



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

Abraham Kuri Cruz¹ · Marina Amaral Alves^{2,3} · Thorkell Andreasson⁴ · Amanda L. Bayless⁵ · Kent J. Bloodsworth⁶ · John A. Bowden⁷ · Kevin Bullock⁸ · Meagan C. Burnet⁶ · Fausto Carnevale Neto⁹ · Angelina Choy⁸ · Clary B. Clish⁸ · Sneha P. Couvillion⁶ · Raquel Cumeras^{10,11} · Lucas Dailey⁸ · Guido Dallmann¹² · W. Clay Davis⁵ · Amy A. Deik⁸ · Alex M. Dickens^{2,13} · Danijel Djukovic⁹ · Pieter C. Dorrestein¹⁴ · Josie G. Eder⁶ · Oliver Fiehn¹⁰ · Roberto Flores¹⁵ · Helen Gika^{16,17} · Kehau A. Hagiwara⁵ · Tuan Hai Pham¹² · James J. Harynuk¹⁸ · Juan J. Aristizabal-Henao^{7,25} · David W. Hoyt⁶ · Focant Jean-François¹⁹ · Matilda Kråkström² · Amit Kumar⁴ · Jennifer E. Kyle⁶ · Santosh Lamichhane² · Yuan Li²⁰ · Seo Lin Nam¹⁸ · Rupasri Mandal²¹ · A. Paulina de la Mata¹⁸ · Michael J. Meehan¹⁴ · Thomas Meikopoulos^{15,17} · Thomas O. Metz⁶ · Thomai Mouskeftara^{16,17} · Nathalie Munoz⁶ · G. A. Nagana Gowda⁹ · Matej Orešič^{2,22} · Morgan Panitchpakdi¹⁴ · Stefanuto Pierre-Hugues¹⁹ · Daniel Raftery⁹ · Blake Rushing²⁰ · Tracey Schock⁵ · Harold Seifried⁴ · Stephanie Servetas²³ · Tong Shen¹⁰ · Susan Sumner²⁰ · Kieran S. Tarazona Carrillo¹⁸ · Dejong Thibaut¹⁹ · Jesse B. Trejo⁶ · Lieven Van Meulebroek²⁴ · Lynn Vanhaecke²⁴ · Christina Virgiliou^{16,17} · Kelly C. Weldon¹⁴ · David S. Wishart²¹ · Lu Zhang²¹ · Jiamin Zheng²¹ · Sandra Da Silva²³

Received: 23 July 2024 / Accepted: 10 October 2024 / Published online: 4 November 2024

This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2024

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 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.

✉ Sandra Da Silva
sandra.dasilva@nist.gov

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

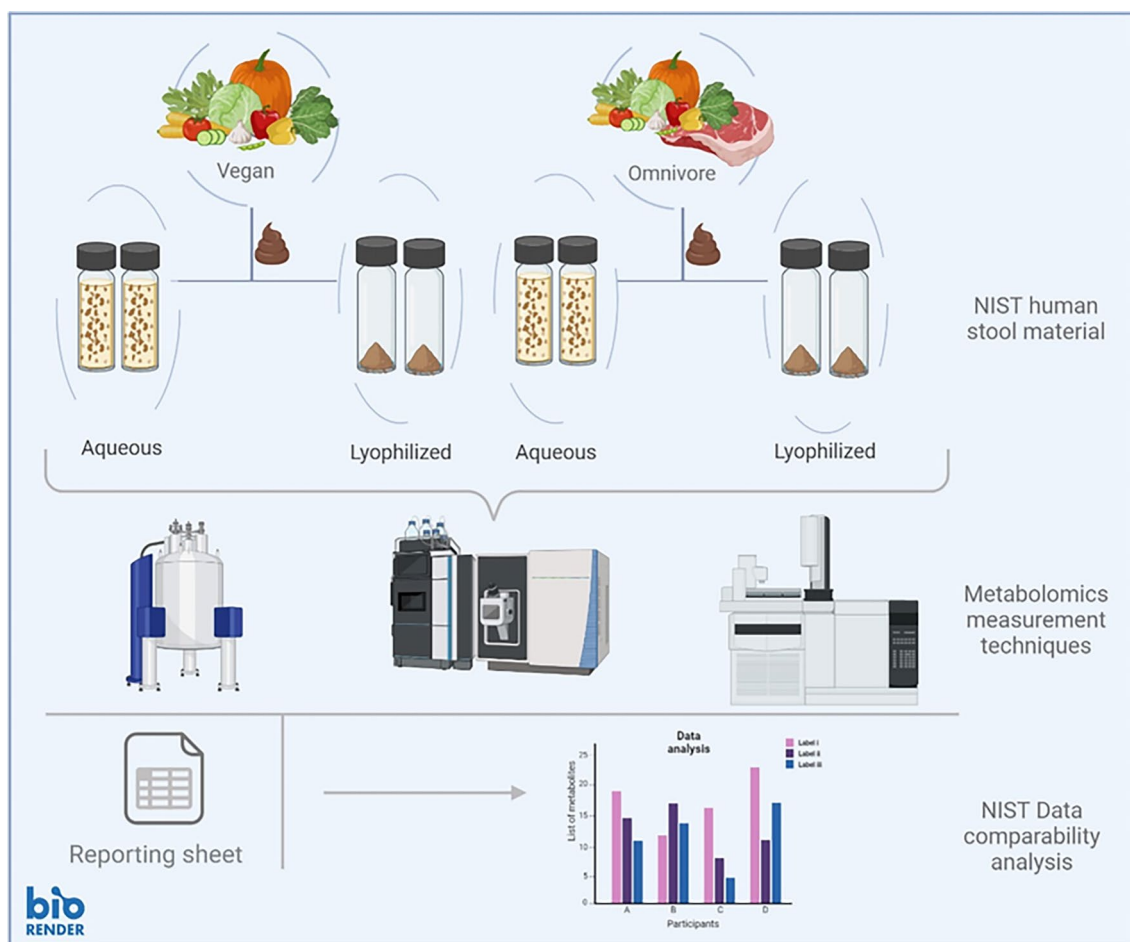
² Turku Bioscience Centre, University of Turku and Åbo Akademi University, Tykistökatu 6, 20520 Turku, Finland

