standard reference material for human plasma (SRM 1950): GC-MS, LC-MS, NMR, and clinical laboratory analyses, libraries, and web-based resources. Analytical Chemistry, 85(24), 11725–11731. 10.1021/ac402503m [PubMed: 24147600]

Sindelar M, & Patti GJ (2020). Chemical Discovery in the Era of Metabolomics. Journal of the American Chemical Society (Vol. 142, Issue 20, pp. 9097–9105). 10.1021/jacs.9b13198 [PubMed: 32275430]

Stephen AM, & Cummings JH (1980). The microbial contribution to human faecal mass. The Pathological Society of Great Britain and Ireland, 13(1), 45–56.

Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TWM, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, … Viant MR (2007). Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics, 3(3), 211–221. 10.1007/s11306-007-0082-2 [PubMed: 24039616]

Sun C, Li A, Xu C, Ma J, Wang H, Jiang Z, & Hou J (2023). Comparative Analysis of Fecal Microbiota in Vegetarians and Omnivores. Nutrients, 15(10). 10.3390/nu15102358

Sun YV, & Hu YJ (2016). Integrative Analysis of Multi-omics Data for Discovery and Functional Studies of Complex Human Diseases. Advances Genetics, 93, 147–190. 10.1016/bs.adgen.2015.11.004

Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, & Raes J (2016). Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut, 65(1), 57–62. 10.1136/gutjnl-2015-309618 [PubMed: 26069274]

Viant MR, Ebbels TMD, Beger RD, Ekman DR, Epps DJT, Kamp H, Leonards PEG, Loizou GD, MacRae JI, van Ravenzwaay B, Rocca-Serra P, Salek RM, Walk T, & Weber RJM (2019). Use cases, best practice and reporting standards for metabolomics in regulatory toxicology. Nature Communications, 10(1). 10.1038/s41467-019-10900-y

Wang K, Mehta RS, Ma W, Nguyen LH, Wang DD, Ghazi AR, Yan Y, Al-Shaar L, Wang Y, Hang D, Fu BC, Ogino S, Rimm EB, Hu FB, Carmody RN, Garrett WS, Sun Q, Chan AT, Huttenhower C, & Song M (2023). The gut microbiome modifies the associations of short- and long-term physical activity with body weight changes. Microbiome, 11(1). 10.1186/s40168-023-01542-w

Wawrzyniak R, Kosnowska A, Macioszek S, Bartoszewski R, & Markuszewski MJ (2018). New plasma preparation approach to enrich metabolome coverage in untargeted metabolomics: Plasma protein bound hydrophobic metabolite release with proteinase K. Scientific Reports, 8(1). 10.1038/s41598-018-27983-0

Wilson ID, Theodoridis G, & Virgiliou C (2021). A perspective on the standards describing mass spectrometry-based metabolic phenotyping (metabolomics/metabonomics) studies in publications. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences (Vol. 1164). Elsevier B.V. 10.1016/j.jchromb.2020.122515

Xing S, Shen S, Xu B, Li X, & Huan T (2023). BUDDY: molecular formula discovery via bottom-up MS/MS interrogation. Nature Methods, 20(6), 881–890. 10.1038/s41592-023-01850-x [PubMed: 37055660]

Zierer J, Jackson MA, Kastenmüller G, Mangino M, Long T, Telenti A, Mohney RP, Small KS, Bell JT, Steves CJ, Valdes AM, Spector TD, & Menni C (2018). The fecal metabolome as a functional readout of the gut microbiome. Nature Genetics, 50(6), 790–795. 10.1038/s41588-018-0135-7 [PubMed: 29808030]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

