**Impact of storage conditions on the human stool metabolome and lipidome: preserving
the most accurate fingerprint**

Manuscript_DeSpiegeleer_ACA-19-3520Rev.Highlighted version

Margot De Spiegeleer^a, Marilyn De Graeve^a, Steve Huysman^a, Arno Vanderbeke^a, Lieven
Van Meulebroek^{a,†} and Lynn Vanhaecke^{a,b,†,*}

^aDepartment of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis,
Salisburylaan 133, B-9820 Merelbeke, Belgium

^bInstitute for Global Food Security, School of Biological Sciences, Queen's University,
University Road, Belfast, United Kingdom

Margot.DeSpiegeleer@UGent.be

Marilyn.DeGraeve@UGent.be

Steve.Huysman@UGent.be

Arno.Vanderbeke@KULeuven.be,

Lieven.VanMeulenbroek@UGent.be

[†]Both shared senior authors contributed equally to this work

***Corresponding author**

Prof. Dr. Lynn Vanhaecke

Ghent University, Faculty of Veterinary Medicine

Department of Veterinary Public Health and Food Safety

Laboratory of Chemical Analysis

Salisburylaan 133, B-9820 Merelbeke, Belgium

Tel: +32 9 264 74 57

Fax: +32 9 264 74 92

27 E-mail: Lynn.Vanhaecke@ugent.be

28 **List of abbreviations**

29 GI Gastrointestinal

30 ¹H-NMR Proton nuclear magnetic resonance

31 BCAA Branched-chain amino acids

32 AAA Aromatic amino acids

33 SCFA Short-chain fatty acids

34 BHT butylated hydroxytoluene

35 FT Freeze-thaw

36 UPLC Ultra-high performance liquid chromatography

37 HRMS High-resolution mass spectrometry

38 HESI Heated electrospray ionisation

39 LOESS Locally estimated scatterplot smoothing

40 QC-RLSC QC-based robust (LOESS) signal correction

41 LOESS Locally estimated scatterplot smoothing

42 TIC Total ion current

43 PCA Principal component analysis

44 PCs Principal components

45 OPLS-DA Orthogonal projection to latent structures discriminant analysis

46 AA Amino acids

47 FA Fatty acids

48 PUFA Poly-unsaturated fatty acids

49

50 **ABSTRACT**

51

Faecal metabolomics markedly emerged in clinical as well as analytical chemistry through the unveiling of aberrations in metabolic signatures as reflection of variance in gut (patho)physiology and beyond. Logistic hurdles, however, hinder the analysis of stool samples immediately following collection, inferring the need of biobanking. Yet, the optimum way of storing stool material remains to be determined, in order to conserve an accurate snapshot of the metabolome and circumvent artifacts regarding the disease and parameter(s) under observation. To address this problem, this study scrutinised the impact of freeze-thaw cycling, storage duration, temperature and aerobicity, thereby using ultra-high performance liquid chromatography-high-resolutionmass spectrometry (UPLC-HRMS)-based polar metabolomics and lipidomics methodologies for faecal metabolomics. Both targeted ($n > 400$) and untargeted approaches were implemented to assess storage effects on individual chemical classes of metabolites as well as the faecal fingerprint. In general, recommendations are that intact stool samples should be divided into aliquots, lyophilised and stored at $-80\text{ }^{\circ}\text{C}$ for a period no longer than 18 weeks, and avoiding any freeze-thawing. The first preservation week exerted the most decisive impact, 12.1% and 6.4% of the polar metabolome experienced a shift at $-20\text{ }^{\circ}\text{C}$ and at $-80\text{ }^{\circ}\text{C}$, respectively, whereas 8.6% and 7.9% was observed to be changed significantly for the lipidome. In addition, aside from the negligible impact of aerobicity, the polar metabolome appeared to be more dependent on the storage conditions applied compared to the lipidome, which emerged as the more stable fraction when assessing the storage duration for 25 weeks. If the interest would greatly align with particular chemical classes, such as branched-chain amino acids or short-chain fatty acids, specific storage duration recommendations are reported. The provided insights on the stability of the faecal metabolome may contribute to a more reasoned design of experiments in biomarker detection or pathway elucidation within the field of faecal metabolomics.

Keywords

Faecal fingerprinting; metabolomics; lipidomics; sample storage; ultra-high performance
liquid chromatography-high-resolution mass spectrometry

1. Introduction

The multidisciplinary “omics” field embraces various research disciplines which all seek to define the bewildering complement of analytes within a biological matrix of interest, ranging from what’s encoded downstream in the genome (genomics) to its real-time functional upstream readout (metabolomics) [1,2]. Consequently, metabolites represent the biochemical end points of the “omics” cascade. As such, metabolomics aids the systemic understanding of mechanisms and pathways underlying disease processes to reveal *e.g.* clinically useful biomarkers for diagnosis, prevention and/or prognosis; and enable analysis of response to treatment [1]. Taken together with the current treatment-failure of evidence-based approaches in clinical practice, metabolomics has gained genuine interest and is moving from a novel into a present day concept, *i.e.* by providing clinicians with ‘personalised profiles’ to evolve towards better patient treatment outcomes [1,3,4]

Gastrointestinal (GI) metabolism of the host as well as resident microbiota and, moreover, their synergism, has remarkably broadened knowledge on a variety of diseases [5]. Consequently, the host-microbiome-metabolome axis has been ascribed a pivotal factor within biomedical and clinical sciences. Therefore, stool has received considerable attention as a valuable alternative to blood or urine, whereby gut metabotyping may unravel the mechanistic and responsive framework of the metabolomic sequelae in disease as a deciphering tool of putative biomarkers in diverse pathologies [6–8].

Biochemical discrepancies observed by metabolomics may arise from a large number of sources other than the intended study factor. In particular, ongoing (bio)chemical reactions such as oxidative processes or bacterial activity following sample collection and preservation,

may introduce metabolite conversion leading to deceptive interpretations. Hereto, adequate preservation guidance is imperative for quality assurance, to avoid erroneous interpretation of analytical data and assure the reliability of revealed candidate biomarkers or potential pathways involved in disease. For blood and urine, several studies have been performed to standardise preservation protocols for metabolomics analyses [9,10], whereas for faeces such protocols are less established [11]. Recently, the lack of comprehensive studies on faecal metabolome stability and its importance with regard to clinical applications has been highlighted [11]. Among the relatively small repertoire of studies that are available to date are that of Saric *et al.* [12] and Gratton *et al.* [13], in which metabolite profile variations were investigated upon freeze-thawing for diverse time periods ranging up to about 1 month using ¹H-NMR. Particularly, it was observed that branched-chain amino acids (BCAA), aromatic amino acids (AAA) and short chain fatty acids (SCFA) were altered.

This study addressed the salient shortcomings of metabolic profiling and fingerprinting studies so far, *i.e.* short-term evaluations and applying ¹H-NMR [12,13]. The latter is a less sensitive analytical technique compared to high-resolution mass spectrometry (HRMS), which is regarded most appropriate to achieve optimal metabolome coverage at the border of the chemical complexity in terms of mass range (between 50 – 2000 Da), concentration range and polarity (from very polar to most apolar metabolites) [14]. As such, this study aimed at providing practical recommendations on how to efficiently preserve the holistic faecal metabolome, based on HRMS and for long-term storage. To this end, human stool samples (n = 9) were analysed to assess the effects of storage duration (up to 25 weeks), temperature (-20 °C and -80 °C), oxygen exposure (aerobicity) and freeze-thawing (up to 2 cycles) [9,15].

Ultra-high-performance liquid chromatography (UPLC) hyphenated with Q-ExactiveTM Orbitrap HRMS was utilised for both polar metabolome and lipidome analyses. Metabolic analysis was performed according to in-house validated protocols [7,8], to provide a view on

the stability upon storage across various metabolite classes (towards interpretation of candidate biomarkers) as well as to evaluate the broadscale impact on the stool metabolome.

2. Materials and Methods

2.1. Chemicals and reagents

Analytical standards have been listed in the research paper of De Paepe *et al.* [7] (291 polar metabolites) and Van Meulebroek *et al.* [8] (128 lipids), selected to represent divergent polar chemistries and attain adequate representation of the LIPID MAPS [16] classification structure, respectively. Internal standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), ICN Biomedicals Inc. (Irvine, CA, USA), TLC PharmChem Inc. (Vaughan, Ontario, Canada), Avanti Polar Lipids (Alabama, USA), Toronto Research Chemicals (North York, Canada) and Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). Organic solvents were all of LC-MS grade and purchased from Thermo Fisher Scientific (Loughborough, UK) and VWR International (Merck, Darmstadt, Germany). Ultrapure water (0.055 $\mu\text{S}/\text{cm}$) was acquired by a purified-water system (Sartorius Lab Instrument GmbH, Göttingen, Germany).

2.2. Biological samples and sample handling

The collection of faecal samples of four male and five female volunteers (between 20 and 50 years of age) was performed at Ghent University (Merelbeke, Belgium) and approved by the medical ethics committee of Ghent University Hospital (EC 2016/0673). The study subjects concerned both sexes to account for gender-dependent variations in the fingerprints generated [13], did not receive antibiotics treatment for at least six months prior to sample donation, nor took any medication for treatment of chronic diseases [8].

2.3. Sample extraction

2.3.1. Polar metabolomics

To evaluate the stability of the polar fraction, the validated method described by De Paepe *et al.* [7] was implemented. Briefly, 4 mL ultrapure water and internal standards were added to the faecal aliquots (200 mg, lyophilized material). Subsequent to 30 s of thoroughly vortexing, 1 mL of an ice-cold (-20°C) mixture of methanol and ultrapure water (80:20, v/v) was added, followed by 1 min of vortexing and 10 min of rotation. Next, samples were centrifuged at 9810 g or 10 min (Hettich Rotanta 460R, Merck, Darmstadt, Germany). The supernatant was filtered using a polyamide filter ($\varnothing$ 25 mm, pore size 0.45 μm) (Machery-Nagel, Düren, Germany) and 200 μL of the extract was transferred to an amber glass LC vial of 2 mL. Subsequently, 400 μL ultrapure water was added and the final mixture was vortexed for 30 s.

2.3.2. Lipidomics

Lipidomic extraction was performed according to the optimised and validated method described by Van Meulebroek *et al.* [8] by adding 1200 μL of methanol containing 0.01% butylated hydroxytoluene (BHT) (w/v) (Thermo Fisher Scientific, San José, CA, USA) and internal standards to the faecal aliquots (200 mg, lyophilized material), followed by vortexing for 30 s. Subsequently, 5.4 mL of methyl-tert-butylether (Thermo Fisher Scientific, San José, CA, USA) containing 0.01% BHT (w/v) was added, again followed by a similar vortex step. Hereafter, the samples were incubated for 20 min while shaking at 200 rpm at 20°C (New Brunswick Innova 42, Eppendorf, Aarschot, Belgium). Next, 3 mL of ultrapure water with 2.5% trichloroacetic acid (w/v) (Thermo Fisher Scientific, San José, CA, USA) was added prior to a centrifugation step of 5 min at 1500 G at 20°C , to induce and enforce phase separation. The upper layer of the phased system, *i.e.* 1 mL of methyl-tert-butylether, was

collected and subjected to a gentle stream of nitrogen at 30 °C. The dry residue was resuspended in 250 µL of chloroform and 650 µL of methanol. A 50 µL sub fraction was then transferred to an amber glass LC vial of 2 mL with insert, followed by addition of 50 µL of methanol.

2.4. Analytical instrumentation and quality assurance

UPLC-Quadrupole-Orbitrap HRMS analysis was based on the validated method of Vanden Bussche *et al.* [6] and Van Meulebroek *et al.* [8]. Chromatographic separation was achieved on a Dionex UltiMate 3000 XRS UPLC system (Thermo Fisher Scientific, San José, CA, USA) which was coupled to a benchtop Q-Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionisation source (HESI-II) operating in polarity switching mode. The LC-system was provided with an Acquity HSS T3 C₁₈ column (150 x 2.1 mm, 1.8 µm particle size) and Acquity ethylene-bridged hybrid phenyl C₁₈ column (150 x 2.1 mm, 1.7 µm particle size) (Waters, Manchester, UK) for the polar metabolome and lipidome analyses, respectively. For the latter, a Hypersil Gold column (50 x 2.1 mm x 1.9 µm, Thermo Fisher Scientific, San José, CA, USA) was installed between the LC pump and injection valve, serving as a delay column with the purpose of retarding lipophilic substances originating from the solvent system.

Prior to analysis, calibration of the MS instrumentation for both ionisation modes was performed by injecting calibration mixtures (Thermo Fisher Scientific, San José, CA, USA), to guarantee the accuracy (mass deviations < 3 ppm) of mass measurements. In quintuplicate, a series of QCs was implemented at the beginning of every analytical run, following a blank sample of methanol to ascertain conditioning and stabilisation of the analytical system. Also, duplicate QCs were incorporated throughout the analytical run to allow for monitoring potential instrument drift and within-batch signal correction. In addition, standard mixtures of

target analytes (291 polar (0.25 – 10 ng/μL) and 128 lipid (2 – 100 ng/μL) metabolites) were injected before and after each batch of samples under consideration to monitor the operational conditions of the LC-MS system.