³ Walter Mors Institute of Research On Natural Products, Federal University of Rio de Janeiro, Rio de Janeiro 21941-599, Brazil

⁴ Division of Cancer Protection, National Institutes of Health, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892, USA

⁵ Chemical Sciences Division, National Institute of Standards and Technology (NIST), 331 Fort Johnson Rd, Charleston, SC 29412, USA

Graphical abstract



Keywords Human stool · Fecal matter · Gut metabolomics · Reference materials · Multiplatform analysis · Metabolomics · Lipidomics

⁶ Pacific Northwest National Laboratory, 902 Battelle Blvd., Richland, WA 99354, USA

⁷ University of Florida, Gainesville, FL 32611, USA

⁸ Broad Institute of MIT and Harvard, Merkin Building, 415 Main St., Cambridge, MA 02142, USA

⁹ Northwest Metabolomics Research Center, University of Washington, Seattle, Gerberding Hall G80, Box 351202, Seattle, WA 98195, USA

¹⁰ West Coast Metabolomics Center, University of California Davis, One Shields Ave., Davis, CA 95616, USA

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, CA 92093, USA

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, USA

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

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 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; Poulsen et al., 2022; Rodríguez-Morató et al., 2018; 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 biotechnological 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 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

²⁰ UNC Chapel Hill's Nutrition Research Institute, 500 Laureate Way, Kannapolis, NC 28081, USA

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, USA

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, USA

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 (Aristizabal-Henao et al., 2021; Cumeras et al., 2023; Gauglitz et al., 2022; Nam et al., 2023).

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 30–50 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 (RGTM) (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.

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

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 ^a	x	x
5	x ^b	x ^b	
6	x ^a		
7	x		
8			x
9	x		
10	x ^c		
11	x		
12	x		
13	x		
14		x	
15	x		x
16	x		x
17			x
18	x	x	

