# Optimization of fecal sample homogenization for untargeted metabolomics

**Kieran Tarazona Carrillo**
University of Alberta

**Seo Lin Nam**
University of Alberta

**A. Paulina de la Mata**
University of Alberta

**Olle M. de Bruin**
DNA Genotek

**Evgueni Doukhanine**
DNA Genotek

**James Harynuk** ( ✉ james.harynuk@ualberta.ca )
University of Alberta

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# Abstract

### Introduction

Fecal samples are highly complex and heterogeneous, containing materials at various stages of digestion. The heterogeneity and complexity of feces make stool metabolomics inherently challenging. The level of homogenization influences the outcome of the study, affecting the metabolite profiles and reproducibility; however, there is no consensus on how fecal samples should be prepared to overcome the topographical discrepancy and obtain data representative of the stool as a whole.

### Objective

Various combinations of homogenization conditions were compared to investigate the effects of bead size, addition of solvents and the differences between wet-frozen and lyophilized feces.

### Methods

The homogenization parameters were systematically altered to evaluate the solvent usage, bead size, and whether lyophilization is required in homogenization. The metabolic coverage and reproducibility were compared among the different conditions.

### Results

The current work revealed that a combination of mechanical and chemical lysis obtained by bead-beating with a mixture of big and small sizes of beads in an organic solvent is an effective way to homogenize fecal samples with adequate reproducibility and metabolic coverage. Lyophilization is required when bead-beating is not available.

### Conclusions

A comprehensive and systematical evaluation of various fecal matter homogenization conditions provides a profound understanding for the effects of different homogenization methods. Our findings would be beneficial to assist with standardization of fecal sample homogenization protocol.

## 1. Introduction

In the world of metabolomics, many biological matrices have been studied, including urine, blood, sweat, and saliva. More recently, metabolomics studies of fecal matter have received increasing attention since the important role the gut microbiome plays in its host's health is being recognized in health science.[1] During the metabolic processing of food and xenobiotics, the host and symbiotic gut microbiota coproduce a significant number of metabolic breakdown products as well as non-metabolized compounds.[2,3] Changes in microbiome composition and metabolic function due to various diseases and stresses can lead to alterations in metabolic profiles.[4,5] Measuring fecal metabolites can provide valuable

insight into the metabolic status, which can be potentially related to the health/disease state of the host.[6] Consequently, accurate measurement of fecal metabolites is crucial for the acquired data to be linked to a trustworthy interpretation.

However, unlike urine or blood that are biofluids, feces is not a homogeneous matrix and the metabolite composition varies across different regions of the fecal sample.[8] Hence, feces require a more rigorous sample preparation given the heterogeneity and complexity of the sample matrix. Feces are composed of partially digested and undigested matter, gut bacteria, water, and metabolic waste, containing an enormously vast range of chemical classes.[3,7] The composition of feces varies greatly from donor to donor and day to day depending on a number of factors, including food consumption, level of hydration, health status, etc. The form and size of human feces also differ widely from sample to sample.[8,9] Typically, it is not feasible to use the entire collected stool with modern sensitive instruments for metabolomics studies. Therefore, spot sampling, which involves scooping a small portion of feces, has been commonly used for fecal analysis.[10] The trouble with a localized sampling method is the heterogeneity of feces in that the fecal metabolome varies across different topographical regions in the same fecal bulk sample.[11] Even with visual observation, undigested food residues can be easily found in some parts of feces. Thus, suitable homogenization is required as the first step post-collection to eliminate bias due to spot sampling of unhomogenized feces and to ensure aliquots are representative of the entire fecal sample.[10] It was also reported that bacterial composition varies throughout the stool sample; thus, homogenizing the sample at the start of sample preparation may decrease the biological signal variability.[12,13] Additionally, it has been claimed that vigorous homogenization may be required in order to lyse the heterogenous variety of bacterial cells sufficiently and without taxonomic bias.[10] The analysis and detection of these intracellular bacterial metabolites can further the understanding of the complex interactions between the gut microbiome and host.

Studies on fecal sample preparation with particular emphasis on homogenization have been previously conducted for microbiome analysis and DNA synthesis applications.[14–17] However, to the best of our knowledge, a comprehensive study on homogenization of fecal samples has not been performed for stool metabolomics studies. Previous fecal microbiome and metabolomics studies primarily relied on stirring, grinding samples under liquid nitrogen, or bead-beating for homogenization of the fecal material; nevertheless, a standard protocol for the homogenization of fecal samples has not yet been established.[18–20] Among different methods, bead-beating is an intense homogenization process, which involves the use of beads for mechanical cell disruption by rapidly and continuously exposing the sample to beads, leading to cell wall rupture and the release of intracellular contents. Santiago *et al.* reported that stool homogenization affects the analysis of the microbial community and bead-beating homogenization is crucial for the reliable detection of Gram-positive bacteria.[21] For the adequate lysis of cells, including the bacterial cells present in feces, bead-beating is considered a suitable homogenization technique for fecal metabolomics.

In this study, we evaluated the bead-beating homogenization method in preparation for the optimal processing of fecal samples for untargeted metabolomics studies. The bead-beating parameters were systematically changed to assess the effects of bead size, the use of solvents, and the sample condition (lyophilized vs wet) on the metabolic coverage and reproducibility. The data were acquired using a powerful separation technique, comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS).[22] The total signal intensities and the variations in metabolites abundances were compared among different bead-beating conditions to investigate the metabolome coverage and reproducibility.