2.4.1. Polar metabolomics analysis

In a randomised order, a 10 μL aliquot per sample was injected for polar metabolomics LC-analysis. A binary solvent system consisting of ultrapure water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, was used at a constant flow rate of 0.4 mL/min. The following proportions (v/v) of solvent A were used to achieve the optimized gradient program: 0-1.5 min at 98%, 1.5-7.0 min from 98% to 75%, 7.0-8.0 min from 75% to 40%, 8.0-12.0 min from 40% to 5%; 12.0-14.0 min at 5%, 14.0-14.1 min from 5 to 98%, followed by 4.0 min of re-equilibration. The column oven temperature was set at 45 °C. MS analysis was performed under the following conditions: m/z scan range of 53.4 - 800 Da, an automatic gain control of 1×10^6 ions, a maximum injection time of 70 ms and mass resolution of 140,000 full width at half maximum. The HESI-II was operated in polarity switching mode (0/B/1 position) with the sheath, auxiliary and sweep gas (N₂) flow rate set at 50, 25 and 5 arbitrary units, the heater and capillary temperature were fixed at 350 °C and 250 °C, and the S-lens RF level and spray voltage were 50 V and +/- 3 kV, respectively. Each spectrum was mass corrected by use of the internal standards, *i.e.* L-alanine-d₃ and D-valine-d₈.

2.4.2. Lipidomics analysis

For lipidomics LC-analysis, a 5 μL aliquot per sample was injected in a randomized order. A binary solvent system of ultrapure water (solvent A) and methanol (solvent B), both acidified with 3.5 mM ammonium acetate, was addressed to establish a gradient elution program at a

constant flow rate of 0.3 mL/min. The following proportions of solvent A (v/v) were applied:
0-1.0 min at 25%, 1.0-2.0 min from 25% to 10%, 2.0-6.0 min from 10% to 2%, 6.0-15.0 min
from 2% to 0%, and 15.0-17.0 min at 0%, followed by 3 min of equilibration at initial
conditions. The column oven temperature was set at 40 °C. MS analysis was performed
scanning the mass range of 67-1000 m/z for both ionisation modes, with an additional scan
range of 1000-2300 Da initiated after 4.5 min to cover high-mass lipid species. For the MS
acquisition, an automatic gain control of 2×10^5 ions, a maximum C-trap injection time of 50
ms, and a mass resolution of 70 000 full width half-maximum peak width were applied. The
HESI-II was operated in polarity switching mode (0/B/1 position), with the sheath, auxiliary
and sweep gas (N_2) flow rate set at 40, 10 and 2 a.u., the heater and capillary temperature
were both fixed at 325 °C and the S-lens RF level and spray voltage were 80 V and +3.5 and -
3.0 kV, respectively. Each mass spectrum was mass corrected by use of the internal standards,
i.e. 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine and palmitic acid-d31.

2.5. Experimental design to study the impact of storage conditions

To reduce the substantial role water activity holds in sample preservation with regard to
microbial and chemical stability, e.g. oxidative and enzymatic reactions, all faecal samples
were routinely lyophilised (removal of $\pm 70\%$ water, Christ 1-4 Alpha LSCplus,) prior to
analysis [8]. Subsequently, in assessing the impact of freeze-thaw (FT) cycling (Figure 1A),
which purports freezing at -80°C for 2 h and subsequent thawing at room temperature for 2.5
h, faecal material was divided into twelve equal aliquots.

In order to evaluate the combined impact of storage temperature and FT cycling, faecal
samples were either analysed immediately (Week 0, W_0) or after four weeks (W_4). Regarding
the long-term storage experimental set-up (Figure 1A), the analysis covered seven subsequent
time-points (W_0 as the reference point, W_1 , W_2 , W_4 , W_8 , W_{18} and W_{25}) during which

(an)aerobic influence was assessed additionally. Anaerobicity was attained by means of vacuum pumping (Alltech). A pooled sample, next to a male and female sample, was included to maximise metabolite coverage without substantially increasing the number of analyses. In this regard, it is to note that the distinct samples were considered ‘biological replicates’ accomodating the inherent biological variation. Thereby, the different samples were not individually compared but the various (combinations of) storage conditions were assessed using mean trends. Upon being subjected to its respective storage condition under consideration, every aliquot was lyophilised (Martin Christ) prior to analysis [8,17]. This step may reduce the substantial role water activity holds in microbial and chemical stability, *e.g.* oxidative and enzymatic reactions.

2.6. Metabolomics data analysis and statistical approaches

Compound Discoverer™ 3.0 and XCalibur 3.0 software (Thermo Fisher Scientific, CA, USA) were used for untargeted and targeted processing of the metabolomics data files, respectively. Multivariate data analysis was performed with R, version 3.4.3 [18].

2.6.1. Multivariate data analysis

As a first step, retention time alignment, feature detection (minimum peak intensity threshold of 1,000,000 a.u. and 2,000,000 a.u. for the polar and lipid analyses, respectively, Supplementary Table S7), and signal drift correction were effectuated [7,8]. The latter relied on the QC-based robust locally estimated scatterplot smoothing (LOESS) signal correction (QC-RLSC) algorithm (NormalizeMets R package) [19]. For the long-term storage data, total ion current (TIC) correction was applied prior to the QC-RLSC algorithm as an additional means of normalisation in counteracting possible time-dependent instrumental variability. Since this stability investigation is, in essence, an untargeted metabolomics study, many

compounds remain unidentified and can, thus, not be included in any standard mixture.
Therefore, normalisation to summed signal or TIC was chosen as this represents the
commonly addressed approach used in metabolomics analyses [20–22].

Multivariate differences between the metabolic fingerprints were assessed by constructing principal component analysis (PCA) models (pcaMethods R package) [23] on the combined positive and negative ionisation data. A variation splitting procedure was performed to separate and assess the true impact of preservation rather than interpersonal variability (mixOmics R package) [24]. Prior to modelling, normality of the metabolic features was assessed using the Shapiro-Wilk test, corrected for multiple comparisons by means of the Holm algorithm [25]. Subsequently, data were log-transformed and pareto-scaled [26]. The optimal number of PCs was determined based on the highest cross-validated (5-fold) Q^2Y after random permutation [27]. Next, pairwise orthogonal projection to latent structures discriminant analysis (OPLS-DA) models were generated (ropls R package) [28]. The construction of OPLS-DA models was divided into three subsequent steps in generating pairwise sub-models concerning the evaluation of aerobicity, storage duration and temperature (elaborated under section 3. Results). During the last step of this modelling strategy, the relative magnitude of change in the metabolic fingerprint between two time-points in each sub-model under consideration was calculated according to a piecewise multivariate modelling approach described by Rantalainen *et al.* [29]. For every univariate paired *t*-tests performed, multiple testing was corrected for by the Benjamini-Hochberg algorithm [30]. The model performance parameters included R^2X , R^2Y , Q^2Y , a permutation test ($n = 100$) and pQ^2 (a measure of over-fit) using a significance level of 5% [31,32]. Lastly, UpSet plots were constructed to visualise combinatorial cumulative storage effects, using the UpSetR R package [32].

2.6.2. Univariate data analysis

Next to the multivariate approach, targeted metabolomics analysis was also performed for which the in-house library of authentic reference standards was used. Target analytes were identified based on retention time relative to that of the authentic reference standard with deviation $\leq 2.5\%$, accurate mass according to m/z -value with mass deviation ≤ 3 ppm and $^{13}\text{C}/^{12}\text{C}$ isotope ratio [33] and can be consulted in Supplementary Figure S1, Table S5 and Supplementary Figure S2, Table S6 for the polar and lipid metabolites, respectively. The normality and homogeneity of variances of each metabolite was controlled by means of the Shapiro-Wilk and Levene's test, respectively (IBM® SPSS® Statistics Version 25). The variability in univariate relative abundance (TIC-normalised) along the applied storage condition was evaluated (presented as fold changes in the Supplementary Tables S1, S2, S3 and S4). The effect of FT cycling was determined by comparing fresh samples (C_0) with samples that had been frozen once or twice (C_0 vs. C_1 and C_0 vs. C_2) by means of paired samples Student's t-tests, for each storage time and temperature (the latter using the samples of W_4 , Figure 1A). For the long-term experimental set-up, all of the pairwise comparisons were obtained through repeated measures ANOVA with subsequent post-hoc tests, *i.e.* paired samples Student's t-tests with Bonferroni adjustment for multiple comparisons. In every comparison made, W_0 served as the reference time-point (inferring no or minimal storage at -80°C).

3. Results

A total number of 6870 and 12250 components for the FT and 13907 and 22469 for the long-term storage experiments were detected for the polar and lipidomic analyses, respectively. The difference in number of extracted components between the two experiments may be

linked to the number of participant donors (faecal pool inclusion in the long-term evaluation) and as such, plausibly introducing more unique (*e.g.* diet-related) metabolites [34].

3.1. Impact of the number of FT cycles

Interpersonal variability represented the dominant fraction of variance for the polar metabolome (Figure 2A), whereas this was not the case for the lipidome ([Figures 2C and 2D](#) and [Figures S3A and S3B](#)). Such source of variance would outperform variation introduced by storage effects on the stool metabolome. After variation splitting, the PCA score plot of the polar metabolome (Figure 2B) clearly revealed that the biological variation between person and associated clustering could almost be completely eliminated. Therefore, the variation splitting procedure was consistently applied prior to modelling. Overall, normal distribution was achieved for at least 77% [components](#) of the polar and lipid fraction of the metabolome. The overall good predictive performance of the OPLS-DA models was indicated by the cumulative Q^2Y metric, inferring that the first FT cycle already substantially impacted the metabolome composition (Table 1). The highest changes observed upon FT cycling corresponded to 10.0% and 16.2% of metabolites across the polar (at W_4 , $-80\text{ }^{\circ}\text{C}$); and 6.8% and 10.5% across the lipid fingerprint (at W_4 , $-20\text{ }^{\circ}\text{C}$) that were significantly affected upon 1 or 2 FT cycles, respectively. Thus, the first FT cycle evoked similar changes in the polar metabolome as did the two FT cycles in the lipidome, concluding greater stability of the latter.

Similarly, the targeted lipids were significantly less affected by FT cycling than the polar profile, *i.e.* 16 out of 62 polar and 3 out of 38 lipid metabolites with P -values < 0.05 , respectively, with more substantial contribution of the second FT cycle ([Table S1](#)). Among the targeted metabolites, an enhancement of the relative peak ratios resulting from both polar metabolomics lipidomics profiling was observed. Hereby, the impact was most obvious for

the amino acids (AA). Nevertheless, taking into consideration the data from the targeted and untargeted approach, the lipidome emerged as the more stable fraction of the metabolome with regard to the number of unaffected analytes, even with repeated FT cycling ([Table 1 and Table S2](#)).

3.2. Impact of long-term storage

For accurate assessment of aerobicity, time and temperature impacts on the metabolome, variation splitting was performed likewise the FT regime. The multivariate data analysis comprised three subsequent steps in order to construct pairwise OPLS-DA sub-models (Figure 1B).

First, pairwise OPLS-DA models per time-point and temperature were established to enable assessment of aerobicity conditions. No significant changes (P -value < 0.05) in the metabolome and lipidome composition were observed upon inducing aerobic or anaerobic conditions, as reinforced by the results of the targeted analyses ([Tables S3 and S4](#)).

The second step involved analogous model building within each time-point to gauge the impact of storage temperature. All OPLS-DA models built were valid and indicated a clear difference in metabolic fingerprints between $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ (Table 2). With regard to the polar metabolome, the first series of change (W_1 , $-20\text{ }^{\circ}\text{C}$ vs. $-80\text{ }^{\circ}\text{C}$) remained decisive during subsequent time-points, whereas a significant change (P -value < 0.05 , 95% CI {1483.26, 1923.74}, based on data from the last column of Table 2) to the number of lipid [components](#) was only noted on W_{25} compared to the first series of changes. Nevertheless, both metabolome fractions experienced a similar impact of storage temperature, *i.e.* metabolome differences varied between 7.0% and 10.8% for the polar metabolome and between 6.9% and 9.4% for the lipidome.