^aNIST 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

^bNIST processed the raw data provided by the participant

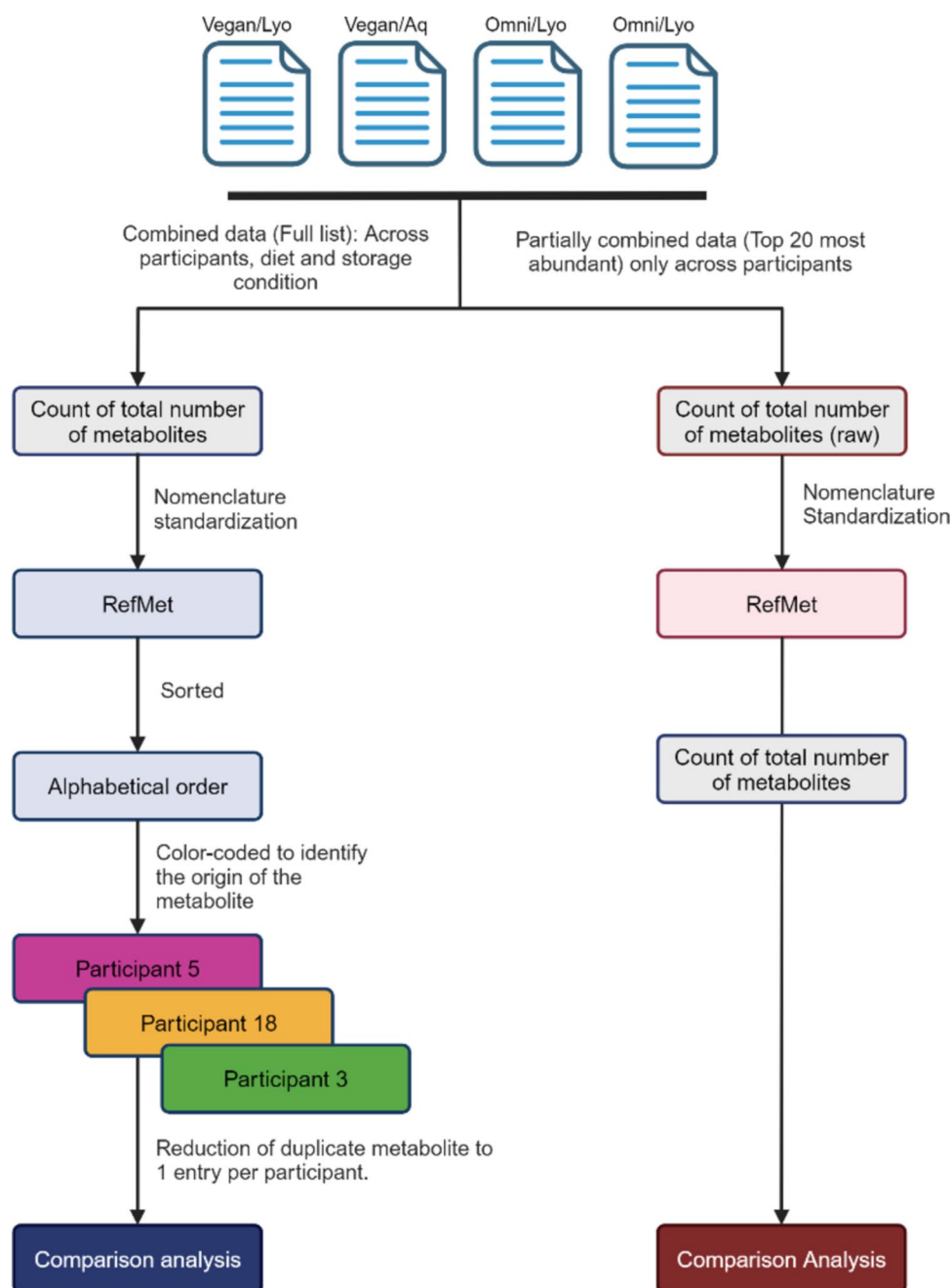
^cThis participant reported only mass-to-charge ratio (*m/z*) values for the Top 20 metabolites. This data was excluded from the analysis

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, 3, 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

Fig. 1 Data analysis workflow



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 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 $\mu\text{L}/\text{min}$.

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 8510 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).

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.