# 2. Materials And Methods

## 2.1 Sample collection and storage

A fecal sample was collected from a healthy volunteer without any known preconditions in a sterile sample collection cup (BioLynx, Brockville, ON, Canada). The sample was divided in half and aliquoted in two 50 mL Nunc™ conical centrifuge tubes (ThermoFisher Scientific, Edmonton, AB, Canada) for freezing at -80 °C and lyophilization. One tube was immediately stored at -80 °C, and another tube for lyophilization was weighed, frozen at -80 °C for 10 h, then lyophilized for 48 h. The lyophilized sample was then stored at -80 °C until the day of sample preparation (extraction and derivatization). The samples were taken out of the freezer and thawed at room temperature (approximately 22 °C) for 2 h before extraction. Fig. 1 shows a simplified sample preparation procedure. The complete procedure schematic is available in SI.

## 2.2 Chemicals

Methanol (>99.9%, HPLC grade), toluene (>99.5%, ACS grade), pyridine (>99.9%, HPLC grade), anhydrous sodium sulfate (>99.0% ACS grade), and methoxyamine hydrochloride (98%) were purchased from Millipore-Sigma (Oakville, ON, Canada). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide + 1 % chlorotrimethylsilane (MSTFA + 1% TMCS) was obtained from Fisher Scientific (Ottawa, ON, Canada). Ultrapure 18.2 MΩ deionized water was supplied from an Elga PURELAB Flex 3 system (VWR International, Edmonton, AB, Canada).

## 2.3 Sample preparation

Different sizes of beads were compared and for each homogenization condition, the samples were prepared in six replicates. The selected beads were big beads (BB) (2.38 mm metal beads, QIAGEN), small beads (SB) (0.1 mm glass beads, QIAGEN), MB representing a mixture of big and small beads, and NB is no beads used at all.  To compare homogenization efficiency in sample preparation, frozen (wet) and lyophilized (dry) feces were used. Water content was determined as described in the literature.[23] The stool sample was weighed before and after lyophilization, and the resulting mass loss was determined to be the amount of water initially in the sample. For clarification of the sample name convention, a table summary was included in SI table 1.

### 2.3.1 Dry sample without solvent or water added (BBdry, NBdry)

120 mg of the lyophilized fecal samples were added to homogenizer tubes containing four BB or NB. Samples were homogenized for 3 min (Vortex Genie2, Fisher Scientific; 2.0 mL tube vortex adapter, QIAGEN). Following the homogenization, 12 mg of the homogenized samples were transferred to 2-mL centrifuge tubes in six replicates. Samples were extracted with 450 μL of 80% MeOH in water (v/v), vortexed for 3 min (Benchmark Scientific Benchmixer V2) and centrifuged at 10,000 g for 10 min. 300 μL aliquots of the supernatants were transferred into GC vials, and the extracts were dried completely at 37 °C under a gentle stream of nitrogen before storage at -80 °C until ready to derivatize.

### 2.3.2 Dry and wet sample with water added (BBwetH$_2$O, BBdryH$_2$O, NBdryH$_2$O, NBwetH$_2$O)

660 mg of wet-frozen fecal samples were added to homogenizer tubes pre-filled with four BB or NB. Due to the nature of the samples (paste-like consistency), water was added to facilitate homogenization with a sample to water ratio of 1:1 (w:v). For the lyophilized sample, 120 mg were added to homogenizer tubes pre-filled with four BB or NB. The same amount of water present in the wet samples was added to reconstitute the lyophilized samples. The amount of water added was equal to the initial water content of the sample determined in Section 2.3 (79% by weight) plus the extra water that was added to the wet-frozen samples for homogenization. The samples were then homogenized for 3 min. 109 mg of the homogenized samples were weighed into 2-mL centrifuge tubes. The extraction was performed as previously described in Section 2.3.1.

### 2.3.3 Dry or wet sample with solvent added (All samples labelled Solv)

36 mg of lyophilized and 166 mg of frozen feces were weighed in duplicate into separate homogenizer tubes pre-filled with BB, SB, MB, or NB. 1350 μL of 80% MeOH was added to each tube and was homogenized for 3 min. Samples were centrifuged at 10,000 g for 10 min. A 300 μL aliquot of each sample supernatant was transferred in triplicate to GC vials. Extracts were dried at 37 °C under a gentle stream of nitrogen then stored at -80 °C until ready to derivatize.

## 2.5 Derivatization

Dried extracts were removed from the -80 °C freezer and 100 μL anhydrous toluene (dried with sodium sulfate) was added to each sample, which was then dried at 50 °C under a gentle stream of nitrogen. A two-step methoxymation/trimethylsilylation derivatization was performed by adding 50 μL methoxyamine hydrochloride solution (20 mg/mL in pyridine) to each sample, incubating at 80 °C for 30 min. Following a 5 min cooling period, 100 μL of MSTFA + 1% TMCS were added to each sample and incubated at 80 °C for another 30 min. The derivatized samples were then cooled for 5 min at room temperature and transferred into GC insert vials for analysis. All derivatized samples were analyzed within 24 hours of derivatization to avoid degradation of analytes.

## 2.6 GC×GC-TOFMS

Sample analyses were performed on an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a LECO quad-jet dual-stage cryogenic modulator and Pegasus 4D Time of Flight mass spectrometer (LECO, St. Joseph, MI, USA). First dimension separation was achieved on an Rxi-5SilMS (60 m, 0.25 mm ID, 0.25 μm df) capillary column, and second dimension used an Rtx-200MS (1.6 m, 0.25 mm ID, 0.25 μm df) column (Chromatographic Specialties, Brockville, ON, Canada). 1 μL splitless injections of each sample were performed by a Gerstel MPS autosampler (Gerstel Inc., Linthicum, MD, USA) into the inlet set at 250 ℃ with 90 s purge time. Helium gas (99.999% pure, Praxair) was used as the carrier gas at a corrected constant flow of 2 mL/min. The GC oven was held at 80 ℃ for 4 min, ramped at 3.50 ℃/min to 315 ℃ and held for 10 min. The modulator and secondary oven were set to +15 ℃ and +10 ℃ relative to the GC oven, respectively. The modulation period was 2.5 s with 0.6 s hot jet and 0.65 s cold jet time. An electron impact energy of -70 eV was used for ionization. The mass spectrometer transfer line and ion source were set to 250 ℃ and 200 ℃, respectively. Data acquisition was done at a rate of 200 Hz in the m/z range of 40 − 800 with the detector voltage offset set to 200 V. An acquisition delay of 525 seconds was used.