Thirdly, OPLS-DA sub-models between subsequent time-points were assembled per temperature to assess the impact of storage duration (Figure 3). Subsequently, UpSet plots revealed the number of significantly altered components and how many thereof recurred significant across the different pair-wise comparisons (Figure 4). The polar metabolome was altered in a more rigorous way at -20 °C, *i.e.* 12.1% when compared to 6.4% at -80 °C during the first week of preservation. In contrast, for the lipidome, the difference according to temperature was less dominant for the first week of preservation, indicated by a 8.6% and 7.9% metabolome alteration at -20 °C and -80 °C, respectively. The cumulative percentage of significantly altered unique polar components after 4 weeks of storage at -20 °C matched 8 weeks of storage at -80 °C, which was 19.9% and 20.0%, respectively. Similarly, for 8 and 18 weeks of storage at -20 °C and -80 °C, 22.2% and 20.4% of change was observed in the lipidome. The holistic impact at W₁₈ and -80 °C was 23.8% for the polar and 20.4% for the lipidome. The holistic impact at W₂₅ this was 25.8% and 30.9%, respectively. Notably, at W₂₅ the lipidome was altered more severely than the polar metabolome. This was in contrast with previous time-points, but clearly coincided with the significant change observed in the number of lipid components when evaluating storage temperature effects (Table 2) and hence inferring serious interaction effects on the holistic metabolome from beyond W₁₈. Furthermore, when compared to storage at -20 °C, changes in the polar metabolome imparted 32.2%, whereas this was 30.6% for the lipidome and hence indicated a confluence in the stability performance of the polar metabolome and lipidome from W₂₅. Yet, bio-analytical recommendations regarding variance in targeted and untargeted metabolomics analyses, which is generally accepted in the range between 20% and 30% [7,8], and the clustering observed in Figure 3 further encouraged faecal samples to be stored no longer than 18 weeks. The targeted profiling strategy (Table S3) revealed that the concentration levels of various AAs and amines did change significantly during the first week of storage, whereas diverse

polar classes (*e.g.* the alcohols, polyols, carbohydrates, and di-and monocarboxylic acids) exhibited prolonged stability (beyond W₈ for storage at -80 °C). Considering the lipidomics analysis, the class of poly-unsaturated fatty acids (PUFAs), including arachidonic acid, linoleic acid, eicosapentaenoic and docosahexaenoic acid was characterized by decreasing concentration levels from W₁₈ when conserved at -80 °C (similar or starting earlier at -20°C), whereas the SCFA were significantly elevated at this timepoint ([Table S4](#)).

4. Discussion

Notwithstanding the relatively small number of study subjects included, previous stability studies were based on a similar number of participants [9,13,35] and the established OPLS-DA models were able to discriminate according to the storage conditions applied. Chiefly, the observed impact of the FT regime on the metabolome was in line with the results of Gratton *et al.* [13], Zivkovic *et al.* [35] and Breier *et al.* [10], by whom other analytical platforms than UPLC-HRMS were used and hence harmonising holistic metabolome stability findings. Even more, similar metabolites were significantly altered as reported in blood by Breier *et al.* [10], albeit in the opposite direction. For instance, (iso)leucine (BCAA) as well as tryptophan were significantly decreased, whereas the targeted analysis of stool samples revealed elevated levels upon repeated FT cycling as further reinforced by Gratton *et al.* [13] through the detection of increased AAA concentrations including tyrosine and phenylalanine in stool samples. The essential role of gut microbiota in AA metabolism [36], *i.e.* the reactivation of microbial mechanisms upon thawing of samples may advocate these findings. Even more, the increasing number of significantly altered metabolites suggest a plausible interaction effect between FT cycling and storage duration. Particularly for the polar metabolites, the number of FT cycles showed stronger impact after 4 weeks of stool preservation and approximately doubled the number of metabolites that were significantly altered when subjected to two FT

cycles. Opposed to the latter, both untargeted and targeted analyses highlighted the lipidome
 as less affected by FT cycling. Among the altered lipids, *e.g.* the n-3 PUFA eicosapentanoic
 acid, the fold change observed could be ascribed to its characteristic low abundance [35]
 inferring some inherent variability in measurement rather than non-enzymatic oxidation
 reinforced by the inability of detecting docosahexanoic acid.

Regarding the targeted profiling as well as untargeted fingerprinting, almost no effect was
 observed when stool samples were stored in an oxygenated environment compared to
 vacuum. Most likely, the performance of an *a priori* lyophilisation step served to efficiently
 quench bacterial activity and limit oxidative reactions as well as to immobilise enzymatic, *e.g.*
 lipases, and non-enzymatic reactions [9,37]. The freezing temperature (-20 °C and -80 °C)
 and storage duration (W_{0-25}), however, did exert significant effects (P -value < 0.05) on the
 polar and lipid fraction of the metabolome. In this regard, all faecal metabolites consistently
 showcased similar or prolonged stability upon freezing at -80 °C compared to -20 °C, which
 is in agreement with broadscale applied storage approaches in faecal metabolomics [17].

Scrutinising the nature of targeted metabolites that were altered, both enzymatic hydrolysis
 and synthesis as well as reactivated microbial effects were hypothesised as plausible causes
 for the differences observed. For instance, the hydrolysis catalysed by lipases may be revived
 following thawing of the frozen samples due to observed decrease and increase of
 phospholipids and FAs serving as the substrate and hydrolysis product, respectively [35]. The
 decrease in PUFAs, which was also noted in the study of Zivkovic *et al.* [35], could be
 ascribed to the presence of methylene bridges between double bonds of long-chain PUFAs,
 which represent a class of lipids that are more prone to oxidation [35,37]. The discrepancies
 noted in sterol lipids most likely resulted from physical changes to the membrane's structural
 constituents occurring during the freeze-thaw process as this class imparts key players in
 membrane dynamics [38]. Thus, in analogy to the FT cycling, metabolites experienced the

influence of the diverse storage conditions applied in a very consistent manner within their respective class. For instance, glycerolipids and glycerophospholipids were profiled as relatively stable classes compared to FAs, in particular the SCFA, during long-term preservation and as such, stressing the crucial role of gut microbiota in quantitative discrepancies observed in the metabolic (in)stability of stool [39].

As demonstrated before in biofluid metabolome stability studies, the changes attributable to storage were small in comparison to interindividual variation [9,12,13,40]). The initial clustering observed in the polar metabolic fingerprints substantiated the undeniable intimate connection of faecal metabolites with one's unique gut microbiome. In particular, molecules involved in bacteria-host metabolic and immunological cross-talk, *e.g.* SCFA, BCAA, AAA, amines and diverse cytokines, share polar characteristics [41]. Evidently, this gut microbial and associated metabolic dysbiosis is of crucial importance in diseases such as diabetes type 2 and colorectal cancer [8,22]. Despite the individuality of one's microbiota, the functionality resulting thereof is very similar over time according to (patho)physiology. In this regard, the almost complete absence of between-person variation in the lipidome, which consists of rather apolar metabolites, is reflective for such unique host-related changes within health and disease. Thus, this study not only reinforced the clinical value of both fractions of the stool metabolome in light of 'personalised therapies', but also showcased the ability of a multilevel statistical approach based on QC-RLSC normalised data as a valid technique in dealing with the issue of interpersonal variability of the metabolome to focus on the parameters under investigation, *e.g.* storage conditions. However, the variation in the biochemical composition of faeces caused by interindividual differences remains rather limited when compared to that induced by diseases, underpinning the importance of identifying the more variable and stable components of the metabolic signature upon storage. This study expanded current knowledge

regarding the optimum way to store and process stool samples according to the research question imposed and concordant nature of the metabolomics analysis performed.

5. Conclusions

In conclusion, considering practical reality and as a corollary to this research's findings, the prompt storage of lyophilised and aliquoted faecal samples is advised, *i.e.* anticipating at a number of research questions going forward. It should, however, be taken into mind that if the interest would greatly align with particular chemical classes such as the AA and amines for polar metabolomics analysis, storage should ideally not range beyond 4 weeks at -20 °C nor 8 weeks at -80 °C. Similarly for the lipidome, best representation of the FAs would result from storage up to 8 weeks at -20 °C or 18 weeks at -80 °C. Thus, striving to uncover metabolic signatures of disease state and relating those in the most reliable and accurate manner with clinical signs is best performed when stool samples are being stored at -80 °C, which would approximately double the storage length up to 18 weeks regarding the holistic nature of the sample, and avoiding any FT cycle.

Acknowledgments

The authors gratefully acknowledge the statistical expertise provided by Prof. Dr. Vander Heyden (VUB, Brussels) as well as the input of Vicky De Clippele and Charlotte Salens (Ghent University) regarding experimental and formal analysis provided during their master dissertation. This work was supported by Flanders Innovation & Entrepreneurship (VLAIO, IM 150740). De Spiegeleer Margot is supported by Research Foundation – Flanders (FWO 1S57920N).

References

- [1] R.D. Beger, W. Dunn, M.A. Schmidt, S.S. Gross, J.A. Kirwan, M. Cascante, L. Brennan, D.S. Wishart, M. Oresic, T. Hankemeier, D.I. Broadhurst, A.N. Lane, K. Suhre, G. Kastenmüller, S.J. Sumner, I. Thiele, O. Fiehn, R. Kaddurah-Daouk, for “Precision Medicine, Metabolomics enables precision medicine: “A White Paper, Community Perspective,” *Metabolomics*. 12 (2016).
- [2] C.P. Wild, The exposome: From concept to utility, *Int. J. Epidemiol.* 41 (2012) 24–32.
- [3] A. Tebani, L. Abily-Donval, C. Afonso, S. Marret, S. Bekri, Clinical metabolomics: The new metabolic window for inborn errors of metabolism investigations in the post-genomic era, *Int. J. Mol. Sci.* 17 (2016) 1–25.
- [4] F.R. Pinu, S.A. Goldansaz, J. Jaïne, Translational metabolomics: Current challenges and future opportunities, *Metabolites*. 9 (2019).
- [5] C. Rombouts, L.Y. Hemeryck, T. Van Hecke, S. De Smet, W.H. De Vos, L. Vanhaecke, Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat

- discriminating metabolites, *Sci. Rep.* 7 (2017) 1–13.
- [6] J. Vanden Bussche, M. Marzorati, D. Laukens, L. Vanhaecke, Validated High Resolution Mass Spectrometry-Based Approach for Metabolomic Fingerprinting of the Human Gut Phenotype, *Anal. Chem.* 87 (2015) 10927–10934.
- [7] E. De Paepe, L. Van Meulebroek, C. Rombouts, S. Huysman, K. Verplanken, B. Lapauw, J. Wauters, L.Y. Hemeryck, L. Vanhaecke, A validated multi-matrix platform for metabolomic fingerprinting of human urine, feces and plasma using ultra-high performance liquid-chromatography coupled to hybrid orbitrap high-resolution mass spectrometry, *Anal. Chim. Acta.* 1033 (2018) 108–118.
- [8] L. Van Meulebroek, E. De Paepe, V. Vercruysse, B. Pomian, S. Bos, B. Lapauw, L. Vanhaecke, Holistic Lipidomics of the Human Gut Phenotype Using Validated Ultra-High-Performance Liquid Chromatography Coupled to Hybrid Orbitrap Mass Spectrometry, *Anal. Chem.* 89 (2017) 12502–12510.
- [9] J. Laparre, Z. Kaabia, M. Mooney, T. Buckley, M. Sherry, B. Le Bizec, G. Dervilly-Pinel, Impact of storage conditions on the urinary metabolomics fingerprint, *Anal. Chim. Acta.* 951 (2016) 99–107.
- [10] M. Breier, S. Wahl, C. Prehn, M. Fugmann, U. Ferrari, M. Weise, F. Banning, J. Seissler, H. Grallert, J. Adamski, A. Lechner, Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples, *PLoS One.* 9 (2014) 1–11.
- [11] J.A. Kirwan, R. Kaddurah-Daouk, T. Mitchell, T. Pischon, M.A. Schmidt, V. Velagapudi, Biobanking for Metabolomics and Lipidomics in Precision Medicine, *Clin. Chem.* (2019) 1–6.
- [12] J. Saric, Y. Wang, J. Li, M. Coen, J. Utzinger, J.R. Marchesi, J. Keiser, K. Veselkov, J.C. Lindon, J.K. Nicholson, E. Holmes, Species Variation in the Fecal Metabolome Gives Insight into Differential Gastrointestinal Function, *J. Proteome Res.* 7 (2007)

- 539 352–360.
- 540 [13] J. Gratton, J. Phetcharaburanin, B.H. Mullish, H.R.T. Williams, M. Thursz, J.K.
 541 Nicholson, E. Holmes, J.R. Marchesi, J. V. Li, Optimized Sample Handling Strategy
 542 for Metabolic Profiling of Human Feces, *Anal. Chem.* 88 (2016) 4661–4668.
- 543 [14] C. Junot, F. François, C. Benoit, B. François, High resolution mass spectrometry based
 544 techniques at the crossroads of metabolic pathways, *Mass Spectrom. Rev.* 33 (2014)
 545 471–500.
- 546 [15] H.G.M. Tiedink, M.B. Katan, Variability in lipoprotein concentrations in serum after
 547 prolonged storage at -20° C, *Clin. Chim. Acta.* 180 (1989) 147–155.
- 548 [16] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy,
 549 C.R.H. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van
 550 Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, A
 551 comprehensive classification system for lipids, *J. Lipid Res.* 46 (2005) 839–862.
- 552 [17] M.M. Ulaszewska, C.H. Weinert, A. Trimigno, R. Portmann, C. Andres Lacueva, R.
 553 Badertscher, L. Brennan, C. Brunius, A. Bub, F. Capozzi, M. Cialiè Rosso, C.E.
 554 Cordero, H. Daniel, S. Durand, B. Egert, P.G. Ferrario, E.J.M. Feskens, P. Franceschi,
 555 M. Garcia-Aloy, F. Giacomoni, P. Giesbertz, R. González-Domínguez, K. Hanhineva,
 556 L.Y. Hemeryck, J. Kopka, S.E. Kulling, R. Llorach, C. Manach, F. Mattivi, C. Migné,
 557 L.H. Münger, B. Ott, G. Picone, G. Pimentel, E. Pujos-Guillot, S. Riccadonna, M.J.
 558 Rist, C. Rombouts, J. Rubert, T. Skurk, P.S.C. Sri Harsha, L. Van Meulebroek, L.
 559 Vanhaecke, R. Vázquez-Fresno, D. Wishart, G. Vergères, *Nutrimetabolomics: An*
 560 *Integrative Action for Metabolomic Analyses in Human Nutritional Studies*, *Mol. Nutr.*
 561 *Food Res.* 1800384 (2018) 1–38.
- 562 [18] R Core Team, R: A language and environment for statistical computing, (2013).
 563 <http://www.r-project.org/>.