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

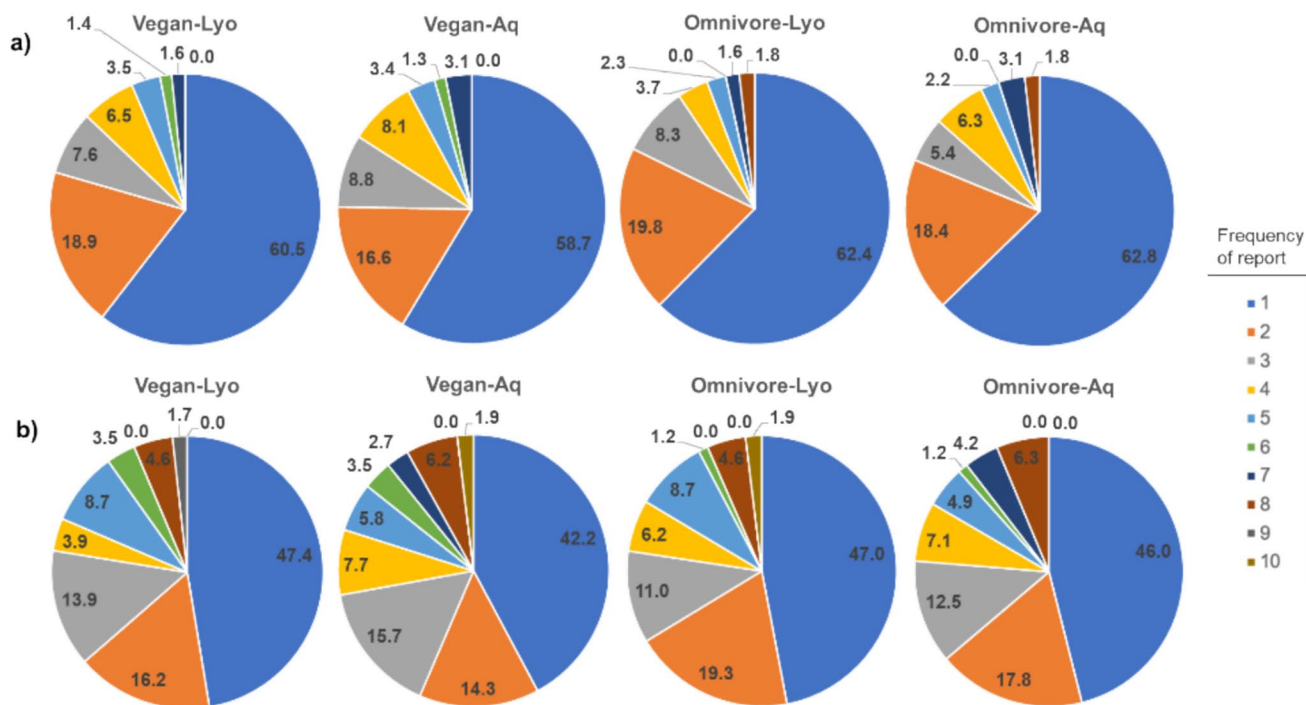
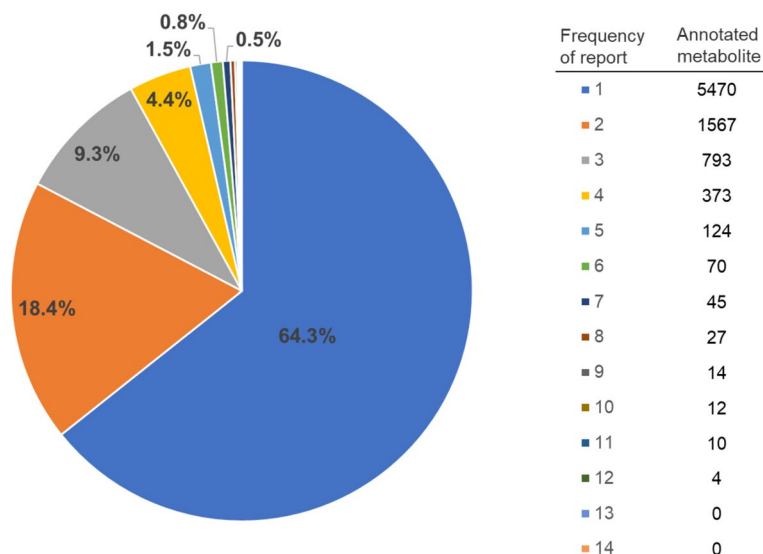


Fig. 3 Frequency (%) of annotated metabolites reported before (A) and after (B) nomenclature standardization by RefMet. The colors indicate the frequency of metabolites reported, ranging from one to ten times. For reference, before standardization 47.4, 42.2, 47 and

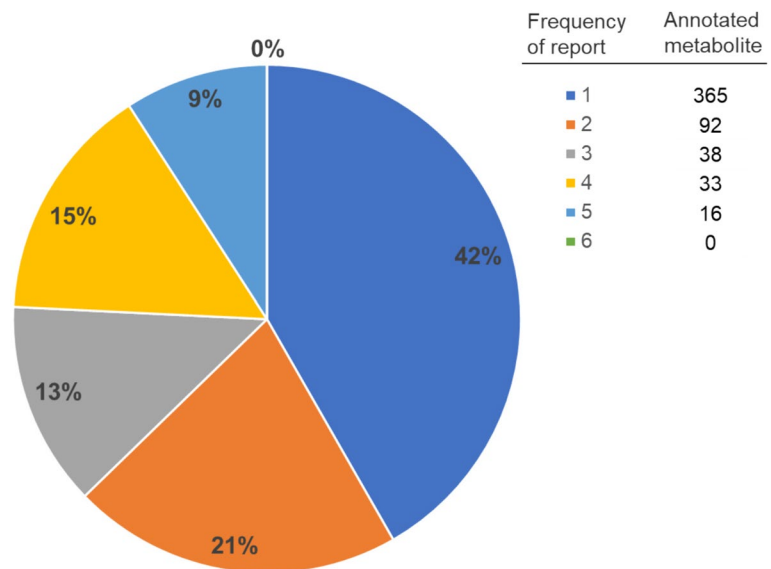
46% of metabolites were reported one time across all samples (blue). After nomenclature standardization these values were 60.5, 58.7, 62.4 and 62.8% respectively, showing a higher frequency of multiple reported metabolites

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

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



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.