## 2.7 Data analysis

Data collection and sample processing was done using ChromaTOF® (v.4.72, LECO, St. Joseph, MI, USA). Data were initially integrated with a baseline offset of 0.9, the first-dimension peak width set to 12 s and the second-dimension width set to 0.15 s. Chromatograms were searched for peaks containing the TMS fragment of m/z 73. The minimum S/N ratio for finding base peaks was set to 100:1 and for sub peaks was set to 6:1. The mass spectral match was set to 650 for subpeaks to be included in the auto-smoothed peak. The column bleed region was excluded from the integration and all chromatographic peaks were searched against the NIST-MS 2017 libraries. Statistical Compare feature from ChromaTOF® was used for peak alignment, and samples were aligned by homogenization condition (i.e. all six replicates of BBwet samples were aligned together but separated from the other conditions). Retention time match criteria for alignment were set to ± 5 modulation periods in the first dimension and 0.2 s in the second dimension allowing for retention time shifting between samples. The lowest mass spectral match similarity to combine sub-peaks was 600 and used all m/z values with greater than 1% abundance of the base peak intensity. The aligned peak areas were normalized by the dry weight of the sample. In the case of wet-frozen stool samples, the equivalent dry weight was calculated using previously determined water content (Section 2.3) and used for normalization.

## 2.8 Assessment criteria

The two main criteria for evaluating the performance of the different bead homogenization conditions were metabolic coverage and reproducibility. In terms of metabolic coverage, a larger quantity of compounds would be desired as it indicates that cells are more efficiently lysed during the homogenization and metabolites are extracted that may not be accessible without adequate lysis. The total number of peaks detected, the total peak area (TPA), and the total useful peak area (TUPA) were employed as metrics for comparing different homogenization methods. TPA refers to the total summed

peak areas of all detected analyte signals. In contrast, TUPA includes only the analyte signals present in all the samples within the same homogenization condition for the summation.[24] For reproducibility, the relative standard deviation (RSD) between replicates for each homogenization method was assessed. Lower RSDs would indicate better homogeneity of the sample.

## 3. Results And Discussion

Figure 2 shows total ion chromatograms (TIC) of two samples for comparison. The remaining chromatograms are available in the Supplementary Information. The samples displayed similar metabolomic profiles. Differences in the profiles and resultant sample composition can be attributed to the differences in homogenization method used.

The quantitative comparison of the total number of peaks detected, TPA and TUPA is shown in Fig. 3 and Table 1 for all homogenization methods. The total number of peaks ranged from 1207 to 1610 while the RSD ranged from 0.70−6.12%. The lowest total number of peaks was found with BBdry with an RSD of 5.33%, while MBwetSolv had the highest total number of peaks with an RSD of 1.04% (SI Table S1). The TPA ranged from $5.01 \times 10^7$ to $2.67 \times 10^8$, while the RSD ranged from 2.10−27.70% (SI Table S2). The TUPA ranged from $3.00 \times 10^7$ to $2.42 \times 10^8$, while the RSD ranged from 2.89−22.70% (SI Table S3). The lowest TPA was resulted from BBdry, whereas the lowest TUPA was found with NBdry. Both the highest TPA and TUPA were found with MBwetSolv, which also yielded the most total number of peaks.

Table 1
Bead-beating homogenization results

| Sample | | Total Number of Peaks | | TPA | | TUPA | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Average | RSD (%) | Average | RSD (%) | Average | RSD (%) |
| NBdry | * | 1334 | 6.12 | 7.96E + 07 | 27.7 | 4.03E + 07 | 22.7 |
| NBdryH$_2$O | *# | 1397 | 4.89 | 1.09E + 08 | 20.6 | 6.22E + 07 | 19.5 |
| NBwetH$_2$O | # | 1381 | 4.58 | 1.29E + 08 | 21.7 | 7.32E + 07 | 16.6 |
| NBdrySolv | *~# | 1436 | 2.16 | 1.61E + 08 | 2.10 | 1.40E + 08 | 3.67 |
| NBwetSolv | ~# | 1416 | 4.99 | 1.13E + 08 | 23.0 | 6.10E + 07 | 18.8 |
| BBdry | * | 1272 | 5.33 | 7.56E + 07 | 21.9 | 4.41E + 07 | 22.0 |
| BBdryH$_2$O | *# | 1451 | 5.48 | 1.06E + 08 | 22.5 | 5.97E + 07 | 18.7 |
| BBwetH$_2$O | # | 1404 | 5.27 | 1.21E + 08 | 26.1 | 7.05E + 07 | 18.2 |
| BBdrySolv | *~# | 1434 | 1.66 | 1.57E + 08 | 4.10 | 1.39E + 08 | 5.55 |
| BBwetSolv | ~# | 1505 | 3.45 | 2.51E + 08 | 4.21 | 2.08E + 08 | 4.70 |
| SBdrySolv | ~# | 1389 | 0.70 | 1.42E + 08 | 2.37 | 1.28E + 08 | 2.89 |
| SBwetSolv | ~# | 1479 | 1.98 | 2.19E + 08 | 13.6 | 1.86E + 08 | 13.5 |
| MBdrySolv | ~# | 1412 | 1.05 | 1.39E + 08 | 4.05 | 1.14E + 08 | 4.07 |
| MBwetSolv | ~# | 1539 | 1.04 | 2.58E + 08 | 3.34 | 2.29E + 08 | 4.00 |

Samples marked with * in Table 1 were used to evaluate the effects of solvents during homogenization (sec. 3.1). Samples marked with ~ were used to evaluate the effects of the use and different sizes of beads during homogenization (sec. 3.2). Samples marked with # were used for comparison between lyophilized (dry) and wet feces (sec 3.3).