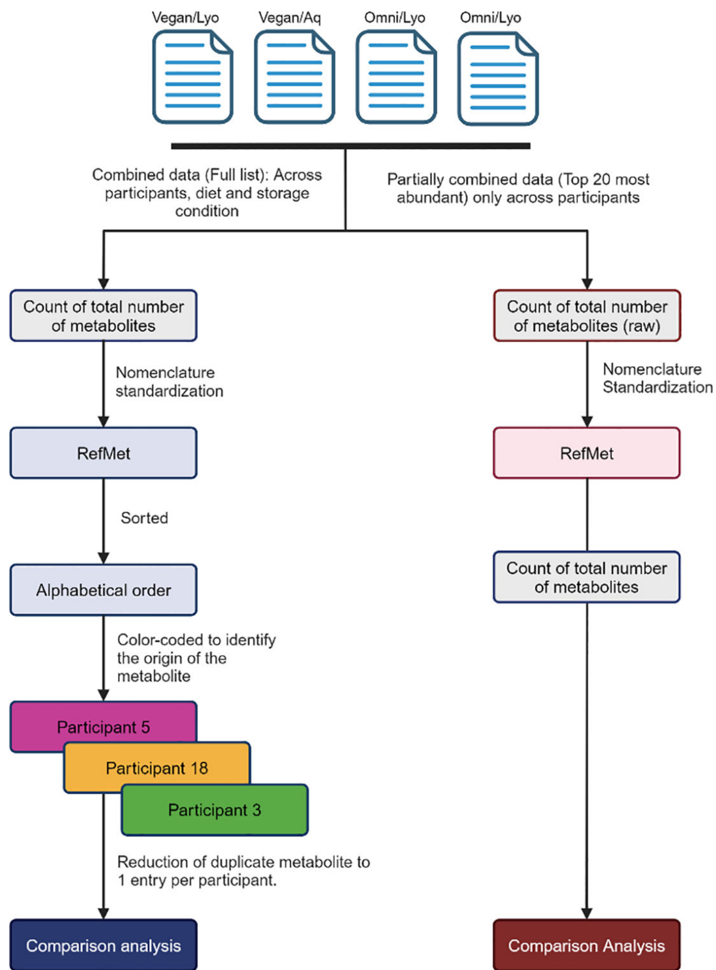


Fig. 1.
Data Analysis Workflow

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

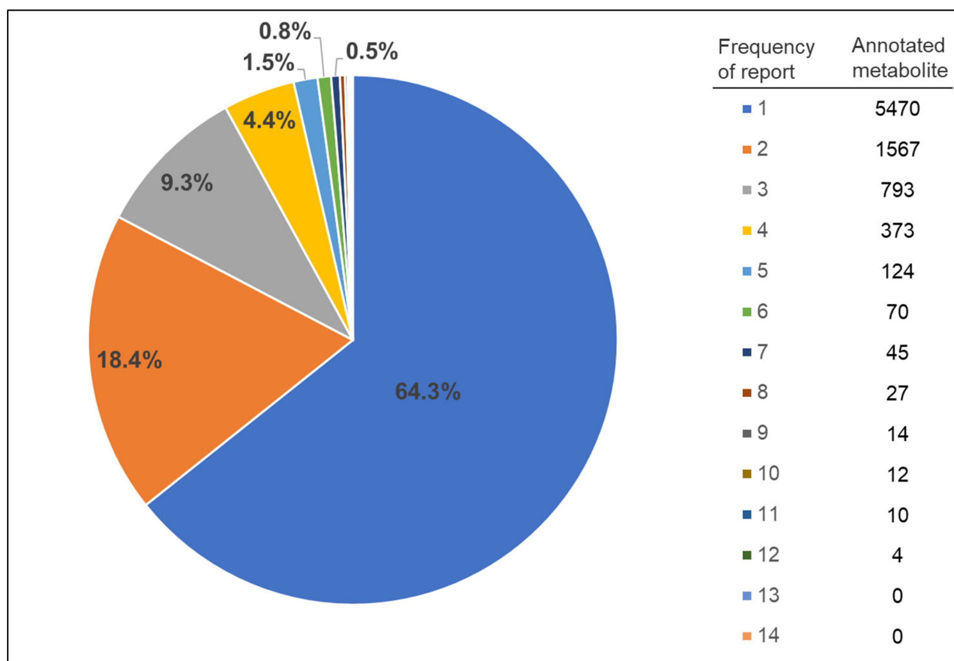


Fig. 2.

Frequency of metabolites (full list) reported by participants using LC-MS. The table denotes how often a metabolite was reported across participants. For instance, four metabolites were similarly reported by 12 participants while no common metabolites (zero) were reported across all 14 participants.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