- [19] A.M. De Livera, G. Olshansk, NormalizeMets: Analysis of Metabolomics Data, (2018). <https://cran.r-project.org/package=NormalizeMets>.
- [20] D. Weindl, A. Wegner, C. Jäger, K. Hiller, Isotopologue ratio normalization for non-targeted metabolomics, *J. Chromatogr. A*. 1389 (2015) 112–119.
- [21] E.A. Palmer, H.J. Cooper, W.B. Dunn, Investigation of the 12-Month Stability of Dried Blood and Urine Spots Applying Untargeted UHPLC-MS Metabolomic Assays, *Anal. Chem.* 91 (2019) 14306–14313.
- [22] C. Rombouts, M. De Spiegeleer, L. Van Meulebroek, W.H. De Vos, L. Vanhaecke, Validated comprehensive metabolomics and lipidomics analysis of colon tissue and cell lines, *Anal. Chim. Acta*. 1066 (2019) 79–92.
- [23] W. Stacklies, H. Redestig, M. Scholz, D. Walther, J. Selbig, pcaMethods - A bioconductor package providing PCA methods for incomplete data, *Bioinformatics*. 23 (2007) 1164–1167.
- [24] L.C. Kim-Anh, F. Rohart, I. Gonzalez, S. Dejean, mixOmics: Omics Data Integration Project, (2017). <https://cran.r-project.org/package=mixOmics>.
- [25] S. Holm, A simple sequentially rejective multiple test procedure, *Scand. J. Stat.* 6 (1979) 65–70.
- [26] D.M. Rocke, B. Durbin, Approximate variance-stabilizing transformations for gene-expression microarray data, *Bioinformatics*. 19 (2003) 966–972.
- [27] D. Giancarlo, T. Chiara, Cross-validation Methods in Principal Component Analysis: A Comparison, *Stat. Methods Appl.* 11 (2002) 71–82.
- [28] E.A. Thévenot, A. Roux, Y. Xu, E. Ezan, C. Junot, Analysis of the Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and Gender by Implementing a Comprehensive Workflow for Univariate and OPLS Statistical Analyses, *J. Proteome Res.* 14 (2015) 3322–3335.

- [29] M. Rantalainen, O. Cloarec, T.M.D. Ebbels, T. Lundstedt, J.K. Nicholson, E. Holmes, J. Trygg, Piecewise multivariate modelling of sequential metabolic profiling data, *BMC Bioinformatics*. 9 (2008) 1–13.
- [30] A. Alonso, S. Marsal, A. Juliá, Analytical Methods in Untargeted Metabolomics: State of the Art in 2015, *Front. Bioeng. Biotechnol.* 3 (2015) 1–20.
- [31] E. Szymańska, E. Saccenti, A.K. Smilde, J.A. Westerhuis, Double-check: Validation of diagnostic statistics for PLS-DA models in metabolomics studies, *Metabolomics*. 8 (2012) 3–16.
- [32] J.R. Conway, A. Lex, N. Gehlenborg, UpSetR: An R Package for the Visualization of Intersecting Sets and their Properties, (2017).
- [33] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W.M. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J.J. Thaden, M.R. Viant, Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI), *Metabolomics*. 3 (2007) 211–221.
- [34] G.D. Wu, C. Compher, E.Z. Chen, S.A. Smith, R.D. Shah, C. Chehoud, L.G. Albenberg, L. Nessel, E. Gilroy, A.M. Weljie, H.J. Flint, D.C. Metz, M.J. Bennett, H. Li, F.D. Bushman, J.D. Lewis, Comparative metabolomics in vegan and omnivores reveal constraints on diet-dependent gut microbiota metabolite production, *Gut*. 65 (2016) 63–72.
- [35] A.M. Zivkovic, M.M. Wiest, U.T. Nguyen, R. Davis, S.M. Watkins, J.B. German, Effects of sample handling and storage on quantitative lipid analysis in human serum, *Metabolomics*. 5 (2009) 507–516.
- [36] L. Zhao, Y. Huang, L. Lu, W. Yang, T. Huang, Z. Lin, C. Lin, H. Kwan, H.L.X. Wong,

- Y. Chen, S. Sun, X. Xie, X. Fang, H. Yang, J. Wang, L. Zhu, Z. Bian, Saturated long-chain fatty acid-producing bacteria contribute to enhanced colonic motility in rats, *Microbiome*. 6 (2018) 1–16.
- [37] M.D. Rudy, M.J. Kainz, M. Graeve, S.M. Colombo, M.T. Arts, Handling and storage procedures have variable effects on fatty acid content in fishes with different lipid quantities, *PLoS One*. 11 (2016).
- [38] E.J. Dufourc, Sterols and membrane dynamics, *J. Chem. Biol.* 1 (2008) 63–77.
- [39] G. den Besten, K. van Eunen, A.K. Groen, K. Venema, D.-J. Reijngoud, B.M. Bakker, The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism, *J. Lipid Res.* 54 (2013) 2325–2340.
- [40] M. Bictash, T.M. Ebbels, Q. Chan, R.L. Loo, I.K.S. Yap, I.J. Brown, M. De Iorio, M.L. Daviglus, E. Holmes, J. Stamler, J.K. Nicholson, P. Elliott, Opening up the “black box”: Metabolic phenotyping and metabolome-wide association studies in epidemiology, *J. Clin. Epidemiol.* 63 (2010) 970–979.
- [41] M.X. Chen, S.Y. Wang, C.H. Kuo, I.L. Tsai, Metabolome analysis for investigating host-gut microbiota interactions, *J. Formos. Med. Assoc.* 118 (2019) S10–S22.

Tables & Figures

Table 1. OPLS-DA model performance parameters for the various combinations of storage duration, temperature and FT cycles for the polar metabolome (above the dotted line) and lipidome (below the dotted line). The number of orthogonal and predictive components was 1 (i.e. $t_p + t_o$; 1 + 1). The last column covers the number of significantly altered [components](#) (paired Student's t -test, P -value < 0.05).

<i>Week</i>	<i>T (°C)</i>	<i>FT cycles</i>	<i>R²X (cum)</i>	<i>R²Y (cum)</i>	<i>Q²Y (cum)</i>	<i>P</i>	<i>pQ²</i>	<i>#significant</i>
<u>Polar metabolome</u>								
0		0 vs. 1	0.541	0.990	0.748	0.147	0.11	611
0		0 vs. 2	0.638	0.996	0.885	0.079	0.07	1025
4	−20	0 vs. 1	0.539	0.993	0.718	0.125	0.09	415
4	−20	0 vs. 2	0.675	0.999	0.939	0.096	0.09	949
4	−80	0 vs. 1	0.484	0.999	0.781	0.102	0.08	688
4	−80	0 vs. 2	0.570	0.999	0.951	0.095	0.09	1113
<u>Lipidome</u>								
0		0 vs. 1	0.493	0.995	0.756	0.132	0.10	812
0		0 vs. 2	0.562	0.996	0.888	0.158	0.14	610
4	−20	0 vs. 1	0.487	0.994	0.807	0.136	0.11	831
4	−20	0 vs. 2	0.594	0.998	0.933	0.150	0.14	1281
4	−80	0 vs. 1	0.440	0.996	0.792	0.101	0.08	637
4	−80	0 vs. 2	0.599	0.999	0.911	0.088	0.08	890

Table 2. OPLS-DA model performance parameters for the comparison of storage temperature (-20 °C vs. -80 °C) for the polar metabolome and lipidome. The number of predictive and orthogonal components was 1 (*i.e.* $t_p + t_o$; 1 + 1). The last column covers the number of significantly altered components (paired Student's *t*-test, *P*-value < 0.05).

<i>Week</i>	<i>R²X (cum)</i>	<i>R²Y (cum)</i>	<i>Q²Y (cum)</i>	<i>P</i>	<i>pQ²</i>	<i>#significant</i>
<u>Polar metabolome</u>						
1	0.227	0.999	0.763	0.013	<0.05	1426
2	0.228	0.999	0.774	0.013	<0.05	1507
4	0.194	0.999	0.605	0.017	<0.05	980
8	0.257	0.999	0.775	0.013	<0.05	1464
18	0.220	0.999	0.757	0.026	<0.05	1318
25	0.347	0.998	0.775	0.013	<0.05	1493
<u>Lipidome</u>						
1	0.268	0.999	0.543	0.018	<0.05	1543
2	0.282	0.999	0.560	0.018	<0.05	1586
4	0.289	0.999	0.598	0.017	<0.05	1637
8	0.289	0.999	0.565	0.018	<0.05	1645
18	0.280	0.999	0.584	0.017	<0.05	1691
25	0.322	0.999	0.758	0.013	<0.05	2119

Figure 1.

Figure 2.

650

651

652 **Figure 3.**

653

654 **Figure 4.**

655

656 **Figure captions**

Figure 1.A. Overview of the workflow for stool preparation and procedures for analysis. Dashed arrows indicate that the applied procedures were the same as in the already depicted workflow. **B.** Data analysis strategy. The single arrows indicate OPLS-DA models within and between time-points to study the effect of aerobic vs. anaerobic storage and to explore the storage duration effects, respectively. The double arrows indicate OPLS-DA models to assess the impact of storage temperature within time-points (W_0 = common reference point).

Figure 2. PCA score plots following polar metabolomics (A & B) and lipidomics (C & D) fingerprinting for all samples (W_0 analysed immediately and W_4 preserved at -20 °C and -80 °C) to visualise freeze-thaw effects. The largest ellipse is a Hotelling's T^2 95% confidence ellipse on all samples, whereas the smaller ellipses represent 95% confidence intervals based on multivariate t -distribution on the samples per person. **A.** Initial PCA score plot without separation of between-person variation, with data preprocessing and pretreatment, i.e. QC-RLSC normalisation, log transformation and pareto scaling. **B.** PCA score plot with between-person variation splitting and subsequent data preprocessing and pretreatment steps. **C.** Initial PCA score plot for W_0 and W_4 without separation of the between-person variation, with data preprocessing and pretreatment, i.e. QC-RLSC normalisation, log transformation and pareto scaling. **D.** PCA score plot for W_0 and W_4 with between-person variation splitting and subsequent preprocessing and pretreatment steps.

Figure 3. PCA score plots to visualise trends of the long-term storage duration separately according to storage temperature and the type of analysis performed. The largest ellipse is a Hotelling's T^2 95% confidence ellipse for all samples whereas the smaller ellipses represent 95% confidence intervals based on the multivariate t -distribution for the samples per week. **A.** Polar metabolomics, at -20 °C, **B.** Polar metabolomics, at -80 °C, **C.** Lipidomics, at -20 °C, **D.** Lipidomics, at -80 °C.

Figure 4. UpSet plots reflecting the intersections of modified components upon storage duration for every combination of preservation temperature and type of analysis, **A.** Polar metabolomics at -20 °C, **B.** Polar metabolomics at -80 °C, **C.** Lipidomics at -20 °C, **D.** Lipidomics at -80 °C. The total number of significantly altered components per pairwise t-test (P-value < 0.05), the latter denoted as ‘a set’, is presented at the left-side of the horizontal bars, bottom left. The number of shared altered components, i.e. those in common between the compared sets, are indicated above the vertical bars and visualised by connecting the respective black dots. Bars that are accompanied by only a single dot represent the number of significantly altered components that are unique for that set.