# 3.1 Effect Of Solvent Addition During Bead-beating Homogenization

Samples labeled with * in Table 1 summarize the results on the effect of the solvent addition. The samples were prepared using the same dry mass. NBdry and BBdry had lower TPA and TUPA with higher RSD compared to when water or organic solvent was added. When water was added to the sample, both NBdryH$_2$O and BBdryH$_2$O had higher TPA and TUPA with lower RSD compared to their respective dry sample, with or without beads. When organic solvents were added for bead-beating homogenization, it resulted in the highest TPA and TUPA with significantly dropped RSD for both cases without or with big beads during homogenization.

The results show that more efficient homogenization is obtained when liquid is added to the sample during bead homogenization. However, because different volumes were used for water and organic solvent, it is inappropriate to interpret the results as organic solvents aid better with homogenization than water. A higher volume of organic solvent (1350 μL of 80% MeOH) was used compared to 990 μL for water. With the 1350 μL of solvents, it filled approximately 80% of the 2-mL Eppendorf tube. It is difficult to draw conclusions on whether aqueous or organic solvents are superior since efficiency of homogenization may depend on the solvent fill-volume of the sample tube. Further research is required to investigate this.

Water was used during homogenization to determine whether its presence would result in improved homogenization efficiency. Enough water was added to ensure the sample would be well mixed during homogenization without clumping or smearing of the fecal material on the walls of the tube which would lead to inadequate homogenization efficiency. The samples homogenized with water were later extracted with 80% MeOH. The samples homogenized with organic solvent involved a homogenization and extraction in a single step and the volume used was the volume of solvent required for extraction.

## 3.2 Use Of Beads During Homogenization And Size Of Beads

Samples labeled with ~ in Table 1 summarize the conditions to examine the effects of beads and the impact of different sizes of beads for homogenization. For the dry samples, when they are homogenized with organic solvents, the use of beads nor the size of beads during homogenization had no significant effects on the performance of homogenization in terms of the total number of peaks, TPA and TUPA. On the contrary, when the fecal sample was wet-frozen, the use of beads during homogenization with solvents significantly increased the TPA and TUPA. Compared to NBwetSolv, there were 2.2, 1.9, and 2.3 times increase for BBwetSolv, SBwetSolv, and MBwetSolv, respectively. Amongst the three different conditions regarding bead sizes (BB, SB, MB), SB offered the least total number of peaks, TPA and TUPA, with the highest RSD (13.5%). This indicates that small beads do not provide an efficient homogenization even with the aid of organic solvent when the sample is not lyophilized. When big beads were used instead, both TPA and TUPA increased approximately 1.1 times relative to SBwetSolv, while the RSD dropped significantly from 13.6–4.21% for TPA and 13.5–4.70% for TUPA. For the homogenization with beads and solvents, the best result was obtained with MBwetSolv demonstrated by the increased TPA and TUPA with lower RSD. When big beads were mixed with small beads, both TPA and TUPA increased compared to big beads only, while RSD for both TPA and TUPA decreased. This indicates that big beads are required to break the wet, chunky fecal material adequately, while small beads help to further lyse cells, including bacterial, fungal, or mammalian cells present within the sample, releasing more cellular contents.

Additionally, it is noteworthy that the lowest RSD for TPA and TUPA were found with SBdrySolv (2.37% and 2.89%, respectively), while much higher RSD (13.6% and 13.5%) were found with the SBwetSolv.

Lyophilized feces are dry, powdery and flaky. It appears that it does not require as much mechanical power to break up the dry feces compared to the wet material, which is more solid and chunky.

## 3.3 Freezing Vs. Lyophilization

Lyophilized samples without the addition of solvents (BBdry and NBdry) resulted in low number of peaks (Table 1). BBdry and NBdry had 1272 and 1334 peaks, respectively, which are the lowest and second-lowest of all the tested conditions. They also resulted in the lowest for TPA ($7.56×10^7$ and $7.96×10^7$, respectively) as well as for TUPA ($4.41×10^7$ and $4.03×10^7$, respectively). Bead-beating homogenization with wet feces without the aid of solvents was omitted in the study since it is practically not valid without beads getting stuck in the wet feces. Thus, the direct comparison between wet-frozen versus lyophilized feces was not available, yet the conditions that used the solvents when homogenizing were compared as alternatives. Samples denoted with # in Table 1 show the comparison between the lyophilized and wet-frozen feces homogenized with either water or organic solvent.

When water was added for homogenization, the lyophilized samples resulted in slightly less TPA and TUPA than their respective wet feces in comparison for both cases of NB and BB. For the cases of homogenizing with organic solvents added, NBdrySolv and NBwetSolv were compared first and observed that around 1.4 times and 2.3 times were extracted more with the lyophilized feces than wet-frozen feces for TPA and TUPA, respectively. In addition, the RSD of TPA for NBdrySolv was 2.10%, whereas it was 22.95% for NBwetSolv. This indicates that lyophilization is more advantageous for homogenization of dry than wet feces, more effectively and reproducibly releasing the metabolites when homogenized with organic solvents when mechanical disruption is not available.