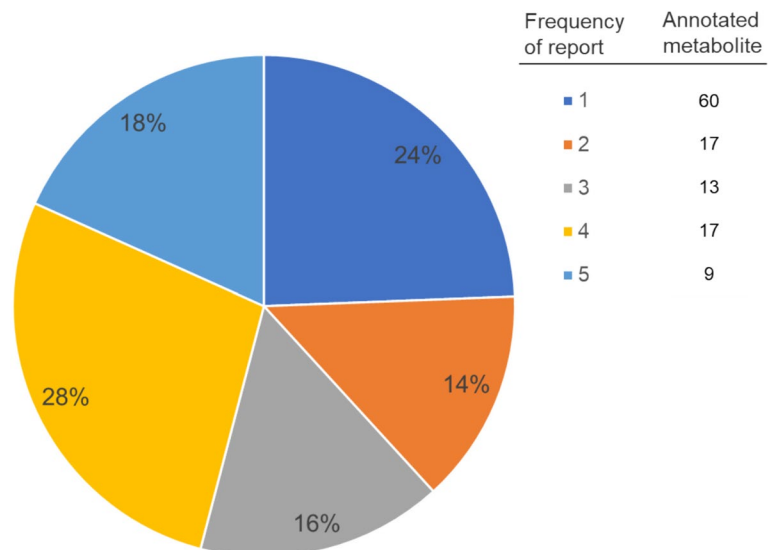
Similarly, to the LC–MS data, the top 20 most abundant metabolites by GC–MS were used to assess the number of times that a metabolite was reported by participants. A total of 120 metabolites were provided, with 98 unique metabolites reported for vegan-lyophilized stool, 88 metabolites for vegan-aqueous 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

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



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).

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

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

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 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 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 Information The online version contains supplementary material available at <https://doi.org/10.1007/s11306-024-02185-0>.

Acknowledgements 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 multi-program national laboratory operated by Battelle for the DOE under Contract DE-AC05-76RLO 1830.

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.

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.I.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. 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 Skłodowska-Curie Grant Agreement No. (798038)". B.R.R. and S. S. would like to declare that this study was supported by the NIEHS through the Human Health Exposure Analysis Resource (HHEAR) program through grant 5U2CES030857 and by NCI through the Metabolomics and Clinical Assay Center of the Nutrition for Precision Health (NPH) program through grant 5U24CA268153.

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

Declarations

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.

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.

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.

References

- Alseekh, S., Aharoni, A., Brotman, Y., Contrepois, K., D'Auria, J., Ewald, J., C. Ewald, J., Fraser, P. D., Giavalisco, P., Hall, R. D., Heinemann, M., Link, H., Luo, J., Neumann, S., Nielsen, J., Perez de Souza, L., Saito, K., Sauer, U., Schroeder, F. C., Fernie, A. R. (2021). Mass spectrometry-based metabolomics: A guide for annotation, quantification and best reporting practices. In *Nature methods* (Vol. 18, Issue 7, pp. 747–756). Nature Research. <https://doi.org/10.1038/s41592-021-01197-1>
- Aristizabal-Henao, J. J., Lemas, D. J., Griffin, E. K., Costa, K. A., Camacho, C., & Bowden, J. A. (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. <https://doi.org/10.1021/jasms.1c00194>
- Bang, G., Park, J. H., Park, C., Kim, K. J., Kim, J. K., Lee, S. Y., Kim, J. Y., & Park, Y. H. (2022). High-resolution metabolomics-based biomarker discovery using exhaled breath condensate from patients with lung cancer. *Journal of Analytical Science and Technology*. <https://doi.org/10.1186/s40543-022-00347-0>
- Barandouzi, Z. A., Lee, J., del Carmen Rosas, M., Chen, J., Henderson, W. A., Starkweather, A. R., & Cong, X. S. (2022). Associations of neurotransmitters and the gut microbiome with emotional distress in mixed type of irritable bowel syndrome. *Scientific Reports*. <https://doi.org/10.1038/s41598-022-05756-0>
- Bayless, A., Da Silva, S., Davis, W. C., Kuri Cruz, A., Piotrowski, P., Schock, T., & Servetas, S. (2023). Multi-omic characterization of human whole stool RGTMs. *NIST Internal Report (IR) 8451*. <https://doi.org/10.6028/NIST.IR.8451>
- Brunmair, J., Gotsmy, M., Niederstaetter, L., Neuditschko, B., Bileck, A., Slany, A., Feuerstein, M. L., Langbauer, C., Janker, L., Zanghellini, J., Meier-Menches, S. M., & Gerner, C. (2021). Finger sweat analysis enables short interval metabolic biomonitoring in humans. *Nature Communications*. <https://doi.org/10.1038/s41467-021-26245-4>
- Cumeras, R., Shen, T., Valdiviez, L., Tippins, Z., Haffner, B. D., & Fiehn, O. (2023). Differences in the stool metabolome between vegans and omnivores: Analyzing the NIST stool reference material. *Metabolites*. <https://doi.org/10.3390/metabo13080921>
- Cunningham, A. L., Stephens, J. W., & Harris, D. A. (2021). Gut microbiota influence in type 2 diabetes mellitus (T2DM). In *Gut pathogens* (Vol. 13, Issue 1). BioMed Central Ltd. <https://doi.org/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-inflammatory functions are linked to pediatric allergy and acquisition of immune tolerance. *Nature Communications*. <https://doi.org/10.1038/s41467-021-26266-z>
- De Leoz, M. L. A., Duewer, D. L., Fung, A., Liu, L., Yau, H. K., Potter, O., Staples, G. O., Furuki, K., Frenkel, R., Hu, Y., Sosis, Z., Zhang, P., Altmann, F., Grunwald-Grube, C., Shao, C., Zaia, J., Evers, W., Pengelley, S., Suckau, D., & Stein, S. E. (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. <https://doi.org/10.1074/mcp.RA119.001677>
- 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. <https://doi.org/10.1016/j.aca.2020.02.046>
- Deda, O., Chatziioannou, A. C., Fasoula, S., Palachanis, D., Raikos, N., Theodoridis, G. A., & Gika, H. G. (2017). Sample preparation optimization in fecal metabolic profiling. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1047, 115–123. <https://doi.org/10.1016/j.jchromb.2016.06.047>
- Elpa, D. P., Chiu, H. Y., Wu, S. P., & Urban, P. L. (2021). Skin metabolomics. In *Trends in endocrinology and metabolism* (Vol. 32, Issue 2, pp. 66–75). Elsevier. <https://doi.org/10.1016/j.tem.2020.11.009>
- Erlandson, K. M., Liu, J., Johnson, R., Dillon, S., Jankowski, C. M., Kroehl, M., Robertson, C. E., Frank, D. N., Tuncil, Y., Higgins, J., Hamaker, B., & Wilson, C. C. (2021). An exercise intervention alters stool microbiota and metabolites among older, sedentary adults. *Therapeutic Advances in Infectious Disease*. <https://doi.org/10.1177/20499361211027067>