Figure
[Click here to download high resolution image](#)

Figure
[Click here to download high resolution image](#)

Figure
[Click here to download high resolution image](#)

Figure

[Click here to download high resolution image](#)

Electronic Supplementary Material (online publication only)

[Click here to download Electronic Supplementary Material \(online publication only\): Supplementary data.docx](#)

Impact of storage conditions on the human stool metabolome and lipidome: preserving the most accurate fingerprint

Margot De Spiegeleer^a, Marilyn De Graeve^a, Steve Huysman^a, Arno Vanderbeke^a, Lieven Van Meulebroek^{a,†} and Lynn Vanhaecke^{a,b,†,*}

^aDepartment of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium
^bInstitute for Global Food Security, School of Biological Sciences, Queen’s University, University Road, Belfast, United Kingdom

Margot.DeSpiegeleer@UGent.be
Marilyn.DeGraeve@UGent.be
Steve.Huysman@UGent.be
Arno.Vanderbeke@KULeuven.be,
Lieven.VanMeulenbroek@UGent.be

[†]Both shared senior authors contributed equally to this work

***Corresponding author**

Prof. Dr. Lynn Vanhaecke
Ghent University, Faculty of Veterinary Medicine
Department of Veterinary Public Health and Food Safety
Laboratory of Chemical Analysis
Salisburylaan 133, B-9820 Merelbeke, Belgium
Tel: +32 9 264 74 57

26 Fax: +32 9 264 74 92

27 E-mail: Lynn.Vanhaecke@ugent.be

28 List of abbreviations

29 GI Gastrointestinal

30 ¹H-NMR Proton nuclear magnetic resonance

31 BCAA Branched-chain amino acids

32 AAA Aromatic amino acids

33 SCFA Short-chain fatty acids

34 BHT butylated hydroxytoluene

35 FT Freeze-thaw

36 UPLC Ultra-high performance liquid chromatography

37 HRMS High-resolution mass spectrometry

38 HESI Heated electrospray ionisation

39 LOESS Locally estimated scatterplot smoothing

40 QC-RLSC QC-based robust (LOESS) signal correction

41 LOESS Locally estimated scatterplot smoothing

42 TIC Total ion current

43 PCA Principal component analysis

44 PCs Principal components

45 OPLS-DA Orthogonal projection to latent structures discriminant analysis

46 AA Amino acids

47 FA Fatty acids

48 PUFA Poly-unsaturated fatty acids

49

50 ABSTRACT

51

1
2 52 Faecal metabolomics markedly emerged in clinical as well as analytical chemistry through the
3
4 53 unveiling of aberrations in metabolic signatures as reflection of variance in gut
5
6
7 54 (patho)physiology and beyond. Logistic hurdles, however, hinder the analysis of stool
8
9
10 55 samples immediately following collection, inferring the need of biobanking. Yet, the optimum
11
12 56 way of storing stool material remains to be determined, in order to conserve an accurate
13
14 57 snapshot of the metabolome and circumvent artifacts regarding the disease and parameter(s)
15
16
17 58 under observation. To address this problem, this study scrutinised the impact of freeze-thaw
18
19 59 cycling, storage duration, temperature and aerobicity, thereby using ultra-high performance
20
21 60 liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS)-based polar
22
23
24 61 metabolomics and lipidomics methodologies for faecal metabolomics. Both targeted ($n > 400$)
25
26 62 and untargeted approaches were implemented to assess storage effects on individual chemical
27
28
29 63 classes of metabolites as well as the faecal fingerprint. In general, recommendations are that
30
31 64 intact stool samples should be divided into aliquots, lyophilised and stored at $-80\text{ }^{\circ}\text{C}$ for a
32
33
34 65 period no longer than 18 weeks, and avoiding any freeze-thawing. The first preservation week
35
36 66 exerted the most decisive impact, 12.1% and 6.4% of the polar metabolome experienced a
37
38
39 67 shift at $-20\text{ }^{\circ}\text{C}$ and at $-80\text{ }^{\circ}\text{C}$, respectively, whereas 8.6% and 7.9% was observed to be
40
41 68 changed significantly for the lipidome. In addition, aside from the negligible impact of
42
43
44 69 aerobicity, the polar metabolome appeared to be more dependent on the storage conditions
45
46 70 applied compared to the lipidome, which emerged as the more stable fraction when assessing
47
48
49 71 the storage duration for 25 weeks. If the interest would greatly align with particular chemical
50
51 72 classes, such as branched-chain amino acids or short-chain fatty acids, specific storage
52
53 73 duration recommendations are reported. The provided insights on the stability of the faecal
54
55
56 74 metabolome may contribute to a more reasoned design of experiments in biomarker detection
57
58 75 or pathway elucidation within the field of faecal metabolomics.

Keywords

Faecal fingerprinting; metabolomics; lipidomics; sample storage; ultra-high performance liquid chromatography-high-resolution mass spectrometry

1. Introduction

The multidisciplinary “omics” field embraces various research disciplines which all seek to define the bewildering complement of analytes within a biological matrix of interest, ranging from what’s encoded downstream in the genome (genomics) to its real-time functional upstream readout (metabolomics) [1,2]. Consequently, metabolites represent the biochemical end points of the “omics” cascade. As such, metabolomics aids the systemic understanding of mechanisms and pathways underlying disease processes to reveal *e.g.* clinically useful biomarkers for diagnosis, prevention and/or prognosis; and enable analysis of response to treatment [1]. Taken together with the current treatment-failure of evidence-based approaches in clinical practice, metabolomics has gained genuine interest and is moving from a novel into a present day concept, *i.e.* by providing clinicians with ‘personalised profiles’ to evolve towards better patient treatment outcomes [1,3,4]

Gastrointestinal (GI) metabolism of the host as well as resident microbiota and, moreover, their synergism, has remarkably broadened knowledge on a variety of diseases [5]. Consequently, the host-microbiome-metabolome axis has been ascribed a pivotal factor within biomedical and clinical sciences. Therefore, stool has received considerable attention as a valuable alternative to blood or urine, whereby gut metabotyping may unravel the mechanistic and responsive framework of the metabolomic sequelae in disease as a deciphering tool of putative biomarkers in diverse pathologies [6–8].

Biochemical discrepancies observed by metabolomics may arise from a large number of sources other than the intended study factor. In particular, ongoing (bio)chemical reactions

such as oxidative processes or bacterial activity following sample collection and preservation, may introduce metabolite conversion leading to deceptive interpretations. Hereto, adequate preservation guidance is imperative for quality assurance, to avoid erroneous interpretation of analytical data and assure the reliability of revealed candidate biomarkers or potential pathways involved in disease. For blood and urine, several studies have been performed to standardise preservation protocols for metabolomics analyses [9,10], whereas for faeces such protocols are less established [11]. Recently, the lack of comprehensive studies on faecal metabolome stability and its importance with regard to clinical applications has been highlighted [11]. Among the relatively small repertoire of studies that are available to date are that of Saric *et al.* [12] and Gratton *et al.* [13], in which metabolite profile variations were investigated upon freeze-thawing for diverse time periods ranging up to about 1 month using ¹H-NMR. Particularly, it was observed that branched-chain amino acids (BCAA), aromatic amino acids (AAA) and short chain fatty acids (SCFA) were altered.

This study addressed the salient shortcomings of metabolic profiling and fingerprinting studies so far, *i.e.* short-term evaluations and applying ¹H-NMR [12,13]. The latter is a less sensitive analytical technique compared to high-resolution mass spectrometry (HRMS), which is regarded most appropriate to achieve optimal metabolome coverage at the border of the chemical complexity in terms of mass range (between 50 – 2000 Da), concentration range and polarity (from very polar to most apolar metabolites) [14]. As such, this study aimed at providing practical recommendations on how to efficiently preserve the holistic faecal metabolome, based on HRMS and for long-term storage. To this end, human stool samples (n = 9) were analysed to assess the effects of storage duration (up to 25 weeks), temperature (-20 °C and -80 °C), oxygen exposure (aerobicity) and freeze-thawing (up to 2 cycles) [9,15].

Ultra-high-performance liquid chromatography (UPLC) hyphenated with Q-ExactiveTM Orbitrap HRMS was utilised for both polar metabolome and lipidome analyses. Metabolic

analysis was performed according to in-house validated protocols [7,8], to provide a view on the stability upon storage across various metabolite classes (towards interpretation of candidate biomarkers) as well as to evaluate the broadscale impact on the stool metabolome.

2. Materials and Methods

2.1. Chemicals and reagents

Analytical standards have been listed in the research paper of De Paepe *et al.* [7] (291 polar metabolites) and Van Meulebroek *et al.* [8] (128 lipids), selected to represent divergent polar chemistries and attain adequate representation of the LIPID MAPS [16] classification structure, respectively. Internal standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), ICN Biomedicals Inc. (Irvine, CA, USA), TLC PharmChem Inc. (Vaughan, Ontario, Canada), Avanti Polar Lipids (Alabama, USA), Toronto Research Chemicals (North York, Canada) and Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). Organic solvents were all of LC-MS grade and purchased from Thermo Fisher Scientific (Loughborough, UK) and VWR International (Merck, Darmstadt, Germany). Ultrapure water (0.055 $\mu\text{S}/\text{cm}$) was acquired by a purified-water system (Sartorius Lab Instrument GmbH, Göttingen, Germany).

2.2. Biological samples and sample handling

The collection of faecal samples of four male and five female volunteers (between 20 and 50 years of age) was performed at Ghent University (Merelbeke, Belgium) and approved by the medical ethics committee of Ghent University Hospital (EC 2016/0673). The study subjects concerned both sexes to account for gender-dependent variations in the fingerprints generated [13], did not receive antibiotics treatment for at least six months prior to sample donation, nor took any medication for treatment of chronic diseases [8].

2.3. Sample extraction

2.3.1. Polar metabolomics

To evaluate the stability of the polar fraction, the validated method described by De Paepe *et al.* [7] was implemented. Briefly, 4 mL ultrapure water and internal standards were added to the faecal aliquots (200 mg, lyophilized material). Subsequent to 30 s of thoroughly vortexing, 1 mL of an ice-cold (−20 °C) mixture of methanol and ultrapure water (80:20, v/v) was added, followed by 1 min of vortexing and 10 min of rotation. Next, samples were centrifuged at 9810 g or 10 min (Hettich Rotanta 460R, Merck, Darmstadt, Germany). The supernatant was filtered using a polyamide filter (ø 25 mm, pore size 0.45 µm) (Machery-Nagel, Düren, Germany) and 200 µL of the extract was transferred to an amber glass LC vial of 2 mL. Subsequently, 400 µL ultrapure water was added and the final mixture was vortexed for 30 s.

2.3.2. Lipidomics

Lipidomic extraction was performed according to the optimised and validated method described by Van Meulebroek *et al.* [8] by adding 1200 µL of methanol containing 0.01% butylated hydroxytoluene (BHT) (w/v) (Thermo Fisher Scientific, San José, CA, USA) and internal standards to the faecal aliquots (200 mg, lyophilized material), followed by vortexing for 30 s. Subsequently, 5.4 mL of methyl-tert-butylether (Thermo Fisher Scientific, San José, CA, USA) containing 0.01% BHT (w/v) was added, again followed by a similar vortex step. Hereafter, the samples were incubated for 20 min while shaking at 200 rpm at 20 °C (New Brunswick Innova 42, Eppendorf, Aarschot, Belgium). Next, 3 mL of ultrapure water with 2.5% trichloroacetic acid (w/v) (Thermo Fisher Scientific, San José, CA, USA) was added prior to a centrifugation step of 5 min at 1500 G at 20 °C, to induce and enforce phase

separation. The upper layer of the phased system, *i.e.* 1 mL of methyl-tert-butylether, was collected and subjected to a gentle stream of nitrogen at 30 °C. The dry residue was resuspended in 250 µL of chloroform and 650 µL of methanol. A 50 µL sub fraction was then transferred to an amber glass LC vial of 2 mL with insert, followed by addition of 50 µL of methanol.

2.4. Analytical instrumentation and quality assurance

UPLC-Quadrupole-Orbitrap HRMS analysis was based on the validated method of Vanden Bussche *et al.* [6] and Van Meulebroek *et al.* [8]. Chromatographic separation was achieved on a Dionex UltiMate 3000 XRS UPLC system (Thermo Fisher Scientific, San José, CA, USA) which was coupled to a benchtop Q-Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionisation source (HESI-II) operating in polarity switching mode. The LC-system was provided with an Acquity HSS T3 C₁₈ column (150 x 2.1 mm, 1.8 µm particle size) and Acquity ethylene-bridged hybrid phenyl C₁₈ column (150 x 2.1 mm, 1.7 µm particle size) (Waters, Manchester, UK) for the polar metabolome and lipidome analyses, respectively. For the latter, a Hypersil Gold column (50 x 2.1 mm x 1.9 µm, Thermo Fisher Scientific, San José, CA, USA) was installed between the LC pump and injection valve, serving as a delay column with the purpose of retarding lipophilic substances originating from the solvent system.