When mechanical disruption was added in homogenization using solvents, interestingly, the opposite was found. When comparing TPA values for BB, SB, and MB, 1.6 times, 1.5 times, and 1.9 times were extracted more with the wet-frozen feces compared to their respective lyophilized feces. Similarly, for TUPA, 1.5 times, 1.5 times, and 2.0 times were extracted more with wet feces. The RSDs were comparable between the lyophilized and wet samples except for the case of SB, and the explanation for the exception was mentioned in Section 3.2.2. Bead-beating homogenization in organic solvents provides two means of cell lysis; chemical and physical/mechanical disruption. Earlier, the comparison of NBdrySolv and NBwetSolv has shown that the chemical lysis alone without the use of beads was not sufficient to effectively release metabolites from wet chunks of feces. However, when mechanical disruption was added using bead-beating, the increased release of metabolites from wet feces in comparison to the dry was observed regardless of the size of the beads. The low RSD values support that the combination of chemical and mechanical disruption offered by bead-beating rin solvent delivers a high performance of homogenization. When competent homogenization has been accomplished with beads in solvents, the increased metabolite abundances for wet feces in comparison to lyophilized samples indicate that a portion of metabolites may have been lost during the lyophilization process.

# 4. Conclusions

In the current work, a thorough study for fecal matter homogenization was conducted, which involved the investigation on the effects of the addition of solvents, the size of beads, and the lyophilization of the sample prior to homogenization. The total number of peaks detected and the total summed analyte signals were compared among different conditions. The results demonstrate the impact of the use of beads to aid homogenization. Increased signal intensities were observed when beads were used for wet-frozen feces with organic solvents, representing an efficient release of metabolites upon mechanical cell lysis via bead-beating. The best homogenization was achieved with respect to the abundances of metabolites and reproducibility when a combination of larger and smaller beads was used with organic solvents on wet-frozen feces. If bead-beating homogenization is available, such as in a commercial stool collection device with stabilization buffer, lyophilization would not be necessary because the additional step risks the loss of metabolites during its lengthy process. However, when bead-beating is not feasible, lyophilized samples showed much improved reproducibility when vortexed with organic solvents compared to wet-frozen feces. The study provides an enhanced understanding of stool homogenization using the bead-beating technique. Our finding suggests that using a combination of different sizes of beads may be an effective method to homogenize fecal samples for untargeted metabolomics, yielding high metabolite abundances and consistency.

# Declarations

## Acknowledgements

## Author contributions

All authors contributed to the study conception and design. Sample preparation and data collection were performed by KT; data processing and analysis were performed by KT and SLN. The first draft of the manuscript was written by KT and all authors commented on previous versions of the manuscript. All authors have read and agreed to the final manuscript.

## Funding

## Ethics Declarations

## Conflicts of Interest

All authors declare that they have no conflict of interest.

## Ethical approval

This study was approved by the University of Alberta Research Ethics Board, under approval number Pro00071285.

## Informed consent

Informed consent was obtained from all individual participants included in this study.

# References

1. Gao, X.; Pujos-Guillot, E.; Sébédio, J.-L. Development of a Quantitative Metabolomic Approach to Study Clinical Human Fecal Water Metabolome Based on Trimethylsilylation Derivatization and GC/MS Analysis. *Anal. Chem.* **2010**, *82* (15), 6447–6456. https://doi.org/10.1021/ac1006552.

2. Vernocchi, P.; Del Chierico, F.; Putignani, L. Gut Microbiota Metabolism and Interaction with Food Components. *Int. J. Mol. Sci.* **2020**, *21* (10), 3688. https://doi.org/10.3390/ijms21103688.

3. Smith, L.; Villaret-Cazadamont, J.; Claus, S. P.; Canlet, C.; Guillou, H.; Cabaton, N. J.; Ellero-Simatos, S. Important Considerations for Sample Collection in Metabolomics Studies with a Special Focus on Applications to Liver Functions. *Metabolites* **2020**, *10* (3), 104. https://doi.org/10.3390/metabo10030104.

4. Vijay, A.; Valdes, A. M. Role of the Gut Microbiome in Chronic Diseases: A Narrative Review. *Eur. J. Clin. Nutr.* **2022**, *76* (4), 489–501. https://doi.org/10.1038/s41430-021-00991-6.

5. Wu, J.; Wang, K.; Wang, X.; Pang, Y.; Jiang, C. The Role of the Gut Microbiome and Its Metabolites in Metabolic Diseases. *Protein Cell* **2021**, *12* (5), 360–373. https://doi.org/10.1007/s13238-020-00814-7.

6. Singh, R. K.; Chang, H.-W.; Yan, D.; Lee, K. M.; Ucmak, D.; Wong, K.; Abrouk, M.; Farahnik, B.; Nakamura, M.; Zhu, T. H.; Bhutani, T.; Liao, W. Influence of Diet on the Gut Microbiome and Implications for Human Health. *J. Transl. Med.* **2017**, *15* (1), 73. https://doi.org/10.1186/s12967-017-1175-y.

7. Rose, C.; Parker, A.; Jefferson, B.; Cartmell, E. The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. *Crit. Rev. Environ. Sci. Technol.* **2015**, *45* (17), 1827–1879. https://doi.org/10.1080/10643389.2014.1000761.

8. Lewis, S. J.; Heaton, K. W. Stool Form Scale as a Useful Guide to Intestinal Transit Time. *Scand. J. Gastroenterol.* **1997**, *32* (9), 920–924. https://doi.org/10.3109/00365529709011203.

9. Heaton, K. W.; Radvan, J.; Cripps, H.; Mountford, R. A.; Braddon, F. E.; Hughes, A. O. Defecation Frequency and Timing, and Stool Form in the General Population: A Prospective Study. *Gut* **1992**, *33* (6), 818–824. https://doi.org/10.1136/gut.33.6.818.