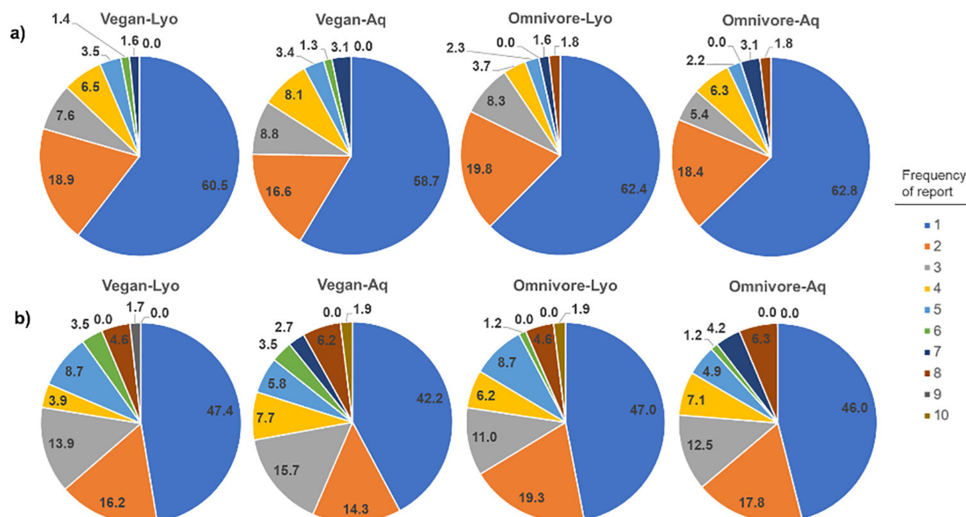


Fig. 3.

Frequency (%) of annotated metabolites reported before (A) and after (B) nomenclature standardization by RefMet. The colors indicate the frequency of metabolites reported, ranging from one to ten times. For reference, before standardization 47.4%, 42.2%, 47% and 46% of metabolites were reported one time across all samples (blue). After nomenclature standardization these values were 60.5%, 58.7%, 62.4% and 62.8% respectively, showing a higher frequency of multiple reported metabolites.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

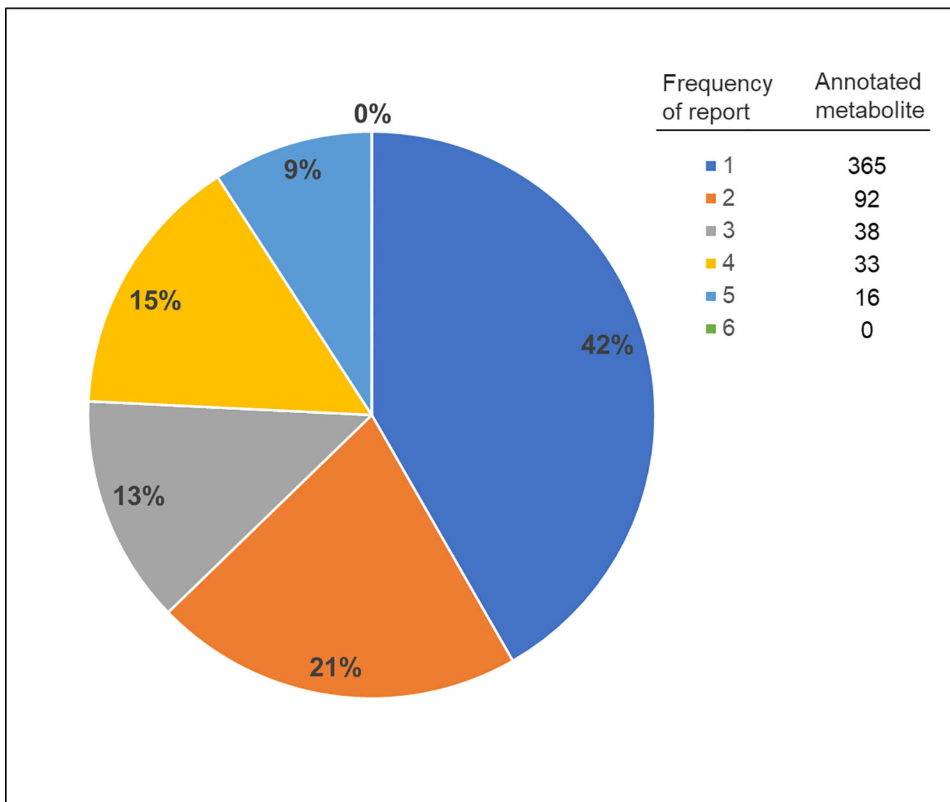


Fig. 4.
Frequency of annotated metabolites (full list, 875 total) reported by participants using GC-MS. The table denotes the number of times a metabolite was reported across participants. For instance, 16 metabolites were reported by five participants, while no single metabolite was consistently reported by all six participants. For example, 21% of metabolites (92 unique metabolites) were reported by two datasets.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