- Fahy, E., & Subramaniam, S. (2020). RefMet: A reference nomenclature for metabolomics. In *Nature methods* (Vol. 17, Issue 12, pp. 1173–1174). *Nature Research*. <https://doi.org/10.1038/s41592-020-01009-y>
- Fiehn, O. (2016). Metabolomics by gas chromatography-mass spectrometry: Combined targeted and untargeted profiling. *Current Protocols in Molecular Biology*. <https://doi.org/10.1002/047142727.mb3004s114>
- Fiehn, O., Robertson, D., Griffin, J., van der Werf, M., Nikolau, B., Morrison, N., Sumner, L. W., Goodacre, R., Hardy, N. W., Taylor, C., Fostel, J., Kristal, B., Kaddurah-Daouk, R., Mendes, P., van Ommen, B., Lindon, J. C., & Sansone, S. A. (2007). The metabolomics standards initiative (MSI). *Metabolomics*, 3(3), 175–178. <https://doi.org/10.1007/s11306-007-0070-6>
- Gardner, A., Carpenter, G., & So, P. W. (2020). Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function. In *Metabolites* (Vol. 10, Issue 2). MDPI AG. <https://doi.org/10.3390/metabo10020047>
- Gauglitz, J. M., West, K. A., Bittremieux, W., Williams, C. L., Weldon, K. C., Panitchpakdi, M., Di Ottavio, F., Aceves, C. M., Brown, E., Sikora, N. C., Jarmusch, A. K., Martino, C., Tripathi, A., Meehan, M. J., Dorrestein, K., Shaffer, J. P., Coras, R., Vargas, F., Goldasich, L. D. R., & Dorrestein, P. C. (2022). Enhancing untargeted metabolomics using metadata-based source annotation. *Nature Biotechnology*, 40(12), 1774–1779. <https://doi.org/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*. <https://doi.org/10.1002/jms.4817>
- Journey, & E. N. K., Ortega-Santos, C. P., Bruening, M., & Whisner, C. M. (2020). Changes in weight status and the intestinal microbiota among College Freshman, aged 18 Years. *Journal of Adolescent Health*, 66(2), 166–171. <https://doi.org/10.1016/j.jadohealth.2019.06.005>
- Kirwan, J. A., Gika, H., Beger, R. D., Bearden, D., Dunn, W. B., Goodacre, R., Theodoridis, G., Witting, M., Yu, L. R., & Wilson, I. D. (2022). Quality assurance and quality control reporting in untargeted metabolic phenotyping: mQACC recommendations for analytical quality management. In *Metabolomics* (Vol. 18, Issue 9). Springer. <https://doi.org/10.1007/s11306-022-01926-3>
- Köfeler, H. C., Eichmann, T. O., Ahrends, R., Bowden, J. A., Danne-Rasche, N., Dennis, E. A., Fedorova, M., Griffiths, W. J., Han, X., Hartler, J., Holčápek, M., Jirásko, R., Koelmel, J. P., Ejsing, C. S., Liebisch, G., Ni, Z., O'Donnell, V. B., Quehenberger, O., Schwudke, D., ... Ekroos, K. (2021). Quality control requirements for the correct annotation of lipidomics data. *Nature Communications*, 12, 4771. <https://doi.org/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. <https://doi.org/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. <https://doi.org/10.3390/metabo10110468>
- Lippa, K. A., Aristizabal-Henao, J. J., Beger, R. D., Bowden, J. A., Broeckling, C., Beecher, C., Clay Davis, W., Dunn, W. B., Flores, R., Goodacre, R., Gouveia, G. J., Harms, A. C., Hartung, T., Jones, C. M., Lewis, M. R., Ntai, I., Percy, A. J., Raftery, D., Schock, T. B., Ubhi, B. K. (2022). Reference materials for MS-based untargeted metabolomics and lipidomics: a review by the metabolomics quality assurance and quality control consortium (mQACC). In *Metabolomics* (Vol. 18, Issue 4). Springer. <https://doi.org/10.1007/s11306-021-01848-6>