10. Karu, N.; Deng, L.; Slae, M.; Guo, A. C.; Sajed, T.; Huynh, H.; Wine, E.; Wishart, D. S. A Review on Human Fecal Metabolomics: Methods, Applications and the Human Fecal Metabolome Database. *Anal. Chim. Acta* **2018**, *1030*, 1–24. https://doi.org/10.1016/j.aca.2018.05.031.

11. Gratton, J.; Phetcharaburanin, J.; Mullish, B. H.; Williams, H. R. T.; Thursz, M.; Nicholson, J. K.; Holmes, E.; Marchesi, J. R.; Li, J. V. Optimized Sample Handling Strategy for Metabolic Profiling of Human Feces. *Anal. Chem.* **2016**, *88* (9), 4661–4668. https://doi.org/10.1021/acs.analchem.5b04159.

12. Gorzelak, M. A.; Gill, S. K.; Tasnim, N.; Ahmadi-Vand, Z.; Jay, M.; Gibson, D. L. Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. *PLOS ONE* **2015**, *10* (8), e0134802. https://doi.org/10.1371/journal.pone.0134802.

13. Liang, Y.; Dong, T.; Chen, M.; He, L.; Wang, T.; Liu, X.; Chang, H.; Mao, J.-H.; Hang, B.; Snijders, A. M.; Xia, Y. Systematic Analysis of Impact of Sampling Regions and Storage Methods on Fecal Gut Microbiome and Metabolome Profiles. *mSphere* **2020**, *5* (1). https://doi.org/10.1128/mSphere.00763-19.

14. Lim, M. Y.; Park, Y.-S.; Kim, J.-H.; Nam, Y.-D. Evaluation of Fecal DNA Extraction Protocols for Human Gut Microbiome Studies. *BMC Microbiol.* **2020**, *20* (1), 212. https://doi.org/10.1186/s12866-020-01894-5.

15. Wu, W.-K.; Chen, C.-C.; Panyod, S.; Chen, R.-A.; Wu, M.-S.; Sheen, L.-Y.; Chang, S.-C. Optimization of Fecal Sample Processing for Microbiome Study — The Journey from Bathroom to Bench. *J. Formos. Med. Assoc.* **2019**, *118* (2), 545–555. https://doi.org/10.1016/j.jfma.2018.02.005.

16. Zhang, B.; Brock, M.; Arana, C.; Dende, C.; Hooper, L.; Raj, P. Impact of Bead-Beating Intensity on Microbiome Recovery in Mouse and Human Stool: Optimization of DNA Extraction. *bioRxiv* **2020**, 2020.06.15.151753. https://doi.org/10.1101/2020.06.15.151753.

17. Gangadoo, S.; Rajapaksha Pathirannahalage, P.; Cheeseman, S.; Dang, Y. T. H.; Elbourne, A.; Cozzolino, D.; Latham, K.; Truong, V. K.; Chapman, J. The Multiomics Analyses of Fecal Matrix and Its Significance to Coeliac Disease Gut Profiling. *Int. J. Mol. Sci.* **2021**, *22* (4), 1965. https://doi.org/10.3390/ijms22041965.

18. Moosmang, S.; Pitscheider, M.; Sturm, S.; Seger, C.; Tilg, H.; Halabalaki, M.; Stuppner, H. Metabolomic Analysis—Addressing NMR and LC-MS Related Problems in Human Feces Sample Preparation. *Clin. Chim. Acta* **2019**, *489*, 169–176. https://doi.org/10.1016/j.cca.2017.10.029.

19. O'Sullivan, V.; Madrid-Gambin, F.; Alegra, T.; Gibbons, H.; Brennan, L. Impact of Sample Storage on the NMR Fecal Water Metabolome. *ACS Omega* **2018**, *3* (12), 16585–16590. https://doi.org/10.1021/acsomega.8b01761.

20. Yang, Y.; Yin, Y.; Chen, X.; Chen, C.; Xia, Y.; Qi, H.; Baker, P. N.; Zhang, H.; Han, T.-L. Evaluating Different Extraction Solvents for GC-MS Based Metabolomic Analysis of the Fecal Metabolome of Adult and Baby Giant Pandas. *Sci. Rep.* **2019**, *9* (1), 12017. https://doi.org/10.1038/s41598-019-48453-1.

21. Santiago, A.; Panda, S.; Mengels, G.; Martinez, X.; Azpiroz, F.; Dore, J.; Guarner, F.; Manichanh, C. Processing Faecal Samples: A Step Forward for Standards in Microbial Community Analysis. *BMC Microbiol.* **2014**, *14* (1), 112. https://doi.org/10.1186/1471-2180-14-112.

22. Higgins Keppler, E. A.; Jenkins, C. L.; Davis, T. J.; Bean, H. D. Advances in the Application of Comprehensive Two-Dimensional Gas Chromatography in Metabolomics. *TrAC Trends Anal. Chem.* **2018**, *109*, 275–286. https://doi.org/10.1016/j.trac.2018.10.015.

23. Bliss, D. Z.; Savik, K.; Jung, H.; Jensen, L.; LeMoine, M.; Lowry, A. Comparison of Subjective Classification of Stool Consistency and Stool Water Content. *J. WOCN* **1999**, *26* (3), 137–141. https://doi.org/10.1016/S1071-5754(99)90031-1.