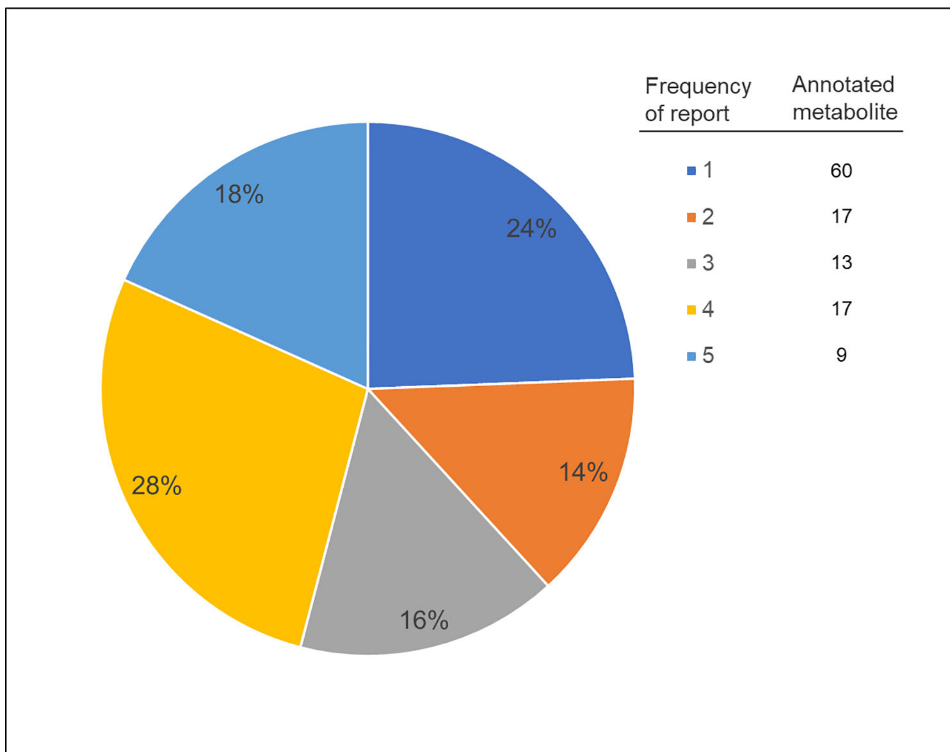


Fig. 5.

Frequency of annotated metabolites (full list, 246 total) reported by participants using NMR. The table shows how often metabolites were recorded across participants. For instance, nine unique metabolites were reported by all five participants and 16% of metabolites were reported by three datasets.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Table 1.

Summary of analytical techniques used in the evaluation of human stool materials.

Participant ID	LC-MS	GC-MS	NMR
1	x	x	
2		x	
3	x		
4	x[1]	x	x
5	x[2]	x[2]	
6	x[1]		
7	x		
8			x
9	x		
10	x[3]		
11	x		
12	x		
13	x		
14		x	
15	x		x
16	x		x
17			x
18	x	x	

[1] NIST reordered the list of the Top 20 metabolites provided by this participant by either the reported peak height or peak area. This participant did not report the data in the requested format.

[2] NIST processed the raw data provided by the participant.

[3] This participant reported only mass-to-charge ratio (m/z) values for the Top 20 metabolites. This data was excluded from the analysis.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2.

Metabolites reported across all analytical techniques, diet and storage conditions used in this study

Sub class	Reported Metabolites
1,2-diols	Glycerol
Amino acids	Alanine
Amino acids	Glutamic acid
Amino acids	Glutamine
Amino acids	Glycine
Amino acids	Isoleucine
Amino acids	Leucine
Amino acids	Methionine
Amino acids	Proline
Amino acids	Threonine
Amino acids	Tryptophan
Amino acids	Valine
Dicarboxylic acids	2-Methylglutaric acid
Dicarboxylic acids	Glutaric acid
Dicarboxylic acids	Malonic acid
Hexoses	Galactose
Hexoses	Glucose
Hexoses	Mannose
Hydroxy FA	3-Hydroxybutyric acid
Other phenols	P-Cresol
Other pyrimidines	Uracil
Pentoses	Xylose
Phenolic acids	3-Hydroxyphenylacetic acid
Pyridine alkaloids	4-Pyridoxic acid
Saturated FA	Acetic acid
Saturated FA	Butyric acid
Saturated FA	Capric acid
Saturated FA	Propionic acid
Saturated FA	Valeric acid
SCFAs	Lactic acid
SCFAs	Pyruvic acid
Short-chain keto acids	3-Methyl-2-oxovaleric acid
Sulfonic acids	Taurine
Trichloroacetic acids	Fumaric acid
Trichloroacetic acids	Succinic acid
Trioses	Dihydroxyacetone
Xanthines	Xanthine