Prior to analysis, calibration of the MS instrumentation for both ionisation modes was performed by injecting calibration mixtures (Thermo Fisher Scientific, San José, CA, USA), to guarantee the accuracy (mass deviations < 3 ppm) of mass measurements. In quintuplicate, a series of QCs was implemented at the beginning of every analytical run, following a blank sample of methanol to ascertain conditioning and stabilisation of the analytical system. Also, duplicate QCs were incorporated throughout the analytical run to allow for monitoring

potential instrument drift and within-batch signal correction. In addition, standard mixtures of target analytes (291 polar (0.25 – 10 ng/μL) and 128 lipid (2 – 100 ng/μL) metabolites) were injected before and after each batch of samples under consideration to monitor the operational conditions of the LC-MS system.

2.4.1. Polar metabolomics analysis

In a randomised order, a 10 μL aliquot per sample was injected for polar metabolomics LC-analysis. A binary solvent system consisting of ultrapure water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, was used at a constant flow rate of 0.4 mL/min. The following proportions (v/v) of solvent A were used to achieve the optimized gradient program: 0-1.5 min at 98%, 1.5-7.0 min from 98% to 75%, 7.0-8.0 min from 75% to 40%, 8.0-12.0 min from 40% to 5%; 12.0-14.0 min at 5%, 14.0-14.1 min from 5 to 98%, followed by 4.0 min of re-equilibration. The column oven temperature was set at 45 °C. MS analysis was performed under the following conditions: m/z scan range of 53.4 - 800 Da, an automatic gain control of 1×10^6 ions, a maximum injection time of 70 ms and mass resolution of 140,000 full width at half maximum. The HESI-II was operated in polarity switching mode (0/B/1 position) with the sheath, auxiliary and sweep gas (N₂) flow rate set at 50, 25 and 5 arbitrary units, the heater and capillary temperature were fixed at 350 °C and 250 °C, and the S-lens RF level and spray voltage were 50 V and +/- 3 kV, respectively. Each spectrum was mass corrected by use of the internal standards, *i.e.* L-alanine-d₃ and D-valine-d₈.

2.4.2. Lipidomics analysis

For lipidomics LC-analysis, a 5 μL aliquot per sample was injected in a randomized order. A binary solvent system of ultrapure water (solvent A) and methanol (solvent B), both acidified

with 3.5 mM ammonium acetate, was addressed to establish a gradient elution program at a constant flow rate of 0.3 mL/min. The following proportions of solvent A (v/v) were applied: 0-1.0 min at 25%, 1.0-2.0 min from 25% to 10%, 2.0-6.0 min from 10% to 2%, 6.0-15.0 min from 2% to 0%, and 15.0-17.0 min at 0%, followed by 3 min of equilibration at initial conditions. The column oven temperature was set at 40 °C. MS analysis was performed scanning the mass range of 67-1000 m/z for both ionisation modes, with an additional scan range of 1000-2300 Da initiated after 4.5 min to cover high-mass lipid species. For the MS acquisition, an automatic gain control of 2×10^5 ions, a maximum C-trap injection time of 50 ms, and a mass resolution of 70 000 full width half-maximum peak width were applied. The HESI-II was operated in polarity switching mode (0/B/1 position), with the sheath, auxiliary and sweep gas (N₂) flow rate set at 40, 10 and 2 a.u., the heater and capillary temperature were both fixed at 325 °C and the S-lens RF level and spray voltage were 80 V and +3.5 and - 3.0 kV, respectively. Each mass spectrum was mass corrected by use of the internal standards, *i.e.* 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine and palmitic acid-d31.

2.5. Experimental design to study the impact of storage conditions

To reduce the substantial role water activity holds in sample preservation with regard to microbial and chemical stability, *e.g.* oxidative and enzymatic reactions, all faecal samples were routinely lyophilised (removal of $\pm 70\%$ water, Christ 1-4 Alpha LSCplus,) prior to analysis [8]. Subsequently, in assessing the impact of freeze-thaw (FT) cycling (Figure 1A), which purports freezing at -80°C for 2 h and subsequent thawing at room temperature for 2.5 h, faecal material was divided into twelve equal aliquots.

In order to evaluate the combined impact of storage temperature and FT cycling, faecal samples were either analysed immediately (Week 0, W₀) or after four weeks (W₄). Regarding the long-term storage experimental set-up (Figure 1A), the analysis covered seven subsequent

time-points (W_0 as the reference point, W_1 , W_2 , W_4 , W_8 , W_{18} and W_{25}) during which (an)aerobic influence was assessed additionally. Anaerobicity was attained by means of vacuum pumping (Alltech). A pooled sample, next to a male and female sample, was included to maximise metabolite coverage without substantially increasing the number of analyses. In this regard, it is to note that the distinct samples were considered ‘biological replicates’ accomodating the inherent biological variation. Thereby, the different samples were not individually compared but the various (combinations of) storage conditions were assessed using mean trends. Upon being subjected to its respective storage condition under consideration, every aliquot was lyophilised (Martin Christ) prior to analysis [8,17]. This step may reduce the substantial role water activity holds in microbial and chemical stability, *e.g.* oxidative and enzymatic reactions.

2.6. Metabolomics data analysis and statistical approaches

Compound Discoverer™ 3.0 and XCalibur 3.0 software (Thermo Fisher Scientific, CA, USA) were used for untargeted and targeted processing of the metabolomics data files, respectively. Multivariate data analysis was performed with R, version 3.4.3 [18].

2.6.1. Multivariate data analysis

As a first step, retention time alignment, feature detection (minimum peak intensity threshold of 1,000,000 a.u. and 2,000,000 a.u. for the polar and lipid analyses, respectively, Supplementary Table S7), and signal drift correction were effectuated [7,8]. The latter relied on the QC-based robust locally estimated scatterplot smoothing (LOESS) signal correction (QC-RLSC) algorithm (NormalizeMets R package) [19]. For the long-term storage data, total ion current (TIC) correction was applied prior to the QC-RLSC algorithm as an additional means of normalisation in counteracting possible time-dependent instrumental variability.

Since this stability investigation is, in essence, an untargeted metabolomics study, many compounds remain unidentified and can, thus, not be included in any standard mixture. Therefore, normalisation to summed signal or TIC was chosen as this represents the commonly addressed approach used in metabolomics analyses [20–22].

Multivariate differences between the metabolic fingerprints were assessed by constructing principal component analysis (PCA) models (pcaMethods R package) [23] on the combined positive and negative ionisation data. A variation splitting procedure was performed to separate and assess the true impact of preservation rather than interpersonal variability (mixOmics R package) [24]. Prior to modelling, normality of the metabolic features was assessed using the Shapiro-Wilk test, corrected for multiple comparisons by means of the Holm algorithm [25]. Subsequently, data were log-transformed and pareto-scaled [26]. The optimal number of PCs was determined based on the highest cross-validated (5-fold) Q^2Y after random permutation [27]. Next, pairwise orthogonal projection to latent structures discriminant analysis (OPLS-DA) models were generated (ropls R package) [28]. The construction of OPLS-DA models was divided into three subsequent steps in generating pairwise sub-models concerning the evaluation of aerobicity, storage duration and temperature (elaborated under section 3. Results). During the last step of this modelling strategy, the relative magnitude of change in the metabolic fingerprint between two time-points in each sub-model under consideration was calculated according to a piecewise multivariate modelling approach described by Rantalainen *et al.* [29]. For every univariate paired *t*-tests performed, multiple testing was corrected for by the Benjamini-Hochberg algorithm [30]. The model performance parameters included R^2X , R^2Y , Q^2Y , a permutation test ($n = 100$) and pQ^2 (a measure of over-fit) using a significance level of 5% [31,32]. Lastly, UpSet plots were constructed to visualise combinatorial cumulative storage effects, using the UpSetR R package [32].

2.6.2. Univariate data analysis

Next to the multivariate approach, targeted metabolomics analysis was also performed for which the in-house library of authentic reference standards was used. Target analytes were identified based on retention time relative to that of the authentic reference standard with deviation $\leq 2.5\%$, accurate mass according to m/z -value with mass deviation ≤ 3 ppm and $^{13}\text{C}/^{12}\text{C}$ isotope ratio [33] and can be consulted in Supplementary Figure S1, Table S5 and Supplementary Figure S2, Table S6 for the polar and lipid metabolites, respectively. The normality and homogeneity of variances of each metabolite was controlled by means of the Shapiro-Wilk and Levene's test, respectively (IBM® SPSS® Statistics Version 25). The variability in univariate relative abundance (TIC-normalised) along the applied storage condition was evaluated (presented as fold changes in the Supplementary Tables S1, S2, S3 and S4). The effect of FT cycling was determined by comparing fresh samples (C_0) with samples that had been frozen once or twice (C_0 vs. C_1 and C_0 vs. C_2) by means of paired samples Student's t-tests, for each storage time and temperature (the latter using the samples of W_4 , Figure 1A). For the long-term experimental set-up, all of the pairwise comparisons were obtained through repeated measures ANOVA with subsequent post-hoc tests, *i.e.* paired samples Student's t-tests with Bonferroni adjustment for multiple comparisons. In every comparison made, W_0 served as the reference time-point (inferring no or minimal storage at -80°C).

3. Results

A total number of 6870 and 12250 components for the FT and 13907 and 22469 for the long-term storage experiments were detected for the polar and lipidomic analyses, respectively. The difference in number of extracted components between the two experiments may be

linked to the number of participant donors (faecal pool inclusion in the long-term evaluation) and as such, plausibly introducing more unique (*e.g.* diet-related) metabolites [34].

3.1. Impact of the number of FT cycles

Interpersonal variability represented the dominant fraction of variance for the polar metabolome (Figure 2A), whereas this was not the case for the lipidome (Figures 2C and 2D and Figures S3A and S3B). Such source of variance would outperform variation introduced by storage effects on the stool metabolome. After variation splitting, the PCA score plot of the polar metabolome (Figure 2B) clearly revealed that the biological variation between person and associated clustering could almost be completely eliminated. Therefore, the variation splitting procedure was consistently applied prior to modelling. Overall, normal distribution was achieved for at least 77% components of the polar and lipid fraction of the metabolome. The overall good predictive performance of the OPLS-DA models was indicated by the cumulative Q^2Y metric, inferring that the first FT cycle already substantially impacted the metabolome composition (Table 1). The highest changes observed upon FT cycling corresponded to 10.0% and 16.2% of metabolites across the polar (at W_4 , $-80\text{ }^{\circ}\text{C}$); and 6.8% and 10.5% across the lipid fingerprint (at W_4 , $-20\text{ }^{\circ}\text{C}$) that were significantly affected upon 1 or 2 FT cycles, respectively. Thus, the first FT cycle evoked similar changes in the polar metabolome as did the two FT cycles in the lipidome, concluding greater stability of the latter.

Similarly, the targeted lipids were significantly less affected by FT cycling than the polar profile, *i.e.* 16 out of 62 polar and 3 out of 38 lipid metabolites with P -values < 0.05 , respectively, with more substantial contribution of the second FT cycle (Table S1). Among the targeted metabolites, an enhancement of the relative peak ratios resulting from both polar metabolomics lipidomics profiling was observed. Hereby, the impact was most obvious for

the amino acids (AA). Nevertheless, taking into consideration the data from the targeted and untargeted approach, the lipidome emerged as the more stable fraction of the metabolome with regard to the number of unaffected analytes, even with repeated FT cycling (Table 1 and Table S2).

3.2. Impact of long-term storage

For accurate assessment of aerobicity, time and temperature impacts on the metabolome, variation splitting was performed likewise the FT regime. The multivariate data analysis comprised three subsequent steps in order to construct pairwise OPLS-DA sub-models (Figure 1B).

First, pairwise OPLS-DA models per time-point and temperature were established to enable assessment of aerobicity conditions. No significant changes (P -value < 0.05) in the metabolome and lipidome composition were observed upon inducing aerobic or anaerobic conditions, as reinforced by the results of the targeted analyses (Tables S3 and S4).

The second step involved analogous model building within each time-point to gauge the impact of storage temperature. All OPLS-DA models built were valid and indicated a clear difference in metabolic fingerprints between $-20\text{ }^{\circ}\text{C}$ and $-80\text{ }^{\circ}\text{C}$ (Table 2). With regard to the polar metabolome, the first series of change (W_1 , $-20\text{ }^{\circ}\text{C}$ vs. $-80\text{ }^{\circ}\text{C}$) remained decisive during subsequent time-points, whereas a significant change (P -value < 0.05 , 95% CI {1483.26, 1923.74}, based on data from the last column of Table 2) to the number of lipid components was only noted on W_{25} compared to the first series of changes. Nevertheless, both metabolome fractions experienced a similar impact of storage temperature, *i.e.* metabolome differences varied between 7.0% and 10.8% for the polar metabolome and between 6.9% and 9.4% for the lipidome.