24. Nam, S. L.; de la Mata, A. P.; Dias, R. P.; Harynuk, J. J. Towards Standardization of Data Normalization Strategies to Improve Urinary Metabolomics Studies by GC×GC-TOFMS. *Metabolites* **2020**, *10* (9). https://doi.org/10.3390/metabo10090376.
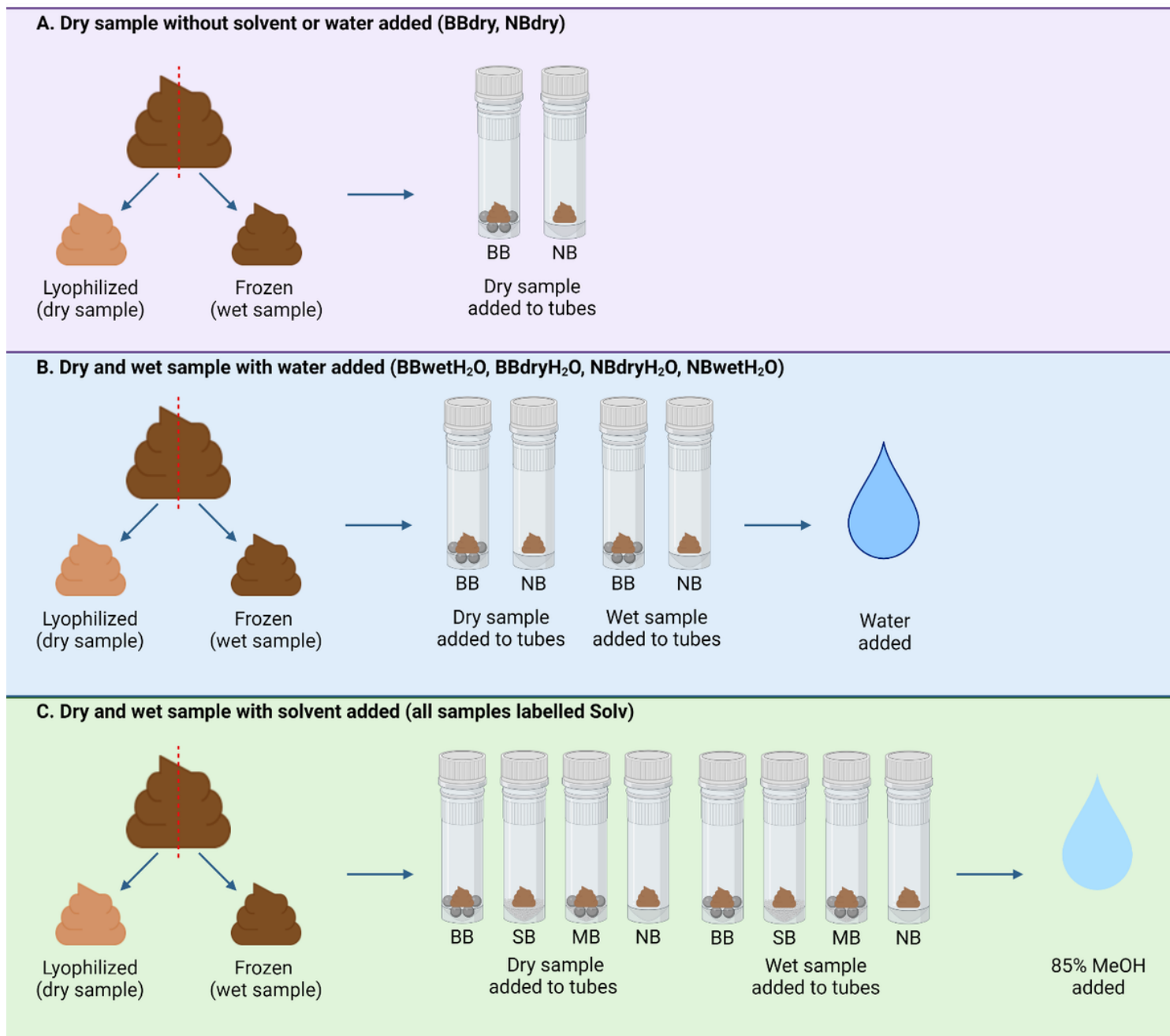
# Figures