- McDonald, J. G., Ejsing, C. S., Kopczynski, D., Holčápek, M., Aoki, J., Arita, M., Arita, M., Baker, E. S., Bertrand-Michel, J., Bowden, J. A., Brügger, B., Ellis, S. R., Fedorova, M., Griffiths, W. J., Han, X., Hartler, J., Hoffmann, N., Koelmel, J. P., Köfeler, H. C., Ekroos, K. (2022). Introducing the Lipidomics Minimal Reporting Checklist. In *Nature Metabolism* (Vol. 4, Issue 9, pp. 1086–1088). *Nature Research*. <https://doi.org/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. In *Oxidative Medicine and Cellular Longevity* (Vol. 2017). Hindawi Limited. <https://doi.org/10.1155/2017/3831972>
- Moosmang, S., Pitscheider, M., Sturm, S., Seger, C., Tilg, H., Hala-balaki, M., & Stuppner, H. (2019). Metabolomic analysis—addressing NMR and LC-MS related problems in human feces sample preparation. *Clinica Chimica Acta*, 489, 169–176. <https://doi.org/10.1016/j.cca.2017.10.029>
- Moreau, R., Clària, J., Aguilar, F., Fenaille, F., Lozano, J. J., Junot, C., Colsch, B., Caraceni, P., Trebicka, J., Pavesi, M., Alessandria, C., Nevens, F., Saliba, F., Welzel, T. M., 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. <https://doi.org/10.1016/j.jhep.2019.11.009>
- Nam, S. L., Tarazona Carrillo, K., de la Mata, A. P., & Harynuk, J. J. (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*. <https://doi.org/10.3390/metabo13070828>
- Neuman, H., & Koren, O. (2016). The gut microbiome. In *Encyclopedia of cell biology* (Vol. 2, pp. 799–808). Elsevier. <https://doi.org/10.1016/B978-0-12-394447-4.20083-7>
- Pantazi, A. C., Mihai, C. M., Balasa, A. L., Chisnoiu, T., Lupu, A., Frecus, C. E., Mihai, L., Ungureanu, A., Kassim, M. A. K., Andrusca, A., Nicolae, M., Cuzic, V., Lupu, V. V., & Cambrea, S. C. (2023). Relationship between gut microbiota and allergies in children: A literature review. In *Nutrients* (Vol. 15, Issue 11). MDPI. <https://doi.org/10.3390/nu15112529>
- Parekh, P. J., Balart, L. A., & Johnson, D. A. (2015). The influence of the gut microbiome on obesity, metabolic syndrome and gastrointestinal disease. In *Clinical and translational gastroenterology* (Vol. 6, Issue 6). Nature Publishing Group. <https://doi.org/10.1038/ctg.2015.16>
- Peter, K. T., Phillips, A. L., Knolhoff, A. M., Gardinali, P. R., Manzano, C. A., Miller, K. E., Pristner, M., Sabourin, L., Sumarah, M. W., Warth, B., & Sobus, J. R. (2021). Nontargeted analysis study reporting tool: A framework to improve research transparency and reproducibility. *Analytical Chemistry*, 93(41), 13870–13879. <https://doi.org/10.1021/acs.analchem.1c02621>
- Poulsen, K. O., Meng, F., Lanfranchi, E., Young, J. F., Stanton, C., Ryan, C. A., Kelly, A. L., & Sundekilde, U. K. (2022). Dynamic changes in the human milk metabolome over 25 weeks of lactation. *Frontiers in Nutrition*. <https://doi.org/10.3389/fnut.2022.917659>
- Prochazkova, M., Budinska, E., Kuzma, M., Pelantova, H., Hradecky, J., Heczkova, M., Daskova, N., Bratova, M., Modos, I., Viden-ska, P., Splichalova, P., Sowah, S. A., 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*. <https://doi.org/10.3389/fnut.2021.783302>
- Radjabzadeh, D., Bosch, J. A., Uitterlinden, A. G., Zwinderman, A. H., Ikram, M. A., van Meurs, J. B. J., Luik, A. I., Nieuwdorp, M., Lok, A., van Duijn, C. M., Kraaij, R., & Amin, N. (2022). Gut microbiome-wide association study of depressive symptoms. *Nature Communications*. <https://doi.org/10.1038/s41467-022-34502-3>
- Rodríguez-Morató, J., Pozo, Ó. J., & Marcos, J. (2018). Targeting human urinary metabolome by LC-MS/MS: A review. In *Bioanalysis* (Vol. 10, Issue 7, pp. 489–516). Future Medicine Ltd. <https://doi.org/10.4155/bio-2017-0285>
- Salek, R. M., Steinbeck, C., Viant, M. R., Goodacre, R., & Dunn, W. B. (2013). The role of reporting standards for metabolite annotation and identification in metabolomic studies. *GigaScience*. <https://doi.org/10.1186/2047-217X-2-13>