Thirdly, OPLS-DA sub-models between subsequent time-points were assembled per temperature to assess the impact of storage duration (Figure 3). Subsequently, UpSet plots revealed the number of significantly altered components and how many thereof recurred significant across the different pair-wise comparisons (Figure 4). The polar metabolome was altered in a more rigorous way at -20 °C, *i.e.* 12.1% when compared to 6.4% at -80 °C during the first week of preservation. In contrast, for the lipidome, the difference according to temperature was less dominant for the first week of preservation, indicated by a 8.6% and 7.9% metabolome alteration at -20 °C and -80 °C, respectively. The cumulative percentage of significantly altered unique polar components after 4 weeks of storage at -20 °C matched 8 weeks of storage at -80 °C, which was 19.9% and 20.0%, respectively. Similarly, for 8 and 18 weeks of storage at -20 °C and -80 °C, 22.2% and 20.4% of change was observed in the lipidome. The holistic impact at W₁₈ and -80 °C was 23.8% for the polar and 20.4% for the lipidome. The holistic impact at W₂₅ this was 25.8% and 30.9%, respectively. Notably, at W₂₅ the lipidome was altered more severely than the polar metabolome. This was in contrast with previous time-points, but clearly coincided with the significant change observed in the number of lipid components when evaluating storage temperature effects (Table 2) and hence inferring serious interaction effects on the holistic metabolome from beyond W₁₈. Furthermore, when compared to storage at -20 °C, changes in the polar metabolome imparted 32.2%, whereas this was 30.6% for the lipidome and hence indicated a confluence in the stability performance of the polar metabolome and lipidome from W₂₅. Yet, bio-analytical recommendations regarding variance in targeted and untargeted metabolomics analyses, which is generally accepted in the range between 20% and 30% [7,8], and the clustering observed in Figure 3 further encouraged faecal samples to be stored no longer than 18 weeks. The targeted profiling strategy (Table S3) revealed that the concentration levels of various AAs and amines did change significantly during the first week of storage, whereas diverse

polar classes (*e.g.* the alcohols, polyols, carbohydrates, and di-and monocarboxylic acids) exhibited prolonged stability (beyond W₈ for storage at -80 °C). Considering the lipidomics analysis, the class of poly-unsaturated fatty acids (PUFAs), including arachidonic acid, linoleic acid, eicosapentaenoic and docosahexaenoic acid was characterized by decreasing concentration levels from W₁₈ when conserved at -80 °C (similar or starting earlier at -20°C), whereas the SCFA were significantly elevated at this timepoint (Table S4).

4. Discussion

Notwithstanding the relatively small number of study subjects included, previous stability studies were based on a similar number of participants [9,13,35] and the established OPLS-DA models were able to discriminate according to the storage conditions applied. Chiefly, the observed impact of the FT regime on the metabolome was in line with the results of Gratton *et al.* [13], Zivkovic *et al.* [35] and Breier *et al.* [10], by whom other analytical platforms than UPLC-HRMS were used and hence harmonising holistic metabolome stability findings. Even more, similar metabolites were significantly altered as reported in blood by Breier *et al.* [10], albeit in the opposite direction. For instance, (iso)leucine (BCAA) as well as tryptophan were significantly decreased, whereas the targeted analysis of stool samples revealed elevated levels upon repeated FT cycling as further reinforced by Gratton *et al.* [13] through the detection of increased AAA concentrations including tyrosine and phenylalanine in stool samples. The essential role of gut microbiota in AA metabolism [36], *i.e.* the reactivation of microbial mechanisms upon thawing of samples may advocate these findings. Even more, the increasing number of significantly altered metabolites suggest a plausible interaction effect between FT cycling and storage duration. Particularly for the polar metabolites, the number of FT cycles showed stronger impact after 4 weeks of stool preservation and approximately doubled the number of metabolites that were significantly altered when subjected to two FT

cycles. Opposed to the latter, both untargeted and targeted analyses highlighted the lipidome as less affected by FT cycling. Among the altered lipids, *e.g.* the n-3 PUFA eicosapentanoic acid, the fold change observed could be ascribed to its characteristic low abundance [35] inferring some inherent variability in measurement rather than non-enzymatic oxidation reinforced by the inability of detecting docosahexanoic acid.

Regarding the targeted profiling as well as untargeted fingerprinting, almost no effect was observed when stool samples were stored in an oxygenated environment compared to vacuum. Most likely, the performance of an *a priori* lyophilisation step served to efficiently quench bacterial activity and limit oxidative reactions as well as to immobilise enzymatic, *e.g.* lipases, and non-enzymatic reactions [9,37]. The freezing temperature (-20 °C and -80 °C) and storage duration (W_{0-25}), however, did exert significant effects (P -value < 0.05) on the polar and lipid fraction of the metabolome. In this regard, all faecal metabolites consistently showcased similar or prolonged stability upon freezing at -80 °C compared to -20 °C, which is in agreement with broadscale applied storage approaches in faecal metabolomics [17].

Scrutinising the nature of targeted metabolites that were altered, both enzymatic hydrolysis and synthesis as well as reactivated microbial effects were hypothesised as plausible causes for the differences observed. For instance, the hydrolysis catalysed by lipases may be revived following thawing of the frozen samples due to observed decrease and increase of phospholipids and FAs serving as the substrate and hydrolysis product, respectively [35]. The decrease in PUFAs, which was also noted in the study of Zivkovic *et al.* [35], could be ascribed to the presence of methylene bridges between double bonds of long-chain PUFAs, which represent a class of lipids that are more prone to oxidation [35,37]. The discrepancies noted in sterol lipids most likely resulted from physical changes to the membrane's structural constituents occurring during the freeze-thaw process as this class imparts key players in membrane dynamics [38]. Thus, in analogy to the FT cycling, metabolites experienced the

influence of the diverse storage conditions applied in a very consistent manner within their respective class. For instance, glycerolipids and glycerophospholipids were profiled as relatively stable classes compared to FAs, in particular the SCFA, during long-term preservation and as such, stressing the crucial role of gut microbiota in quantitative discrepancies observed in the metabolic (in)stability of stool [39].

As demonstrated before in biofluid metabolome stability studies, the changes attributable to storage were small in comparison to interindividual variation [9,12,13,40]). The initial clustering observed in the polar metabolic fingerprints substantiated the undeniable intimate connection of faecal metabolites with one's unique gut microbiome. In particular, molecules involved in bacteria-host metabolic and immunological cross-talk, *e.g.* SCFA, BCAA, AAA, amines and diverse cytokines, share polar characteristics [41]. Evidently, this gut microbial and associated metabolic dysbiosis is of crucial importance in diseases such as diabetes type 2 and colorectal cancer [8,22]. Despite the individuality of one's microbiota, the functionality resulting thereof is very similar over time according to (patho)physiology. In this regard, the almost complete absence of between-person variation in the lipidome, which consists of rather apolar metabolites, is reflective for such unique host-related changes within health and disease. Thus, this study not only reinforced the clinical value of both fractions of the stool metabolome in light of 'personalised therapies', but also showcased the ability of a multilevel statistical approach based on QC-RLSC normalised data as a valid technique in dealing with the issue of interpersonal variability of the metabolome to focus on the parameters under investigation, *e.g.* storage conditions. However, the variation in the biochemical composition of faeces caused by interindividual differences remains rather limited when compared to that induced by diseases, underpinning the importance of identifying the more variable and stable components of the metabolic signature upon storage. This study expanded current knowledge

regarding the optimum way to store and process stool samples according to the research question imposed and concordant nature of the metabolomics analysis performed.

5. Conclusions

In conclusion, considering practical reality and as a corollary to this research's findings, the prompt storage of lyophilised and aliquoted faecal samples is advised, *i.e.* anticipating at a number of research questions going forward. It should, however, be taken into mind that if the interest would greatly align with particular chemical classes such as the AA and amines for polar metabolomics analysis, storage should ideally not range beyond 4 weeks at -20 °C nor 8 weeks at -80 °C. Similarly for the lipidome, best representation of the FAs would result from storage up to 8 weeks at -20 °C or 18 weeks at -80 °C. Thus, striving to uncover metabolic signatures of disease state and relating those in the most reliable and accurate manner with clinical signs is best performed when stool samples are being stored at -80 °C, which would approximately double the storage length up to 18 weeks regarding the holistic nature of the sample, and avoiding any FT cycle.

Acknowledgments

The authors gratefully acknowledge the statistical expertise provided by Prof. Dr. Vander Heyden (VUB, Brussels) as well as the input of Vicky De Clippele and Charlotte Salens (Ghent University) regarding experimental and formal analysis provided during their master dissertation. This work was supported by Flanders Innovation & Entrepreneurship (VLAIO, IM 150740). De Spiegeleer Margot is supported by Research Foundation – Flanders (FWO 1S57920N).

References

- [1] R.D. Beger, W. Dunn, M.A. Schmidt, S.S. Gross, J.A. Kirwan, M. Cascante, L. Brennan, D.S. Wishart, M. Oresic, T. Hankemeier, D.I. Broadhurst, A.N. Lane, K. Suhre, G. Kastenmüller, S.J. Sumner, I. Thiele, O. Fiehn, R. Kaddurah-Daouk, for “Precision Medicine, Metabolomics enables precision medicine: “A White Paper, Community Perspective,” *Metabolomics*. 12 (2016).
- [2] C.P. Wild, The exposome: From concept to utility, *Int. J. Epidemiol.* 41 (2012) 24–32.
- [3] A. Tebani, L. Abily-Donval, C. Afonso, S. Marret, S. Bekri, Clinical metabolomics: The new metabolic window for inborn errors of metabolism investigations in the post-genomic era, *Int. J. Mol. Sci.* 17 (2016) 1–25.
- [4] F.R. Pinu, S.A. Goldansaz, J. Jaïne, Translational metabolomics: Current challenges and future opportunities, *Metabolites*. 9 (2019).
- [5] C. Rombouts, L.Y. Hemeryck, T. Van Hecke, S. De Smet, W.H. De Vos, L. Vanhaecke, Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat

- discriminating metabolites, *Sci. Rep.* 7 (2017) 1–13.
- [6] J. Vanden Bussche, M. Marzorati, D. Laukens, L. Vanhaecke, Validated High Resolution Mass Spectrometry-Based Approach for Metabolomic Fingerprinting of the Human Gut Phenotype, *Anal. Chem.* 87 (2015) 10927–10934.
- [7] E. De Paepe, L. Van Meulebroek, C. Rombouts, S. Huysman, K. Verplanken, B. Lapauw, J. Wauters, L.Y. Hemeryck, L. Vanhaecke, A validated multi-matrix platform for metabolomic fingerprinting of human urine, feces and plasma using ultra-high performance liquid-chromatography coupled to hybrid orbitrap high-resolution mass spectrometry, *Anal. Chim. Acta.* 1033 (2018) 108–118.
- [8] L. Van Meulebroek, E. De Paepe, V. Vercruysse, B. Pomian, S. Bos, B. Lapauw, L. Vanhaecke, Holistic Lipidomics of the Human Gut Phenotype Using Validated Ultra-High-Performance Liquid Chromatography Coupled to Hybrid Orbitrap Mass Spectrometry, *Anal. Chem.* 89 (2017) 12502–12510.
- [9] J. Laparre, Z. Kaabia, M. Mooney, T. Buckley, M. Sherry, B. Le Bizec, G. Dervilly-Pinel, Impact of storage conditions on the urinary metabolomics fingerprint, *Anal. Chim. Acta.* 951 (2016) 99–107.
- [10] M. Breier, S. Wahl, C. Prehn, M. Fugmann, U. Ferrari, M. Weise, F. Banning, J. Seissler, H. Grallert, J. Adamski, A. Lechner, Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples, *PLoS One.* 9 (2014) 1–11.
- [11] J.A. Kirwan, R. Kaddurah-Daouk, T. Mitchell, T. Pischon, M.A. Schmidt, V. Velagapudi, Biobanking for Metabolomics and Lipidomics in Precision Medicine, *Clin. Chem.* (2019) 1–6.
- [12] J. Saric, Y. Wang, J. Li, M. Coen, J. Utzinger, J.R. Marchesi, J. Keiser, K. Veselkov, J.C. Lindon, J.K. Nicholson, E. Holmes, Species Variation in the Fecal Metabolome Gives Insight into Differential Gastrointestinal Function, *J. Proteome Res.* 7 (2007)

- 539 352–360.
- 540 [13] J. Gratton, J. Phetcharaburanin, B.H. Mullish, H.R.T. Williams, M. Thursz, J.K.
 541 Nicholson, E. Holmes, J.R. Marchesi, J. V. Li, Optimized Sample Handling Strategy
 542 for Metabolic Profiling of Human Feces, *Anal. Chem.* 88 (2016) 4661–4668.
- 543 [14] C. Junot, F. François, C. Benoit, B. François, High resolution mass spectrometry based
 544 techniques at the crossroads of metabolic pathways, *Mass Spectrom. Rev.* 33 (2014)
 545 471–500.
- 546 [15] H.G.M. Tiedink, M.B. Katan, Variability in lipoprotein concentrations in serum after
 547 prolonged storage at -20° C, *Clin. Chim. Acta.* 180 (1989) 147–155.
- 548 [16] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy,
 549 C.R.H. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van
 550 Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, A
 551 comprehensive classification system for lipids, *J. Lipid Res.* 46 (2005) 839–862.
- 552 [17] M.M. Ulaszewska, C.H. Weinert, A. Trimigno, R. Portmann, C. Andres Lacueva, R.
 553 Badertscher, L. Brennan, C. Brunius, A. Bub, F. Capozzi, M. Cialiè Rosso, C.E.
 554 Cordero, H. Daniel, S. Durand, B. Egert, P.G. Ferrario, E.J.M. Feskens, P. Franceschi,
 555 M. Garcia-Aloy, F. Giacomoni, P. Giesbertz, R. González-Domínguez, K. Hanhineva,
 556 L.Y. Hemeryck, J. Kopka, S.E. Kulling, R. Llorach, C. Manach, F. Mattivi, C. Migné,
 557 L.H. Münger, B. Ott, G. Picone, G. Pimentel, E. Pujos-Guillot, S. Riccadonna, M.J.
 558 Rist, C. Rombouts, J. Rubert, T. Skurk, P.S.C. Sri Harsha, L. Van Meulebroek, L.
 559 Vanhaecke, R. Vázquez-Fresno, D. Wishart, G. Vergères, *Nutrimetabolomics: An*
 560 *Integrative Action for Metabolomic Analyses in Human Nutritional Studies*, *Mol. Nutr.*
 561 *Food Res.* 1800384 (2018) 1–38.
- 562 [18] R Core Team, R: A language and environment for statistical computing, (2013).
 563 <http://www.r-project.org/>.