Figure 1

Bead types and sizes used for fecal sample preparation. Frozen (wet) and lyophilized (dry) samples were homogenized with big beads (BB), small beads (SB), mixed beads (MB), or no beads (NB)
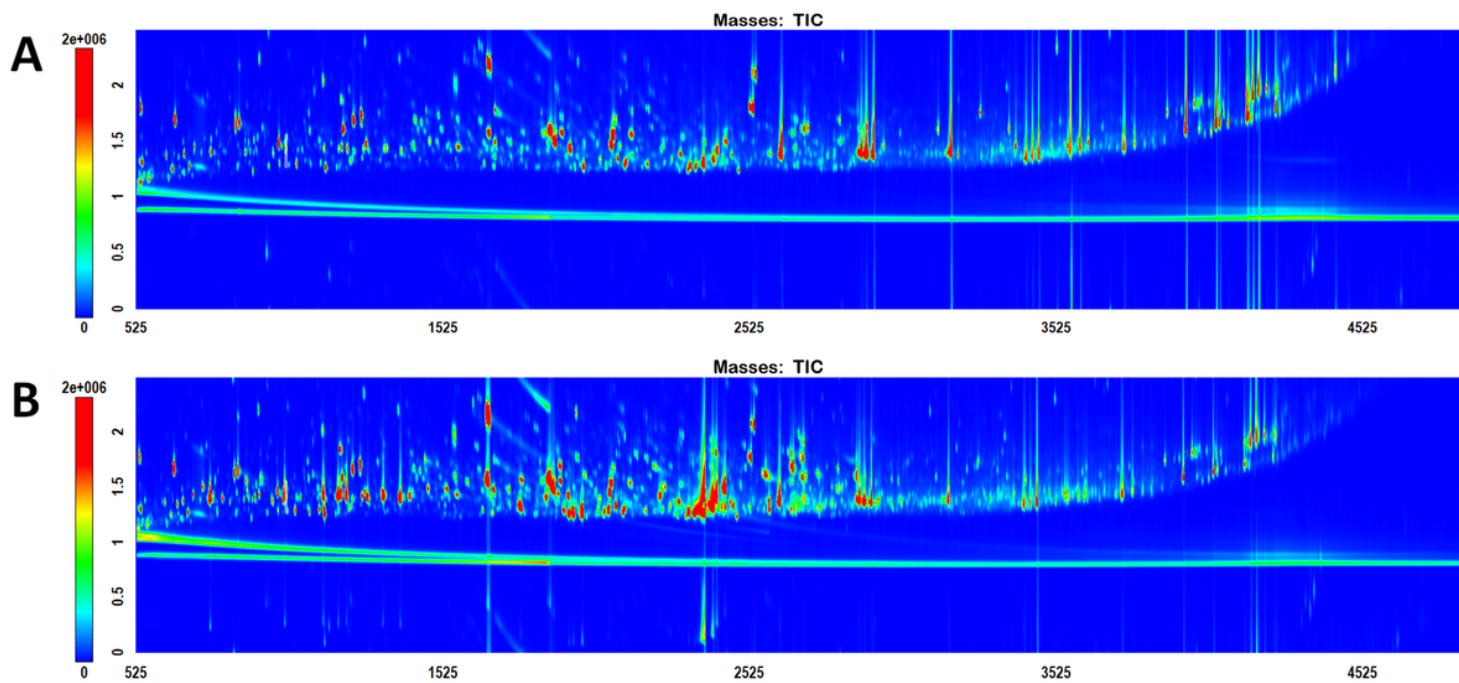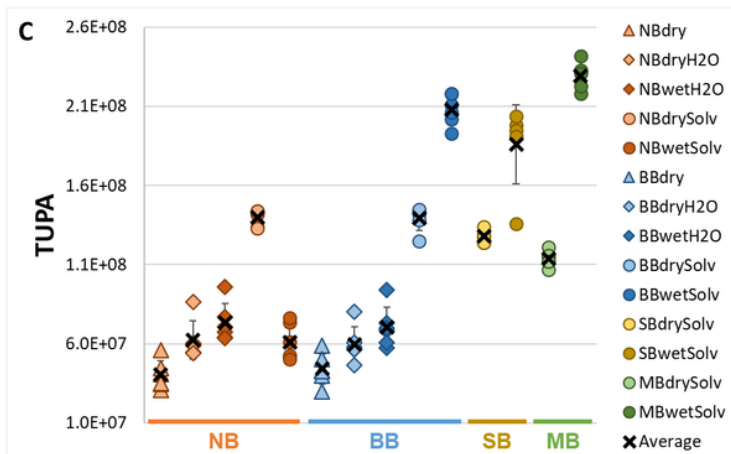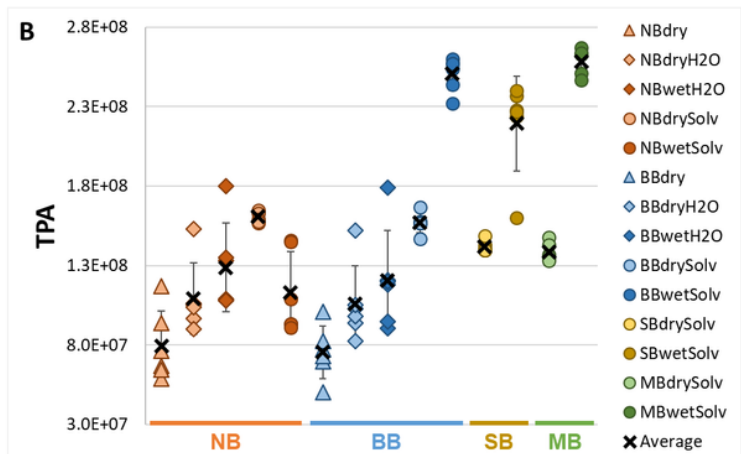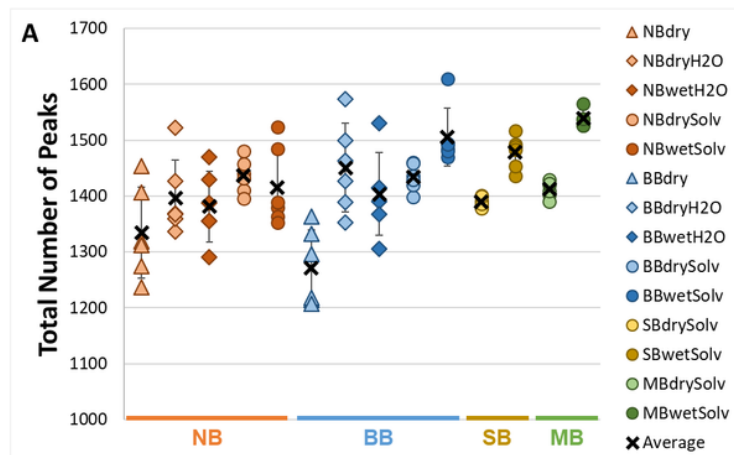


## Figure 2

GC×GC-TOFMS chromatograms **a)** NBdry and **b)**MBwetSolv

**Figure 3**

Scatter plots of **a)** total number of peaks detected, **b)** TPA, **c)** TUPA (*n*=6). Colours were used to differentiate between bead types used: orange – no beads, blue – big beads, yellow – small beads, and green – mixed beads. Dry samples are denoted by lighter shades while wet samples are in darker shades of the respective colour. Shapes were used to differentiate between the solvent used during homogenization: ▲ – no solvent, ◆ – water, ● – 80% MeOH (extraction solvent).

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- BeadHomogenizationSI.xlsx