- Simón-Manso, Y., Lowenthal, M. S., Kilpatrick, L. E., Sampson, M. L., Telu, K. H., Rudnick, P. A., Mallard, W. G., Bearden, D. W., Schock, T. B., Tchekhovskoi, D. V., Blonder, N., Yan, X., Liang, Y., Zheng, Y., Wallace, W. E., Neta, P., Phinney, K. W., Remaley, A. T., & Stein, S. E. (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. <https://doi.org/10.1021/ac402503m>
- Sindelar, M., & Patti, G. J. (2020). Chemical discovery in the era of metabolomics. *Journal of the American Chemical Society*, 142(20), 9097–9105. <https://doi.org/10.1021/jacs.9b13198>
- Stephen, A. M., & Cummings, J. H. (1980). The microbial contribution to human faecal mass. *The Pathological Society of Great Britain and Ireland*, 13(1), 45–56.
- Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W. M., Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., & Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis: Chemical analysis working group (CAWG) metabolomics standards initiative (MSI). *Metabolomics*, 3(3), 211–221. <https://doi.org/10.1007/s11306-007-0082-2>
- 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*. <https://doi.org/10.3390/nu15102358>
- Sun, Y. V., & Hu, Y. J. (2016). Integrative analysis of multi-omics data for discovery and functional studies of complex human diseases. *Advances Genetics*, 93, 147–190. <https://doi.org/10.1016/bs.adgen.2015.11.004>
- Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R. Y., 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. <https://doi.org/10.1136/gutjnl-2015-309618>
- Viant, M. R., Ebbels, T. M. D., Beger, R. D., Ekman, D. R., Epps, D. J. T., Kamp, H., Leonards, P. E. G., Loizou, G. D., MacRae, J. I., van Ravenzwaay, B., Rocca-Serra, P., Salek, R. M., Walk, T., & Weber, R. J. M. (2019). Use cases, best practice and reporting standards for metabolomics in regulatory toxicology. *Nature Communications*. <https://doi.org/10.1038/s41467-019-10900-y>
- Wang, K., Mehta, R. S., Ma, W., Nguyen, L. H., Wang, D. D., Ghazi, A. R., Yan, Y., Al-Shaar, L., Wang, Y., Hang, D., Fu, B. C., Ogino, S., Rimm, E. B., Hu, F. B., Carmody, R. N., Garrett, W. S., Sun, Q., Chan, A. T., Huttenhower, C., & Song, M. (2023). The gut microbiome modifies the associations of short- and long-term physical activity with body weight changes. *Microbiome*. <https://doi.org/10.1186/s40168-023-01542-w>
- Wawrzyniak, R., Kosnowska, A., Macioszek, S., Bartoszewski, R., & Markuszewski, M. J. (2018). New plasma preparation approach to enrich metabolome coverage in untargeted metabolomics: Plasma protein bound hydrophobic metabolite release with proteinase K. *Scientific Reports*. <https://doi.org/10.1038/s41598-018-27983-0>
- Wilson, I. D., Theodoridis, G., & Virgiliou, C. (2021). A perspective on the standards describing mass spectrometry-based metabolic phenotyping (metabolomics/metabonomics) studies in publications. In *Journal of chromatography B: Analytical technologies in the biomedical and life sciences* (Vol. 1164). Elsevier. <https://doi.org/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. <https://doi.org/10.1038/s41592-023-01850-x>
- Zierer, J., Jackson, M. A., Kastenmüller, G., Mangino, M., Long, T., Telenti, A., Mohny, R. P., Small, K. S., Bell, J. T., Steves, C. J., Valdes, A. M., Spector, T. D., & Menni, C. (2018). The fecal metabolome as a functional readout of the gut microbiome. *Nature Genetics*, 50(6), 790–795. <https://doi.org/10.1038/s41588-018-0135-7>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.