- [19] A.M. De Livera, G. Olshansk, NormalizeMets: Analysis of Metabolomics Data, (2018). <https://cran.r-project.org/package=NormalizeMets>.
- [20] D. Weindl, A. Wegner, C. Jäger, K. Hiller, Isotopologue ratio normalization for non-targeted metabolomics, *J. Chromatogr. A*. 1389 (2015) 112–119.
- [21] E.A. Palmer, H.J. Cooper, W.B. Dunn, Investigation of the 12-Month Stability of Dried Blood and Urine Spots Applying Untargeted UHPLC-MS Metabolomic Assays, *Anal. Chem.* 91 (2019) 14306–14313.
- [22] C. Rombouts, M. De Spiegeleer, L. Van Meulebroek, W.H. De Vos, L. Vanhaecke, Validated comprehensive metabolomics and lipidomics analysis of colon tissue and cell lines, *Anal. Chim. Acta*. 1066 (2019) 79–92.
- [23] W. Stacklies, H. Redestig, M. Scholz, D. Walther, J. Selbig, pcaMethods - A bioconductor package providing PCA methods for incomplete data, *Bioinformatics*. 23 (2007) 1164–1167.
- [24] L.C. Kim-Anh, F. Rohart, I. Gonzalez, S. Dejean, mixOmics: Omics Data Integration Project, (2017). <https://cran.r-project.org/package=mixOmics>.
- [25] S. Holm, A simple sequentially rejective multiple test procedure, *Scand. J. Stat.* 6 (1979) 65–70.
- [26] D.M. Rocke, B. Durbin, Approximate variance-stabilizing transformations for gene-expression microarray data, *Bioinformatics*. 19 (2003) 966–972.
- [27] D. Giancarlo, T. Chiara, Cross-validation Methods in Principal Component Analysis: A Comparison, *Stat. Methods Appl.* 11 (2002) 71–82.
- [28] E.A. Thévenot, A. Roux, Y. Xu, E. Ezan, C. Junot, Analysis of the Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and Gender by Implementing a Comprehensive Workflow for Univariate and OPLS Statistical Analyses, *J. Proteome Res.* 14 (2015) 3322–3335.

- [29] M. Rantalainen, O. Cloarec, T.M.D. Ebbels, T. Lundstedt, J.K. Nicholson, E. Holmes, J. Trygg, Piecewise multivariate modelling of sequential metabolic profiling data, *BMC Bioinformatics*. 9 (2008) 1–13.
- [30] A. Alonso, S. Marsal, A. Juliá, Analytical Methods in Untargeted Metabolomics: State of the Art in 2015, *Front. Bioeng. Biotechnol.* 3 (2015) 1–20.
- [31] E. Szymańska, E. Saccenti, A.K. Smilde, J.A. Westerhuis, Double-check: Validation of diagnostic statistics for PLS-DA models in metabolomics studies, *Metabolomics*. 8 (2012) 3–16.
- [32] J.R. Conway, A. Lex, N. Gehlenborg, UpSetR: An R Package for the Visualization of Intersecting Sets and their Properties, (2017).
- [33] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W.M. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reilly, J.J. Thaden, M.R. Viant, Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI), *Metabolomics*. 3 (2007) 211–221.
- [34] G.D. Wu, C. Compher, E.Z. Chen, S.A. Smith, R.D. Shah, C. Chehoud, L.G. Albenberg, L. Nessel, E. Gilroy, A.M. Weljie, H.J. Flint, D.C. Metz, M.J. Bennett, H. Li, F.D. Bushman, J.D. Lewis, Comparative metabolomics in vegan and omnivores reveal constraints on diet-dependent gut microbiota metabolite production, *Gut*. 65 (2016) 63–72.
- [35] A.M. Zivkovic, M.M. Wiest, U.T. Nguyen, R. Davis, S.M. Watkins, J.B. German, Effects of sample handling and storage on quantitative lipid analysis in human serum, *Metabolomics*. 5 (2009) 507–516.
- [36] L. Zhao, Y. Huang, L. Lu, W. Yang, T. Huang, Z. Lin, C. Lin, H. Kwan, H.L.X. Wong,

- Y. Chen, S. Sun, X. Xie, X. Fang, H. Yang, J. Wang, L. Zhu, Z. Bian, Saturated long-chain fatty acid-producing bacteria contribute to enhanced colonic motility in rats, *Microbiome*. 6 (2018) 1–16.
- [37] M.D. Rudy, M.J. Kainz, M. Graeve, S.M. Colombo, M.T. Arts, Handling and storage procedures have variable effects on fatty acid content in fishes with different lipid quantities, *PLoS One*. 11 (2016).
- [38] E.J. Dufourc, Sterols and membrane dynamics, *J. Chem. Biol.* 1 (2008) 63–77.
- [39] G. den Besten, K. van Eunen, A.K. Groen, K. Venema, D.-J. Reijngoud, B.M. Bakker, The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism, *J. Lipid Res.* 54 (2013) 2325–2340.
- [40] M. Bictash, T.M. Ebbels, Q. Chan, R.L. Loo, I.K.S. Yap, I.J. Brown, M. De Iorio, M.L. Daviglus, E. Holmes, J. Stamler, J.K. Nicholson, P. Elliott, Opening up the “black box”: Metabolic phenotyping and metabolome-wide association studies in epidemiology, *J. Clin. Epidemiol.* 63 (2010) 970–979.
- [41] M.X. Chen, S.Y. Wang, C.H. Kuo, I.L. Tsai, Metabolome analysis for investigating host-gut microbiota interactions, *J. Formos. Med. Assoc.* 118 (2019) S10–S22.

Tables & Figures

Table 1. OPLS-DA model performance parameters for the various combinations of storage duration, temperature and FT cycles for the polar metabolome (above the dotted line) and lipidome (below the dotted line). The number of orthogonal and predictive components was 1 (i.e. $t_p + t_o$; 1 + 1). The last column covers the number of significantly altered components (paired Student's t -test, P -value < 0.05).

<i>Week</i>	<i>T (°C)</i>	<i>FT cycles</i>	<i>R²X (cum)</i>	<i>R²Y (cum)</i>	<i>Q²Y (cum)</i>	<i>P</i>	<i>pQ²</i>	<i>#significant</i>
<u>Polar metabolome</u>								
0		0 vs. 1	0.541	0.990	0.748	0.147	0.11	611
0		0 vs. 2	0.638	0.996	0.885	0.079	0.07	1025
4	−20	0 vs. 1	0.539	0.993	0.718	0.125	0.09	415
4	−20	0 vs. 2	0.675	0.999	0.939	0.096	0.09	949
4	−80	0 vs. 1	0.484	0.999	0.781	0.102	0.08	688
4	−80	0 vs. 2	0.570	0.999	0.951	0.095	0.09	1113
<u>Lipidome</u>								
0		0 vs. 1	0.493	0.995	0.756	0.132	0.10	812
0		0 vs. 2	0.562	0.996	0.888	0.158	0.14	610
4	−20	0 vs. 1	0.487	0.994	0.807	0.136	0.11	831
4	−20	0 vs. 2	0.594	0.998	0.933	0.150	0.14	1281
4	−80	0 vs. 1	0.440	0.996	0.792	0.101	0.08	637
4	−80	0 vs. 2	0.599	0.999	0.911	0.088	0.08	890

Table 2. OPLS-DA model performance parameters for the comparison of storage temperature (-20 °C vs. -80 °C) for the polar metabolome and lipidome. The number of predictive and orthogonal components was 1 (*i.e.* $t_p + t_o$; 1 + 1). The last column covers the number of significantly altered components (paired Student's *t*-test, *P*-value < 0.05).

<i>Week</i>	<i>R²X (cum)</i>	<i>R²Y (cum)</i>	<i>Q²Y (cum)</i>	<i>P</i>	<i>pQ²</i>	<i>#significant</i>
<u>Polar metabolome</u>						
1	0.227	0.999	0.763	0.013	<0.05	1426
2	0.228	0.999	0.774	0.013	<0.05	1507
4	0.194	0.999	0.605	0.017	<0.05	980
8	0.257	0.999	0.775	0.013	<0.05	1464
18	0.220	0.999	0.757	0.026	<0.05	1318
25	0.347	0.998	0.775	0.013	<0.05	1493
<u>Lipidome</u>						
1	0.268	0.999	0.543	0.018	<0.05	1543
2	0.282	0.999	0.560	0.018	<0.05	1586
4	0.289	0.999	0.598	0.017	<0.05	1637
8	0.289	0.999	0.565	0.018	<0.05	1645
18	0.280	0.999	0.584	0.017	<0.05	1691
25	0.322	0.999	0.758	0.013	<0.05	2119

Figure 1.

Figure 2.

650

651

652

Figure 3.

653

654

Figure 4.

655

656

Figure captions

Figure 1. A. Overview of the workflow for stool preparation and procedures for analysis. Dashed arrows indicate that the applied procedures were the same as in the already depicted workflow. **B.** Data analysis strategy. The single arrows indicate OPLS-DA models within and between time-points to study the effect of aerobic vs. anaerobic storage and to explore the storage duration effects, respectively. The double arrows indicate OPLS-DA models to assess the impact of storage temperature within time-points (W_0 = common reference point).

Figure 2. PCA score plots following polar metabolomics (A & B) and lipidomics (C & D) fingerprinting for all samples (W_0 analysed immediately and W_4 preserved at -20 °C and -80 °C) to visualise freeze-thaw effects. The largest ellipse is a Hotelling's T^2 95% confidence ellipse on all samples, whereas the smaller ellipses represent 95% confidence intervals based on multivariate t -distribution on the samples per person. **A.** Initial PCA score plot without separation of between-person variation, with data preprocessing and pretreatment, *i.e.* QC-RLSC normalisation, log transformation and pareto scaling. **B.** PCA score plot with between-person variation splitting and subsequent data preprocessing and pretreatment steps. **C.** Initial PCA score plot for W_0 and W_4 without separation of the between-person variation, with data preprocessing and pretreatment, *i.e.* QC-RLSC normalisation, log transformation and pareto scaling. **D.** PCA score plot for W_0 and W_4 with between-person variation splitting and subsequent preprocessing and pretreatment steps.

Figure 3. PCA score plots to visualise trends of the long-term storage duration separately according to storage temperature and the type of analysis performed. The largest ellipse is a Hotelling's T^2 95% confidence ellipse for all samples whereas the smaller ellipses represent 95% confidence intervals based on the multivariate t -distribution for the samples per week. **A.** Polar metabolomics, at -20 °C, **B.** Polar metabolomics, at -80 °C, **C.** Lipidomics, at -20 °C, **D.** Lipidomics, at -80 °C.

Figure 4. UpSet plots reflecting the intersections of modified components upon storage duration for every combination of preservation temperature and type of analysis, **A.** Polar metabolomics at -20 °C, **B.** Polar metabolomics at -80 °C, **C.** Lipidomics at -20 °C, **D.** Lipidomics at -80 °C. The total number of significantly altered components per pairwise t-test (P-value < 0.05), the latter denoted as ‘a set’, is presented at the left-side of the horizontal bars, bottom left. The number of shared altered components, i.e. those in common between the compared sets, are indicated above the vertical bars and visualised by connecting the respective black dots. Bars that are accompanied by only a single dot represent the number of significantly altered components that are unique for that set.